

# BLIMP1 regulates cell growth through repression of p53 transcription

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**Tight regulation of p53 is essential for maintaining normal cell growth. Here we report that BLIMP1 acts in an autoregulatory feedback loop that controls p53 activity through repression of p53 transcription. p53 binds to and positively regulates *BLIMP1*, which encodes for a known B cell transcriptional repressor. Knockdown of BLIMP1 by siRNA results in both apoptosis and growth arrest in human colon cancer cells and cell-cycle arrest in primary human fibroblasts. Interestingly, the levels of both p53 mRNA and protein are substantially increased after BLIMP1 depletion, which is accompanied by the induction of p53 target genes. Importantly, the apoptosis induced by BLIMP1 depletion in HCT116 cells is largely abrogated in cells lacking p53 or in cells depleted in p53 by siRNA. We further demonstrate that BLIMP1 binds to the p53 promoter and represses p53 transcription, and this provides a mechanistic explanation for the induction of p53 response in cells depleted of BLIMP1. Hence, suppression of p53 transcription is a crucial function of endogenous BLIMP1 and is essential for normal cell growth.**

apoptosis | growth arrest | transcription regulation

**B**LIMP1 is a zinc-finger-containing DNA-binding transcriptional repressor and plays essential roles in both embryonic development and adult tissues. BLIMP1 was initially identified as a postviral induction repressor of transcription of *IFNB1* (1) and later was shown to recruit the histone methyltransferase G9a to the promoter of *IFN-β* gene to shut down transcription (2). Blimp1 plays an essential role in the terminal differentiation of B cells into Ig-secreting plasma cell through direct transcriptional silencing of several key transcription activators such as Pax5 and Bcl6 (3–5). In the differentiation of myeloid lineage, Blimp1 also is a key regulator (6). Study on *Blimp1* knockout mice demonstrates that Blimp1 is a critical determinant of the germ cell lineage (7, 8), and it is crucial for consistent repression of homeobox genes that normally accompany specification of primordial germ cells (PGCs) (7). In zebrafish, Blimp1 promotes differentiation of the embryonic slow muscle lineage (9) and specifies neural crest and sensory neuron progenitors (10). Collectively, these studies indicate that Blimp1 plays a key role in the cellular differentiation process.

In addition, a number of reports suggest that Blimp1 might regulate diverse cellular processes including cell growth or survival. The PGC-like cells in Blimp1 mutant embryos failed to show the characteristic proliferation and migration (7). Blimp1 mutant embryos also display apoptosis in multiple cell types, most notably the mesenchyme cells, which express high levels of Blimp1 (8). Recent studies of Blimp1 in T cells demonstrate that mice lacking Blimp1 develop inflammatory disease and show a decrease in survival of T cells in thymocytes (11, 12). However, no studies to date have directly defined the role of Blimp1 in regulating cell proliferation and survival. Furthermore, the upstream transcription regulator of Blimp1 is also not known.

The tumor suppressor p53 responds to a variety of intrinsic and extrinsic stress signals to trigger several cellular programs, including cell-cycle arrest, apoptosis, inhibition of angiogenesis/metastasis, and DNA repair (13–16). p53 regulates the expression of downstream target genes, which serve as mediators of p53 functions (17–19). For example, *p21*, *Bax*, and *Puma* are direct transcriptional

targets of p53, and they play critical role in the p53 pathway (20–23). Our previous study that coupled ChIP with the paired-end ditag technologies for mapping the p53 binding sites in the human genome uncovered many putative p53 target genes (24). One of these candidate genes is *BLIMP1*. In this study we show that *BLIMP1* is a bona fide p53 target gene and, more importantly, that it acts in an autoregulatory feedback loop that controls p53 activity through repression of p53 transcription. Our study uncovers a function of BLIMP1 in regulating cell survival and demonstrates the involvement of p53 in this process.

