Published in final edited form as:

Leuk Res. 2013 December ; 37(12): 1680–1689. doi:10.1016/j.leukres.2013.09.009.

Homeostatic defects in B cells deficient in the E3 ubiquitin ligase ARF-BP1 are restored by enhanced expression of MYC

Chen-Feng Qi^{1,*}, Ruihua Zhang², Jiafang Sun¹, Zhaoyang Li³, Dong-Mi Shin^{1,4}, Hongsheng Wang¹, Alexander L. Kovalchuk¹, Tomomi Sakai¹, Huabao Xiong², Ning Kon², Wei Gu^{5,6}, and Herbert C. Morse III^{1,*}

¹Virology and Cellular Immunology Section, Laboratory of Immunogenetics, National Institutes of Allergy and Infectious Diseases, National Institutes of Health, Rockville, MD

²Immunology Institute, Department of Medicine, Mount Sinai School of Medicine, New York, NY

³Oncology Program, University of Maryland Greenebaum Cancer Center, Baltimore, MD

⁴Department of Food and Nutrition, Seoul National University, Seoul, Korea

⁵Institute for Cancer Genetics, Columbia University College of Physicians and Surgeons, New York, NY

⁶Department of Pathology and Cell Biology, Columbia University College of Physicians and Surgeons, New York, NY

Abstract

The E3 ligase ARF-BP1 governs the balance of life and death decisions by directing the degradation of p53 and enhancing the transcriptional activity of MYC. We find B cells selectively deficient in ARF-BP1 have many defects in developing and mature B cells associated with increased expression of p53 and reduced expression of Myc. Overexpression of Myc results in suppression of p53 and complete reversal of defects induced by ARF-BP1 deficiency. These

© 2013 The Authors. Published by Elsevier Ltd. All rights reserved.

*Corresponding authors, Herbert C. Morse III, MD, Virology and Cellular Immunology Section, Laboratory of Immunogenetics, National Institute of Allergy and Infectious Diseases, National Institutes of Health, 5640 Fishers Lane, Room 1421, Rockville, MD 20852, Tel: 301-496-6379, Fax: 301-402-0077, hmorse@niaid.nih.gov, Chen-Feng Qi, MD, PhD, Head of the Pathology Core, Senior Pathology Research Specialist, Laboratory of Immunogenetics, National Institute of Allergy and Infectious Diseases, National Institutes of Health, 5640 Fishers Lane, Room 1528, Rockville, MD 20852, Tel: 301-402-2698, Fax: 301-402-0077, cqi@niaid.nih.gov.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Author contributions

Conception and design: CF Qi, HC Morse; Development of methodology: CF Qi, R Zhang, J Sun, Z Li, DM Shin, H Wang, A Kovalchuk, T Sakai, N Kon; Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): CF Qi, R Zhang, J Sun, Z Li, DM Shin, H Wang, A Kovalchuk, T Sakai, N Kon; Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): CF Qi, R Zhang, J Sun, Z Li, DM Shin, H Wang, A Kovalchuk, T Sakai, N Kon, H Xiong, W Gu, HC Morse; Writing, review, and/or revision of the manuscript: CF Qi, HC Morse; Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): CF Qi, R Zhang, J Sun, Z Li, DM Shin, H Wang, A Kovalchuk, T Sakai, N Kon.

Conflict of interest disclosure

The authors declare no competing financial interests.

findings indicate that the dynamic balance between MYC and p53 required for normal B cell maturation and function is finely tuned and critically dependent on the activities of ARF-BP1.

Keywords

ARF-BP1; MYC; p53; B cell development

Introduction

ARF-BP1, previously called MULE and HUWE1, is a member of the HECHT-type E3 family of ligases and was initially found to have a crucial role in cancer.[1, 2] Subsequent studies showed that it contributed to fine-tuning of the balance of mechanisms governing proliferation and apoptosis in neuronal cells.[3–5] This concept has been reinforced and extended by more recent studies using conditional alleles of ARF-BP1 that identified critical roles in novel molecular networks governing cellular homeostasis in pancreatic β cells[6] and B lymphocytes.[7] Most recently, it was suggested that ARF-BP1 may be operative in B cell transformation as part of a multimolecular complex that includes CTCF, MYC, ARF and p53.[8]