## Results

**p53 Positively Regulates BLIMP1 Transcription.** The identification of p53 binding in the *BLIMP1* genomic locus suggests that *BLIMP1* could be regulated by p53. The p53 binding locus was located downstream of the *BLIMP1* transcription start site and within the third intron (Fig. 1A). A short sequence containing a p53 consensus site was identified within the paired-end ditag cluster (Fig. 1A). We first verified whether the p53 binding motif within the *BLIMP1* genomic locus determined by ChIP paired-end ditag analysis is associated with p53 interaction *in vivo*. We examined a 2,200-bp region encompassing the p53 binding motif using ChIP followed by quantitative real-time PCR (ChIP-qPCR) in HCT116 cells with p53 activated by treatment with 5-fluorouracil (5-FU). ChIP-qPCR confirmed p53 binding with the highest level of occupancy coinciding with the location of the predicted p53 binding motif (Fig. 1B). Interestingly, we also observed p53 occupancy in untreated HCT116 cells, suggesting that p53 may also regulate *BLIMP1* transcription even in the absence of genotoxic stress (Fig. 1C).

To determine whether the p53 binding motif present in *BLIMP1* intron 3 verified above could mediate p53 responsiveness, two tandem copies of this binding site (p53 wt*BLIMP1*) or its mutants (p53 mut*BLIMP1*) [Fig. 1A, and see supporting information (SI) *Methods*] were inserted upstream of the luciferase coding sequence in pGL3 basic vector. HCT116 p53<sup>-/-</sup> cells were transfected with either p53wt*BLIMP1-luc* or p53mut*BLIMP1-luc* along with plasmids expressing wild-type p53. As shown in Fig. 1D and SI Fig. 6, p53 induced luciferase expression from p53wt*BLIMP1-luc* in a dose-dependent manner whereas no transcriptional activation was observed from p53mut*BLIMP1-luc*. This indicates that the identi-

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The authors declare no conflict of interest.

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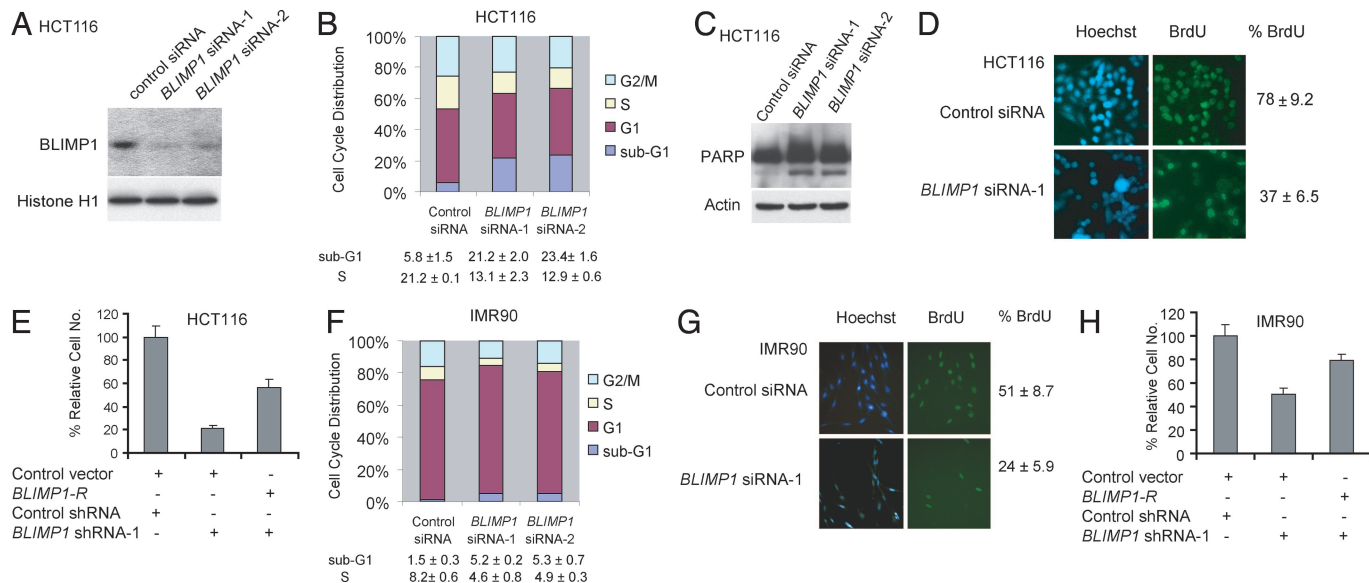
Abbreviations: qPCR, quantitative real-time PCR; 5-FU, 5-fluorouracil; shRNA, short hairpin RNA; PGC, primordial germ cell.

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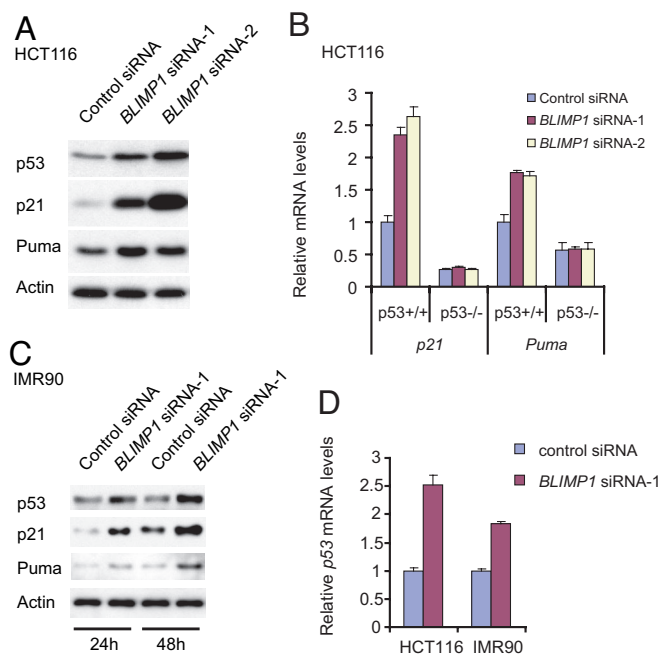




**Fig. 2.** BLIMP1 depletion by siRNA activates apoptosis and/or cell-cycle arrest. (A) The efficiency of the two independent *BLIMP1* siRNAs was tested. HCT116 cells were transfected with *BLIMP1* siRNA-1, *BLIMP1* siRNA-2, or *GFP* siRNA as a control. Nuclear extracts (100  $\mu$ g in each lane) were loaded to detect endogenous BLIMP1 protein, which is expressed at low levels in HCT116 cells. (B) Cell-cycle distribution of HCT116 cells transfected with control or *BLIMP1* siRNAs. The cells were stained with propidium iodide (PI) for DNA content and analyzed by FACS. (C) Immunoblots of total lysates of control siRNA-transfected HCT116 cells and *BLIMP1* siRNA-transfected cells with the indicated antibodies. (D) Quantification of BrdU-positive cells in HCT116 treated with *BLIMP1* siRNA-1 or control siRNA. (E) Cell number count to show that the growth-inhibitory effect induced by loss of BLIMP1 in HCT116 cells could be rescued by BLIMP1-R. Transfected cells were selected with puromycin for 2 days. (F) Cell-cycle distribution of IMR90 cells transfected with control or *BLIMP1* siRNAs. The cells were stained with PI for DNA content and analyzed by FACS. (G) Quantification of BrdU-positive cells in IMR90 treated with *BLIMP1* siRNA-1 or control siRNA. (H) Cell number count to show that the growth defects induced by BLIMP1 depletion in IMR90 cells could be rescued by BLIMP1-R. Transfected cells were selected with puromycin for 2 days.