The contributions of ARF-BP1 to the maintenance of cellular homeostasis have been clearly associated with its ability to bind and ubiquitylate p53, resulting in its proteosomal degradation,[1] and to ubiquitylate MYC through a lysine 63-linked polyubiquitin chain that enhances its transcriptional activity.[9] Interestingly, it was also found that ARF-BP1 also ubiquitinates itself but is stabilized via deubiquitination by the ubiquitin specific protease, USP4.[10] Analyses of mice with B cells selectively deficient in ARF-BP1 from the earliest stages of B cell development uncovered a large number of defects in B cell development and function.[7] As expected, ARF-BP1-deficient B cells showed signs of increased p53 activity as indicated by increased levels of transcripts for a series of p53 target genes, even though p53 proteins levels were undetectable in cells of mutant or control mice.[7] Mice that were doubly deficient in B cells only for both ARF-BP1 and p53 showed that genetic ablation of p53 resulted in restoration of most but not all of these defects. Mice with doubly deficient B cells continued to exhibit a marked depletion of peritoneal B-1a cells, had reduced serum Ig levels and were deficient in CSR.[7]

The understanding that ARF-BP1 plays an important role in governing the activity of MYC as well as p53 prompted us to ask if some or all of the defects identified in mice with ARF-BP1-deficient B cells might also be normalized by enhancing MYC activity. To answer this question, we introduced a MYC transgene spliced into the Ig heavy chain locus with expression controlled by the E μ enhancer that results in high-level MYC expression throughout B cell development.[11] Studies of these mice revealed a near-complete disappearance of all the B cell abnormalities otherwise present in mice with ARF-BP1-deficient B cells.

Methods

Mice

C57BL/6 (B6) mice bearing a conditional *ARF-BP1* allele with exon 11 flanked by loxP sites (*ARF-BP1^{FLY}*) were generated as described previously[6] to allow deletion of exon 11 on expression of a Cre recombinase. Mice with a B cell-specific deficiency for ARF-BP1 (conditional knockout mice, CKO) were generated by crossing *ARF-BP1^{FLY}* with mice heterozygous for a *Cd19-Cre* knock-in allele,[12] backcrossed onto a B6 background and then intercrossing to generate mice with B cells sufficient or deficient in ARF-BP1. Specific deletion of *ARF-BP1* in B cells was documented by qPCR analyses showing a lack of transcripts in B cells but normal levels of transcripts in thymocytes (Figure S1A). In addition, western blot analyses showed that purified splenic B cells from ARF-BP1 CKO CD19-Cre mice had no detectable ARF-BP1 protein while levels in thymocytes of the same mice were normal (Figure S1B). Mice with a human *MYC* cDNA spliced into the IgH locus under the regulatory control of the intronic Eμ enhancer, described previously[11] and abbreviated here as iMYC, were then crossed with the above mice to generate animals with ARF-BP1-deficient B cells that express *MYC* transcripts and protein at high levels. Genotyping was performed using the primers described previously.[6] At necropsy, selected tissues were fixed in formalin for subsequent staining with H&E and immunohistochemical studies, and frozen in OCT for staining and analyses by confocal microscopy. Other samples were frozen for later preparation of RNA. All mouse studies were performed under Protocol LIG14E approved by the NIAID ACUC.

Protein extraction, western blotting, and immunoprecipitation

Nuclear and cytoplasmic protein fractions were extracted as previously described.[13] For western blotting, 30μg of protein per lane was separated on 10% sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) and transferred to a nitrocellulose membrane (Amersham). The membrane was blocked and then incubated with anti-ARF-BP1 (Neomarkers,) or other antibodies for 1 hr at room temperature. After washing, horseradish-peroxidase-conjugated donkey anti-rabbit or anti-mouse secondary antibody (Amersham) was added at a 1:1000 dilution and the mixture incubated for 1 hour at room temperature. Reactive proteins were detected by the enhanced chemiluminescence (ECL) system (GE Healthcare, Piscataway, NJ) according to the manufacturer's instructions.

For immunoprecipitation studies, 300μg of protein in 500μl of 2X immunoprecipitation buffer (2% Triton X-100, 300 mM NaCl, 20 mM Tris [pH7.4], 2 mM EDTA, 2 mM EGTA, pH8.0, 0.4 mM sodium orthovanadate, 0.4 mM PMSF and 1.0% NP-40) and 400μl H₂O were incubated with anti-ARF-BP1 or other antibodies on beads (NeoMarkers), according to the manufacturer's instructions. The immune complexes were collected and analyzed by SDS-PAGE as described above.

Flow Cytometry

Cells were prepared and stained using a standard procedure as previously reported.[14] For cell proliferation assays, splenic B cells were enriched using anti-CD43 magnetic beads (Life Technologies, Grand Island, NY) according to manufacturer's instructions. The cells

were labeled with carboxyfluoresceinsuccinimidyl ester (CFSE) and cultured with complete RPMI1640 medium containing 10% FBS plus 10 µg/ml of anti-IgM (F(ab')₂ (Jackson ImmunoResearch Laboratories, West Grove, PA), 1 µg/ml of anti-CD40 (SouthernBiotech, Birmingham, AL), 10 ng/ml of IL-4 (Peprotech, Rocky Hill, NJ), 20 µg/ml of LPS (Sigma-Aldrich, St. Louis, MO), or 1 µg/ml of CpG (InvivoGen, San Diego, CA) for 3 days. For class switch recombination assays, purified B cells were cultured with 10 µg/ml of LPS plus 10 ng/ml of IL-4 for 4 days. Cells were then stained and analyzed by flow cytometry.

Serum immunoglobulin levels

Circulating serum IgM and IgG titers in WT, ARF-BP1 and ARF-BP1+iMYC mice were detected by ELISA. Briefly, 100ul diluted affinity purified mouse IgM or IgG coating antibody were added to each well for 1 hour at room temperature. After addition of blocking solution, 100ul of sample or standard were added to well, then 100ul horseradish-peroxidase-conjugated mouse IgM detection antibody (Bethyl Laboratories, Montgomery, TX) or horseradish-peroxidase-conjugated mouse IgG detection antibody (Bethyl Laboratories) were added. Wells were washed 5 times after each step. Results are expressed as ng/mL following the value of OD450 and calculated according to a standard curve.

RNA isolation and analysis by microarray and quantitative RT-PCR (qPCR)

RNA prepared from frozen lymphoid tissues or lymphocyte suspensions using the RNeasy Mini kit (Qiagen, Germantown, MD) was examined for quality using a Bioanalyzer (Agilent, Santa Clara, CA). cDNA was synthesized according to the manufacturer's protocol (MessageSensor RT kit, Life Technologies).

Sample preparation and hybridization were performed as described.[15] After the raw data were normalized with loess smoothing function, significant genes were identified by significance analysis of microarrays (SAM) and gene set enrichment analysis (GSEA) was performed. Reverse transcription was performed using 1 µg of RNA, random hexamer primers and Superscript II (Life Technologies). qPCR reactions were performed in a mix of 10 µl reaction mixtures containing 50ng of cDNA, 2X SYBR Green PCR Master Mix (Life Technologies), and 3 µM of each primer. The reaction mixture was denatured and incubated using an ABI PRISM 7900HT (Life Technologies). All samples were tested in triplicate. The comparative CT method was used for quantification of gene expression. Statistical analysis was performed using SDS v2.1 software (Life Technologies).

Results

Developmental and homeostatic defects of B cells in ARF-BP1 CKO mice are normalized by overexpression of Myc

Previous studies of mice with a B cell-specific defect in ARF-BP1 expression identified developmental blocks in the generation of pre-B and mature B cell populations in the bone marrow (BM) due to increased apoptosis in these populations that were restored to normal by the simultaneous abrogation of p53 expression.[7] To determine if overexpression of MYC might have similar or different effects, we first examined B cell development in the BM using the gating parameters illustrated in Figure 1A to study mice of four "B cell

genotypes” - +/+, *ARF-BP1^{FL/Y}*/Cd19-Cre (*ARF-BP1* CKO), iMYC, and *ARF-BP1* CKO + iMYC. The frequencies of pro-B and immature B cells in the BM did not differ significantly for mice of any of the four genotypes (Figure 1B). The frequencies of pre-B cells were slightly but not significantly decreased in *ARF-BP1* CKO mice but were markedly increased in both iMYC and *ARF-BP1* CKO + iMYC mice. Finally, while the frequency of mature recirculating B cells was reduced by about 50% in the BM of *ARF-BP1* CKO mice, and even more so for iMYC mice, the frequency in *ARF-BP1* CKO + iMYC mice did not differ significantly from that of +/+ mice (Figure 1B). These results indicated that overexpression of MYC more than compensated for the reduced levels of pre-B cells seen in the BM of *ARF-BP1* CKO mice and resulted in normalization of the frequency of mature BM B cells.