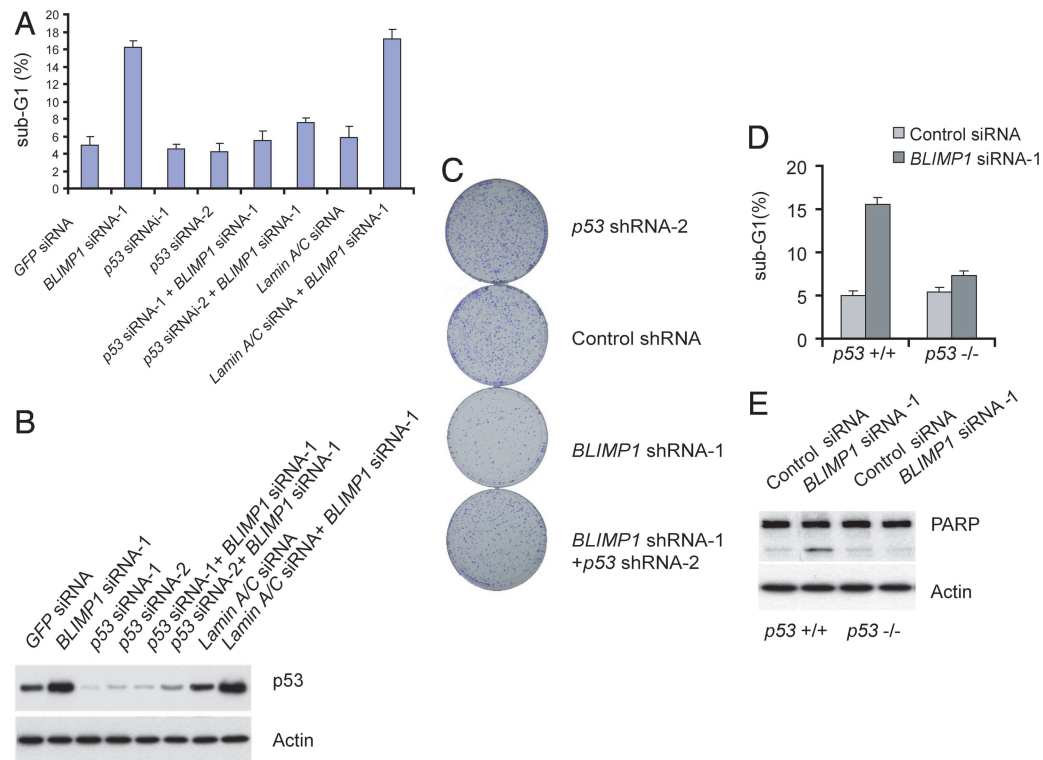
tained with HCT116 cells, IMR90 cells depleted of BLIMP1 also exhibited a decrease in the proportion of cells in S phase (Fig. 2F and SI Fig. 7A). The proportion of cells in sub-G<sub>1</sub> phase after BLIMP1 depletion in IMR90 cells is less than HCT116 cells, suggesting that the apoptotic effect may be cell-type-specific. The growth arrest was substantiated by a decrease in BrdU incorporation in BLIMP1-depleted IMR90 cells (Fig. 2G). Also, we observed that the growth of IMR90 cells was strongly inhibited as a result of *BLIMP1* knockdown by lentiviral transduction (SI Fig. 7B). Importantly, the *BLIMP1-R* was able to prevent the reduction in IMR90 cell numbers caused by *BLIMP1* siRNA (Fig. 2H), confirming that the cell growth inhibition is specifically caused by the BLIMP1 depletion. Taken together, these data indicate that the constitutive expression of *BLIMP1*, which itself is a p53 target gene, is essential for proliferation or survival in both HCT116 and IMR90 cells.

BLIMP1 depletion triggers a p53 response. Because *BLIMP1* is positively regulated by p53 and depletion of BLIMP1 triggers cell apoptosis or growth arrest, which is a hallmark of p53-mediated response, we asked whether removal of BLIMP1 may affect the p53 pathway. Interestingly, the depletion of BLIMP1 in HCT116 cells resulted in an elevation of p53 expression at protein levels (Fig. 3A). More importantly, the induction of p53 was accompanied by an increase in the protein level of its target genes, *p21* (20, 21) and *Puma* (22, 23) (Fig. 3A), which may account for the observed cell growth defects in BLIMP1-depleted HCT116 cells. Furthermore, *p21* and *Puma* mRNAs were observed to be up-regulated in response to *BLIMP1* knockdown only in *p53*<sup>+/+</sup> HCT116 cells, but not in *p53*<sup>-/-</sup> HCT116 cells (Fig. 3B). We also observed a similar result in IMR90 cells with an increase in p53 as well as its downstream target genes, *p21* and *Puma*, at the protein levels upon depletion of BLIMP1 by RNAi (Fig. 3C). These findings identify a role of BLIMP1 in regulating the p53 pathway. Because BLIMP1 is known to function as a transcriptional repressor, we examined whether *p53* transcription is affected by BLIMP1 depletion. Indeed,



**Fig. 3.** Depletion of BLIMP1 elevates p53 and induces the expression of p53 target genes. Lysates were immunoblotted with the indicated antibodies. RNA was isolated, reverse-transcribed, subjected to qPCR, and normalized with *GAPDH* mRNA. (A) Immunoblots of total lysates of HCT116 cells transfected with control siRNA or *BLIMP1* siRNAs. (B) RT-PCR analysis of mRNA levels in HCT116 *p53*<sup>+/+</sup> and *p53*<sup>-/-</sup> cells transfected with *BLIMP1* siRNAs or control siRNA. (C) Immunoblots of total lysates of IMR90 cells transfected with control siRNA or *BLIMP1* siRNAs. (D) RT-PCR analysis of *p53* mRNA levels in HCT116 and IMR90 cells transfected with *BLIMP1* siRNA-1 or control siRNA.





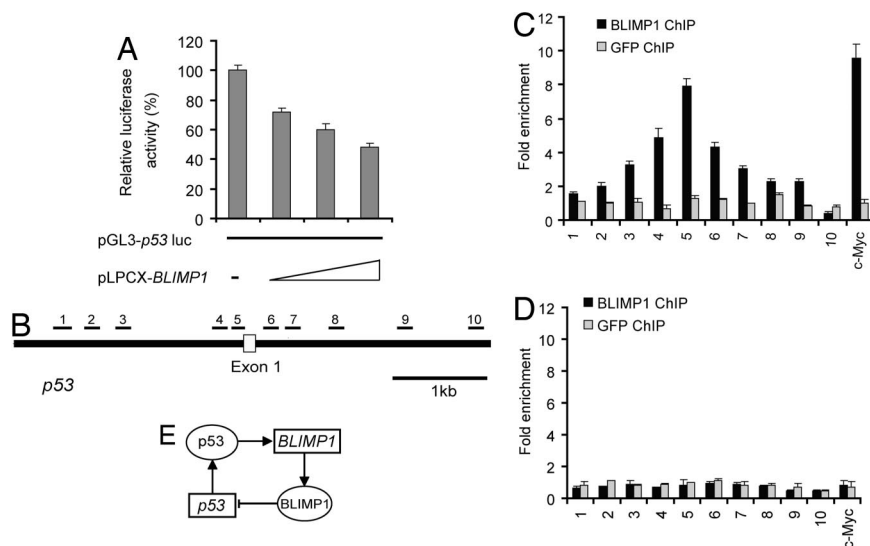
**Fig. 4.** p53 is an important mediator of apoptosis induced by BLIMP1 knockdown in HCT116 cells. (A) Cotransfection of p53 siRNA with BLIMP1 siRNA prevented the induction in apoptotic cell population. Cells were analyzed for apoptotic cells (sub-G<sub>1</sub>) according to PI staining followed by FACS analysis. (B) Protein lysates of cells from A were immunoblotted with antibody against p53. (C) Restoration of surviving colony numbers of HCT116 cells exposed to both p53 shRNA (LV-p53 shRNA) and BLIMP1 shRNA compared with that of cells with BLIMP1 depletion alone. Cells were split at 24 h after transfection and selected with puromycin for 10 days. Plates were stained with crystal violet. (D) In the absence of p53, the apoptosis induced by BLIMP1 depletion is largely abrogated. The proportion of apoptotic cells was determined by PI staining followed by FACS analysis (sub-G<sub>1</sub>). (E) In the absence of p53, the increase in the PARP cleavage induced by BLIMP1 depletion is abrogated. Protein lysates of cells were immunoblotted with antibody against PARP.

qPCR indicated that p53 mRNA levels increased substantially after BLIMP1 RNAi treatment in both HCT116 and IMR90 cells (Fig. 3D). Taken together, these results demonstrate that depletion of BLIMP1 triggers a p53 response and suggest that endogenous BLIMP1 may be involved in suppressing p53 expression at the transcriptional level.

**p53 Is an Important Mediator of Apoptosis Induced by BLIMP1 Knockdown in HCT116 Cells.** The results shown above suggest that p53 appears to be responsible for altered cell proliferation and cell survival ability in BLIMP1 knockdown cells. To determine the contribution of endogenous p53 in apoptosis that is instigated by BLIMP1 depletion in HCT116 cells, we cotransfected HCT116 cells with BLIMP1 siRNA together with either control or p53 siRNA. Control transfections included a GFP siRNA and lamin A/C siRNA, respectively. BLIMP1 reduction resulted in a 3- to 4-fold increase in the proportion of apoptotic cells; this effect was abrogated by the depletion of p53 but not by control siRNA against lamin A/C (Fig. 4A). We confirmed that p53 siRNA did not compromise the efficacy of the BLIMP1 RNAi by using qPCR to check BLIMP1 mRNA levels (data not shown). p53 siRNA-1, which was more efficient at suppressing p53 protein levels, was able to abrogate BLIMP1 RNAi-induced apoptosis more efficiently (Fig. 4A and B). Moreover, we observed an increase in the number of surviving colonies of HCT116 cells coexpressing with p53 shRNA and BLIMP1 shRNA as compared with cells with BLIMP1 depletion alone or shRNA against lamin A/C plus Blimp1 shRNA (Fig. 4C and SI Fig. 8). To seek more evidence that p53 is an important mediator of apoptosis in HCT116 cells induced by BLIMP1 knockdown, we compared the extent of apoptosis between wild-type and