We next performed histologic and immunohistochemical analyses of spleens from +/+ and *ARF-BP1* CKO mice (Figure S2). Staining with H&E showed marked reductions in the size of the white pulp, and germinal centers (GCs), identified by staining with PNA, were fewer in number and size in spleens of *ARF-BP1* CKO mice. Although the distribution and organization of B220⁺ B cells and CD3⁺ T cells in splenic follicles was normal, the size of the B cell population was clearly reduced while the relative proportion of T cells to B cells was seemingly maintained (Figure S2).

Flow cytometric studies were performed with spleen cells from mice of all four “B cell genotypes” using the gating parameters shown in Figure 2A. The results showed that both the frequency and total numbers of splenic B cells were significantly reduced in *ARF-BP1* CKO mice (Figure 2A-C). While the reduced frequency of B cells was not restored by overexpression of MYC the marked reduction in the total number of splenic B cells was almost totally compensated in *ARF-BP1* CKO + iMYC mice (Figure 2C). As described previously, this defect in splenic B cell numbers was also erased by abolishing B cell expression of p53.[7] Interestingly, the normally small splenic population of B-1a cells was further reduced in spleens of *ARF-BP1* CKO mice, but was completely normalized by overexpression of MYC (Figure 2B,C). This effect can probably be explained by the striking increase in B-1a cells seen in MYC TG mice. These data demonstrated that the impaired homeostasis of splenic B cells caused by a B cell-specific deficiency in *ARF-BP1* could be restored to normal either by overexpressing MYC as well as by abolishing expression of p53.

Flow cytometric analyses of peritoneal B cell populations using the gating parameters shown in Figure 3A revealed that a B cell-specific deficiency in *ARF-BP1* was associated with significant reductions in the frequencies of total B cells and B-1a cells but not B-1b or B-2 cells (Figure 3A,B). The frequencies of total peritoneal B cells were significantly increased for iMYC mice due almost entirely to greatly increased proportions of B-1a cells, a finding reminiscent of the increased frequencies of B-1a cells in the spleens of iMYC mice. Remarkably, the frequencies of total B cells and B-1a cells were normalized in the peritoneal cavities of *ARF-BP1* CKO + iMYC mice (Figure 3A,B). It is noteworthy that the frequencies of B-1a cells in the peritoneal cavities of *ARF-BP1* CKO mice with p53-deficient B cells were higher than for +/+ mice[7] indicating an important interplay between the levels of MYC, p52 and *ARF-BP1* in controlling the size of the peritoneal B-1a B cell population.

Taken together, our results and those of previous studies of *ARF-BP1* CKO mice[7] showed that abnormalities in early B cell development and homeostasis of splenic B cells were similarly normalized by deletion of p53 and overexpression of MYC while altered homeostasis of peritoneal B cells was nearly normalized only by overexpressing MYC (Figure 7B).

Overexpression of MYC overcomes defective in vitro proliferative responses and class switch recombination of splenic B cells from *ARF-BP1* CKO mice

Paralleling a previous study [7], analyses of purified splenic B cells from *ARF-BP1* CKO mice revealed significantly reduced in vitro proliferative responses to stimulation with anti-IgM or anti-IgM plus anti-CD40 (Figure 4A). Proliferative responses to stimulation with the TLR4 ligand, LPS, and the TLR9 ligand, CpG, were also markedly impaired. The responsiveness of B cells from iMYC mice to all stimuli but CpG was similar to that of cells from +/+ mice. Interestingly, the impaired proliferative responses of B cells from *ARF-BP1* CKO mice to stimulation with anti-IgM plus anti-CD40, LPS and CpG were restored by overexpression of iMYC while the response to stimulation with anti-IgM alone was only partially restored (Figure 4A). These data demonstrated that high-level expression of MYC compensated for impaired signaling responses downstream of the BCR, similar to the effects of abrogating p53 expression, as well as downstream of TLR4 and TLR9.

We continued by investigating the responsiveness of purified splenic B cells to induction of CSR to IgG1 following in vitro stimulation with LPS + IL4, a response that was previously shown to be greatly reduced for cells from *ARF-BP1* CKO mice[7]. These results were again paralleled in our studies (Figure 4B). Although the ability of B cells from *ARF-BP1* CKO mice to undergo CSR was not restored by B cell-specific deletion of p53[7], it was fully compensated by overexpression of MYC (Figure 4B). The fact that activated B cells require at least two rounds of cell division to undergo CSR to IgG[16] suggests that the proliferative stimulus provided by heightened expression of MYC contributes to this recovery, a suggestion supported by the observation of increased CSR by iMYC B cells stimulated with LPS + IL4 (Figure 4B).

Reduced Ig levels in sera from *ARF-BP1* CKO mice are normalized by overexpression of MYC

An additional comparison between our studies and the prior report is based on analyses of serum levels of IgM and IgG. As reported previously[7], levels of both Ig isotypes were significantly reduced in sera from *ARF-BP1* CKO mice (Figure 4C). While normal levels were not restored in mice with B cells rendered deficient in p53[7] levels of both IgM and IgG were significantly restored towards normal in sera of *ARF-BP1* CKO + iMYC mice (Figure 4C).

Altered gene expression patterns in B cells from *ARF-BP1* CKO mice

To develop a mechanistic basis for understanding the B cell defects characteristic of *ARF-BP1* CKO mice, we performed microarray-based gene expression profiling of purified splenic B cells from WT and CKO mice. A t-test analysis identified 512 differentially expressed genes (Table S1). A principal component analysis (Figure S3A) demonstrated that

the samples from mice of the two genotypes comprised readily distinguishable populations. Categorization of these differentially expressed genes by Gene Ontology (GO) identified significant associations with a range of biologic functions including hematopoietic development and function, cell death, cell cycle regulation and DNA replication and repair among others (Figure S3B). We chose to focus on cell cycle regulation because of the critical involvement of both MYC and p53 in this process and the understanding that both are influenced by the activities of ARF-BP1. A hierarchical clustering algorithm was used to group the cell cycle-related genes that significantly distinguished the two groups (Figure 5). Among other genes, the levels of *Myc* transcripts were significantly lower in B cells from *ARF-BP1* CKO mice (Figure 5) in keeping with the finding that MYC is stabilized by ARF-BP1-mediated ubiquitylation.

To determine if these differences in transcript levels would also be mirrored in changes in gene expression at the protein level, we performed western blot analyses of protein extracts from purified B cells from WT and *ARF-BP1* CKO mice (Figure 6A). The results showed that levels of FOS, KLF4, JUNB, PIM3 and, as expected, ARF-BP1 and MYC, were significantly lower in cells from CKO mice. Parallel studies of B cells purified from spleens of *iMYC* and *ARF-BP1* CKO + *iMYC* mice showed that overexpression of MYC resulted in partial to near complete restitution of the defects in expression of FOS, KLF4, JUNB and PIM3 seen in the CKO mice.

MYC and p53 are master gene regulators controlling multiple cellular pathways by activating or repressing downstream genes. p53 has been shown to be a negative regulator of MYC by inducing miR-145, which directly targets MYC [17]. However, very recent studies suggest that persistent expression of MYC results in a constitutive proliferative state that reduces the responsiveness of cells to signals, such as those initiated by p53, that normal drive growth arrest and terminal differentiation [17,18]. These considerations left open the possibility that the restorative influences of MYC overexpression might be due to effects of MYC on expression of p53 and/or its target genes. To examine this possibility, we used qPCR to quantify transcript levels for *MycTp53* and the p53 target genes *BaxMdm2Pmaip1* (Noxa)*Cdkn1a* (p21) and *Bbc3* (Puma) in purified splenic B cells from mice of all four genotypes. The results of these studies (Figure 6B) showed that overexpression of MYC in B cells from CKO mice resulted in significant reductions in expression of *TP53* and equally marked reductions in expression of all the p53 target genes tested except for p21, which was essentially unaffected. A remarkably similar result – marked reduction in expression of Bax, Mdm2, Noxa and Puma, but not p21 – was also observed following genetic ablation of p53 [7]. These results indicated the ability of MYC to almost completely reverse nearly all the B cell abnormalities identified in *ARF-BP1* CKO mice could be ascribed in large part to its suppression of *TP53* transcription.

Discussion

A rapidly expanding series of recent investigations into the biology of ARF-BP1 has identified a wide range of functions in different normal cell types and cancers. Much of the attention in these studies has been on the role of ARF-BP1 in governing the ubiquitylation and stability of p53 and the inhibition of ARF-BP1 activity mediated by ARF (Figure 7A).