p53<sup>-/-</sup> HCT116 cells after BLIMP1 depletion. Consistent with the above results, the induction of apoptosis by BLIMP1 siRNA was strikingly prohibited in p53<sup>-/-</sup> HCT116 cells relative to p53<sup>+/+</sup> cells (Fig. 4D). We observed a slight increase of apoptosis in p53<sup>-/-</sup> cells, suggesting that a p53-independent apoptosis pathway might be involved (Fig. 4D). Moreover, we saw an increase in PARP cleavage in BLIMP1 siRNA-treated p53<sup>+/+</sup> HCT116 cells but not in p53<sup>-/-</sup> HCT116 cells (Fig. 4E). In conclusion, p53 is, indeed, responsible for the induced apoptosis observed in HCT116 cells depleted of BLIMP1. Thus, negatively regulating p53 is a crucial function of endogenous BLIMP1 and is essential for the survival of HCT116 cells.

**BLIMP1 Directly Suppresses p53 Transcription by Binding to Its Promoter.** In light of the significant biological consequences of BLIMP1-mediated regulation of p53 in unstressed cells, we explored the molecular mechanism. Having shown that BLIMP1 depletion results in up-regulation of p53 mRNA level (Fig. 3D), we next assessed whether BLIMP1 can suppress the activity of p53 promoter. Cotransfection of a STAT1 or IRF-7 expression vector with pGL3p53 reporter into HCT116 cells had no appreciable effect on the p53 promoter (SI Fig. 9A). In contrast, cotransfection of the BLIMP1 expression vector with the pGL3p53 reporter resulted in considerable inhibition of p53 promoter activity (Fig. 5A). Next, we addressed whether BLIMP1 binds to endogenous p53 promoter using ChIP-qPCR assays. Because of low endogenous levels of BLIMP1 in HCT116 cells, ChIP-qPCR was not sensitive enough to locate BLIMP1 binding within the p53 promoter in control vector-transfected cells (Fig. 5D). However, ectopically expressing BLIMP1 in HCT116 cells resulted in sufficient enrichment of



**Fig. 5.** BLIMP1 directly suppresses *p53* transcription by binding to its promoter. (A) Overexpression of BLIMP1 suppressed *p53* promoter activity. Vectors expressing BLIMP1 (pLPCX-BLIMP1) and pGL3-p53luc were transiently cotransfected into HCT116 cells, and luciferase activity was measured 30 h after transfection. (B) Mapping of BLIMP1 binding across the *p53* promoter using 10 primer sets. The locations of the amplified products of the primer sets used to detect the ChIP-enriched DNA fragments are shown within the context of the genomic structure of human *p53*. Amplicons are numbered in order relative to their sites along the gene. ChIP assays were performed by using HCT116 cells transiently transfected with pLPCX-BLIMP1 (C) or vector pLPCX alone (D). Real-time PCR was performed with immunoprecipitated chromatin fragments obtained by using anti-BLIMP1 antibody or an irrelevant antibody (anti-GFP) as control. A known BLIMP1 binding site in the promoter region of *c-Myc* gene was amplified as a positive control for the ChIP assays. (E) A model for the negative feedback loop between *p53* and BLIMP1. The loop creates a circuit composed of BLIMP1, whose mRNA synthesis is influenced by the activation of *p53*, and this in turn results in the alteration of *p53* activity in cells.