MYC has been identified as a p53-independent substrate of ARF-BP1 with the interaction resulting in addition of a K63-linked Ub chain thereby regulating a switch between the repressed and activated states of the MYC protein (Figure 7A).[9]

Previous studies showed ablation of p53 expression in B cells partially restored the developmental and functional defects in ARF-BP1 deficient mice (summarized in Figure 7B).[7] Our results complement those of a previous study[7] in demonstrating that ARF-BP1 is critically involved in the regulation of B cell differentiation and function by controlling the expression and activities of both MYC and p53. B cells of mice deficient in ARF-BP1 exhibited enhanced expression of p53 and reduced levels of MYC and correction of either abnormality – by p53 deletion or overexpression of MYC - resulted in reversal of most B cell defects but with some exceptions. Reduced proliferative responses, CSR and serum Ig levels were not corrected by deletion of p53 but were largely normalized by overexpression of MYC (Figure 7B). The only proliferative response that was not fully restored was the response to crosslinking the B cell receptor (BCR) with anti-IgM. The addition to anti-IgM of CD40L, which activates the non-canonical NF- κ B signaling pathway, fully normalized the response suggesting cooperativity between signaling initiated by BCR ligating an CD40 engagement. The ability of MYC to overcome the proliferative defect can be ascribed to the MYC-driven induction of several genes involved in cell cycle regulation (Figure 6A) in addition to MYC-induced suppression of *Tp53* transcription (Figure 6B).

Previous studies indicated that some of the effects of p53 deletion could be ascribed in part to the reversal of blocks to B cell development and homeostasis caused by p53-driven apoptosis[7]. However, the exact mechanisms by which enhanced expression of MYC resulted in disappearance of the many B cell lineage defects beyond downregulation of p53 and its target genes remains to be determined. Very recent studies suggest that persistent expression of MYC results in a constitutive proliferative state that reduces the responsiveness of cells to signals, such as those initiated by p53, that normally drive growth arrest and terminal differentiation.[18, 19] Given the large numbers of genes affected at the transcriptional levels by both MYC and p53, crosstalk between the signaling pathways they govern will unavoidably be very noisy. This will likely have contributed to the apparent redundancy of activities they exhibit in the regulation of B cell development and function identified here and previously[7]. In this regard, it is noteworthy that neither p53 deletion nor MYC overexpression overcame the elevated expression of p21 either at the transcriptional level[7] (Figure 6) or the protein level (not shown). Determinants of p21 transcription, mRNA stability, translation and protein stability and activity are known to be complex [20] but how they subvert control in the absence or near absence of p53 remains to be determined.

The task of identifying critical target genes of MYC has become more complex than was previously appreciated in view of recent findings indicating that MYC does not act to govern the activity of specific genes in an on or off manner, but rather functions as a nonlinear amplifier of expression from actively transcribed genes[18, 19]. Since these aspects of MYC activity were garnered primarily from studies of ES and tumor cells as well as activated B cells and some effects of ARF-BP1 are manifested in a cell type-specific

manner, it will be important in the future to pursue these issues by analyzing purified resting and activated B cell subpopulations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported in part by the Intramural Research Program of the NIH, National Institute of Allergy and Infectious Diseases and NIH grant RO1CA169246 to WG. We are indebted to Dr. Alfonso Macias for his contributions to animal management and Dr. Zohreh Naghashfar for her contributions to microarray studies.