BLIMP1–DNA complexes. Ten pairs of primers, located sequentially along the proximal promoter, first exon, and intron 1 of *p53* were used to quantify the ChIP-enriched DNA by real-time PCR (Fig. 5B). A peak representing BLIMP1 binding was observed ( $\approx 8$ -fold above background) at a region very close to the transcriptional start site of *p53* (Fig. 5C). Identical results were obtained when we used another polyclonal antibody raised against a different epitope of BLIMP1 (data not shown). ChIP using a control antibody of either GFP or E2F1 showed no significant enrichment over the entire surveyed region (Fig. 5C and SI Fig. 9B). In summary, a ChIP assay shows that BLIMP1 binds to a specific region within the *p53* loci. Together with BLIMP1 suppression of *p53* promoter activity, these data provide strong evidence that *p53* is a direct transcription target of BLIMP1 repression.

## Discussion

Our findings have uncovered a crucial role for BLIMP1 in the control of cell proliferation and survival through the negative regulation of *p53* at the transcriptional level. The results presented here highlight a previously unrecognized link between BLIMP1 and *p53*-mediated response. The data demonstrate that the expression of *p53* is suppressed by BLIMP1 in normal unstressed cells and that release of this repression leads to growth arrest or apoptosis.

A number of E3 ubiquitin ligases, such as Mdm2 (29, 30), Pirh2 (31), and Cop1 (32), have been shown to be transcriptionally regulated by *p53* and form an autoregulatory loop with *p53*. To our knowledge, our results show for the first time that *p53* expression is down-regulated by a transcriptional repressor that is itself up-regulated by *p53*, thereby forming a negative feedback loop that serves to maintain low *p53* expression and prevent inappropriate activation of a *p53* response in unstressed cells (Fig. 5E). It has been recently reported that *Blimp1*-deficient mice exhibit a severe reduction in the number of PGCs (7, 8). We wish to highlight an interesting correlation between *Blimp1* expression and the proliferation status of PGCs. After the specification event of  $\approx 40$  founder PGCs, a rapid proliferation event is required so that a large mass of PGCs is attained. Intriguingly, the expression of *Blimp1* in

PGCs is first detected at embryonic day 6.5 and remains expressed throughout the phase of proliferation. This observation, together with the findings from our study, hints at the possibility that *Blimp1* expression in PGCs after embryonic day 6.5 keeps PGCs in the proliferative state by maintaining a low level of *p53*. Another group also reported that *Blimp1* knockout mice display apoptosis in branchial arch and heart tissue (8). However, it remains unclear how *Blimp1* deletion triggers apoptosis in these tissues. In line with our observations, we speculate that the apoptosis observed in branchial arch and heart tissue is due to the activation of the *p53* pathway as a consequence of *Blimp1* deletion.

The discovery of frequent inactivation of BLIMP1 in activated B cell-like diffuse large B-cell lymphoma (DLBCL) indicates that BLIMP1 has an important role in the pathogenesis of this lymphoma subtype (33) and possibly functions as a tumor suppressor gene. However, a recent report shows that the presence of variable percentages of BLIMP1-positive tumor cells in approximately half of DLBCL cases (34) and patients with higher BLIMP1 expression are significantly correlated with shorter failure-free survival (34). This raises the possibility that higher BLIMP1 expression could possibly promote the development of lymphoma. Deregulated *BCL-6* expression contributes to lymphomagenesis in part by functional inactivation of *p53* (32). Our finding that BLIMP1 represses *p53* expression suggests that high expression of BLIMP1 might also promote the development of tumor by suppression of *p53* activation. The exact nature of how BLIMP1 contributes to lymphomagenesis is unclear, and it would be of interest to study the relationship between BLIMP1 and *p53* status along with patient survival in DLBCL.

Long-lived plasma cells are critical arm of humoral memory and are the important source of antibodies that protect us for future encounters with the pathogens. Calame and coworkers (35) have previously shown that the expression of *Blimp1* in long-lived plasma cell is indispensable for their long-term maintenance; however, the molecular mechanism of *Blimp1*'s role in long-lived plasma cells is not clear. The finding of *p53* activation after *Blimp1* depletion in this study suggests that one possible mechanism by which *Blimp1*