References

1. Chen D, Kon N, Li M, Zhang W, Qin J, Gu W. ARF-BP1/Mule is a critical mediator of the ARF tumor suppressor. *Cell*. 2005; 121:1071–1083. [PubMed: 15989956]
2. Zhong Q, Gao W, Du F, Wang X. Mule/ARF-BP1, a BH3-only E3 ubiquitin ligase, catalyzes the polyubiquitination of Mcl-1 and regulates apoptosis. *Cell*. 2005; 121:1085–1095. [PubMed: 15989957]
3. Medrano S, Scrabble H. Maintaining appearances--the role of p53 in adult neurogenesis. *Biochemical and biophysical research communications*. 2005; 331:828–833. [PubMed: 15865938]
4. Zhao X, D DA, Lim WK, Brahmachary M, Carro MS, Ludwig T, et al. The N-Myc-DLL3 cascade is suppressed by the ubiquitin ligase Huwe1 to inhibit proliferation and promote neurogenesis in the developing brain. *Developmental cell*. 2009; 17:210–221. [PubMed: 19686682]
5. Zhao X, Heng JI, Guardavaccaro D, Jiang R, Pagano M, Guillemot F, et al. The HECT-domain ubiquitin ligase Huwe1 controls neural differentiation and proliferation by destabilizing the N-Myc oncoprotein. *Nature cell biology*. 2008; 10:643–653.
6. Kon N, Zhong J, Qiang L, Accili D, Gu W. Inactivation of arf-bp1 induces p53 activation and diabetic phenotypes in mice. *J Biol Chem*. 2012; 287:5102–5111. [PubMed: 22187431]
7. Hao Z, Duncan GS, Su YW, Li WY, Silvester J, Hong C, et al. The E3 ubiquitin ligase Mule acts through the ATM-p53 axis to maintain B lymphocyte homeostasis. *J Exp Med*. 2012; 209:173–186. [PubMed: 22213803]
8. Qi CF, Kim YS, Xiang S, Abdullaev Z, Torrey TA, Janz S, et al. Characterization of ARF-BP1/HUWE1 Interactions with CTCF, MYC, ARF and p53 in MYC-Driven B Cell Neoplasms. *Int J Mol Sci*. 2012; 13:6204–6219. [PubMed: 22754359]
9. Adhikary S, Marinoni F, Hock A, Hulleman E, Popov N, Beier R, et al. The ubiquitin ligase HectH9 regulates transcriptional activation by Myc and is essential for tumor cell proliferation. *Cell*. 2005; 123:409–421. [PubMed: 16269333]
10. Zhang X, Berger FG, Yang J, Lu X. USP4 inhibits p53 through deubiquitinating and stabilizing ARF-BP1. *The EMBO journal*. 2011; 30:2177–2189. [PubMed: 21522127]
11. Park SS, Kim JS, Tessarollo L, Owens JD, Peng L, Han SS, et al. Insertion of c-Myc into Igh induces B-cell and plasma-cell neoplasms in mice. *Cancer Res*. 2005; 65:1306–1315. [PubMed: 15735016]
12. Rickert RC, Roes J, Rajewsky K. B lymphocyte-specific, Cre-mediated mutagenesis in mice. *Nucleic Acids Res*. 1997; 25:1317–1318. [PubMed: 9092650]
13. Qi CF, Xiang S, Shin MS, Hao X, Lee CH, Zhou JX, et al. Expression of the cyclin-dependent kinase inhibitor p27 and its deregulation in mouse B cell lymphomas. *Leuk Res*. 2006; 30:153–163. [PubMed: 16122798]
14. Wang H, Ye J, Arnold LW, McCray SK, Clarke SH. A VH12 transgenic mouse exhibits defects in pre-B cell development and is unable to make IgM+ B cells. *J Immunol*. 2001; 167:1254–1262. [PubMed: 11466341]

15. Shin DM, Shaffer DJ, Wang H, Roopenian DC, Morse HC 3rd. NOTCH is part of the transcriptional network regulating cell growth and survival in mouse plasmacytomas. *Cancer Res.* 2008; 68:9202–9211. [PubMed: 19010892]
16. Hodgkin PD, Lee JH, Lyons AB. B cell differentiation and isotype switching is related to division cycle number. *J Exp Med.* 1996; 184:277–281. [PubMed: 8691143]
17. Sachdeva M, Zhu S, Wu F, Wu H, Walia V, Kumar S, et al. p53 represses c-Myc through induction of the tumor suppressor miR-145. *Proceedings of the National Academy of Sciences of the United States of America.* 2009; 106:3207–3212. [PubMed: 19202062]
18. Lin CY, Loven J, Rahl PB, Paranal RM, Burge CB, Bradner JE, et al. Transcriptional amplification in tumor cells with elevated c-Myc. *Cell.* 2012; 151:56–67. [PubMed: 23021215]
19. Nie Z, Hu G, Wei G, Cui K, Yamane A, Resch W, et al. c-Myc is a universal amplifier of expressed genes in lymphocytes and embryonic stem cells. *Cell.* 2012; 151:68–79. [PubMed: 23021216]
20. Jung YS, Qian Y, Chen X. Examination of the expanding pathways for the regulation of p21 expression and activity. *Cellular signalling.* 2010; 22:1003–1012. [PubMed: 20100570]

A

B

Figure 1.