maintains long-lived plasma cells is by repressing p53 activation that can induce apoptosis and/or growth arrest. Generation of knockout mice deficient for both Blimp1 and p53 might rescue the loss of maintenance of long-lived plasma cells. Recently, two groups demonstrated that Blimp1 is expressed in T lymphocytes and is essential for T cell homeostasis and self-tolerance (11, 12). Interestingly, the group led by Calame (11) showed that CD4<sup>+</sup> CD8<sup>+</sup> (double-positive) thymocytes in mice with T cell-restricted *Blimp1* deletion were severely reduced to ≈36% of the control littermates and that, furthermore, *Blimp1*-deficient double-positive thymocytes were more susceptible to apoptosis (11). The link between BLIMP1 and p53 presented here probably gives some insight into its mechanism. DNA double-strand break is one important process in T cell lineage maturation and is essential for T cell receptor rearrangement. BCL6 is known to suppress p53 expression in germinal-center B cells (33). Similarly, by suppressing p53 expression in thymocytes, Blimp1 may allow these cells to sustain the physiological genomic stress without eliciting a p53 response during expansion. Together with Calame's study (11), we hypothesize that double-positive thymocytes lacking *Blimp1* expression are not able to reach normal numbers because an inappropriate p53 response prevents their proliferative expansion.

## Materials and Methods

**Cell Lines and Transfection.** The human colorectal cancer cell lines *p53*<sup>+/+</sup> and *p53*<sup>-/-</sup> HCT116 were provided by Bert Vogelstein (The Johns Hopkins University). The human fibroblast IMR90 cells were obtained from American Type Culture Collection. siRNA duplexes and plasmids were transiently transfected by using Lipofectamine 2000 following the manufacturer's procedures (Invitrogen).

**RNAi.** siRNA duplexes targeting the *BLIMP1*, *p53*, and GFP mRNAs were chemically synthesized by Dharmacon Research. Their target sequences are as follows: *BLIMP1*, 5'-GATCTGACCGAATCAATG-3' (*BLIMP1* siRNA-1) and 5'-GCACTGCGATGCTATGT-3' (*BLIMP1* siRNA-2); *p53*, 5'-CAGTCTACCTCCCGCCATA-3' (*p53* siRNA-1) and 5'-GAAGAAACCACTGGATGGA-3' (*p53* siRNA-2). The control siRNAs are as follows: *GFP*, 5'-GGCTACGTCCAGGAGCGCACC-3'; *Lamin A/C*, 5'-GGTGGTGACGATCTGGGCT-3'.

For vector-based RNAi (shRNA), pSUPER.puro (Oligo-engine) was used for *BLIMP1* and *Lamin A/C*. The H1 promoter cassette in pLVTH lentivirus vector was replaced by the H1-shRNA

cassette excised from pSUPER-shRNA, generating LV-shRNA (LV-control shRNA, LV-*BLIMP1* shRNA, and LV-*p53* shRNA), respectively.

**BLIMP1 Expression Constructs.** The full-length *BLIMP1* vector was provided by Tom Maniatis (Harvard University), cloned into pLPCX vector (Clontech), and confirmed by sequencing. For *BLIMP1* shRNA-1 immune rescue construct (*BLIMP1*-R), there are six silent mutations (5'-gacctactcgcattaacg-3' as underlined) in the target sequence.

**ChIP Assays.** ChIP assays were carried out as described (24, 34, 36). Briefly, cells were cross-linked with 1% formaldehyde for 10 min at room temperature, and formaldehyde was then inactivated by the addition of 125 mM glycine. Chromatin extracts containing DNA fragments with an average size of 500 bp were immunoprecipitated by using anti-BLIMP1 polyclonal antibody (ab13700; Abcam), control GFP antibody (Santa Cruz Biotechnology), or control E2F1 antibody (Upstate Biotechnology). For all ChIP experiments, qPCR analyses were performed in real time by using ABI PRISM 7900 Sequence Detection System and SYBR Green Master Mix. Threshold cycles (Ct) were determined for both immunoprecipitated DNA and a known amount of DNA from the input sample for different primer pairs. Relative occupancy values (also known as fold enrichments) were calculated by determining the IP efficiency (ratios of the amount of immunoprecipitated DNA to that of the input sample) and normalized to the level observed at a control region, which was defined as 1.0. The control region is a 279-bp region on chromosome 22 and is amplified by using the primers 5'-GGACTCGGAAGAGGTTACCTTCGG-3' and 5'-GTCGCCTCCGCTTGCTGAACTCAATGC-3'. For all of the primers used, each gave a single product of the right size, as confirmed by agarose gel electrophoresis and dissociation curve analysis.

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