Effects of a B cell specific deficiency in ARF-BP1 on early B cell development in the bone marrow. (A) The frequencies of cells in the pro-B, pre-B, immature B and mature B cell compartments were determined for mice of the indicated genotypes using the gating scheme shown here. (B) The frequencies of cells in each of these compartments were determined for 3–5 individual mice. Data indicated the mean $\pm$ S.E.M. NS, not significant; *, $P < 0.05$.

Figure 2.

Effects of a B cell specific deficiency in ARF-BP1 on the distribution of B cell subsets in the spleen. (A) The frequencies of total B cells (left side), transitional (T1, T2, T3), marginal zone (MZ), follicular (FO) and B-1a cells were determined for mice of the indicated genotypes using the gating schemes shown here. (B) The frequencies of cells in each of these compartments were determined for 3–5 individual mice. Data indicated the mean $\pm$ S.E.M. NS, not significant; *, $P < 0.05$.

A **B** **Figure 3.**

Effects of a B cell specific deficiency in ARF-BP1 on the distribution of B cell subsets in the peritoneal cavity. (A) The frequencies of total B cells and the B-1a, B-1b, and B2 subsets was determined for mice of the indicated genotypes using the gating scheme shown here. (B) The frequencies of cells in each of these compartments were determined for 3–5 individual mice. Data indicated the mean $\pm$ S.E.M. NS, not significant; *, $P < 0.05$.

Figure 4.

Effects of a B cell specific deficiency in ARF-BP1 on proliferative responses, induction of class switch recombination of purified B cells, and serum immunoglobulin levels. (A) Purified B cells from mice of the indicated genotypes were labeled with CFSE and cultured in medium alone or in the presence of the various stimulants for 72h before being analyzed by flow cytometry. Numbers indicate the frequency of cells that had undergone division. (B) Purified B cells from mice of the indicated genotypes were stimulated in vitro with LPS or LPS + IL4 for 72h and then analyzed by flow cytometry to identify class switched IgM⁺

IgG1⁺ cells. The numbers indicate the frequencies of switched cells. (C) Levels of IgM (a) and total IgG (b) in sera of mice of the indicated genotypes determined by ELISA.

Figure 5.
Analyses of gene expression in spleens of ARF-BP1 CKO and wild type mice. Hierarchical clustering of differentially expressed genes.

A

C

Figure 6.

A. Western blot analyses of cell cycle-related and other genes in purified splenic B cells of 8 week old WT, ARF-BP1 CKO and ARF-BP1 CKO + iMYC and iMYC mice. B. Quantitation by qRT-PCR of transcript levels for *Myc* and *p53*-regulated genes in purified splenic B cells of mice of all 4 B cell "genotypes".

A

B

B cell Genotype	ARF-BP1 ^{Fl/Y} /Cd19-Cre	+	+	+
	p53 ^{-/-}	-	+	-
B cell Abnormality	iMYC	-	-	+
	Early B cell development	Decreased	Normalized	Normalized
	Splenic B cell homeostasis	Decreased	Normalized	Normalized
	Peritoneal B-1a cells	Decreased	Decreased	Normalized
	Proliferative response <i>in vitro</i>	Decreased	Normalized	Normalized
	Class switch recombination <i>in vitro</i>	Decreased	Decreased	Normalized
	Serum immunoglobulins	Decreased	Decreased	Normalized

Figure 7. Regulation of p53 and MYC activities by ubiquitylation and effects of ARF-BP1 deficiency in B cells. (A) MYC is polyubiquitylated by ARF-BP1 through K63 linkage leading to its activation while p53 is polyubiquitylated by ARF-BP1 through K48 linkage signaling its degradation. Alternative ubiquitylation pathways for both p53 and MYC mediated by other E3 ligases result in K48-linked Ub chains that signal proteasomal degradation. (B) Mice with a B cell-specific deficiency in ARF-BP1 induced either by expression of a Mb1-Cre [7] or a CD19-Cre (this study) exhibit a range of B cell lineage defects common to mice of both

origins. The effects of abrogating p53 expression [7] or introducing a MYC transgene (this study) resulted in partial or complete normalization of these abnormalities in the two strains.