Inhibition of the Single Downstream Target BAG1 Activates the Latent Apoptotic Potential of MYC[▽]

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Aberrant MYC expression is a common oncogenic event in human cancer. Paradoxically, MYC can either drive cell cycle progression or induce apoptosis. The latent ability of MYC to induce apoptosis has been termed “intrinsic tumor suppressor activity,” and reactivating this apoptotic function in tumors is widely considered a valuable therapeutic goal. As a transcription factor, MYC controls the expression of many downstream targets, and for the majority of these, it remains unclear whether or not they play direct roles in MYC function. To identify the subset of genes specifically required for biological activity, we conducted a screen for functionally important MYC targets and identified *BAG1*, which encodes a prosurvival chaperone protein. Expression of *BAG1* is regulated by MYC in both a mouse model of breast cancer and transformed human cells. Remarkably, *BAG1* induction is essential for protecting cells from MYC-induced apoptosis. Ultimately, the synthetic lethality we have identified between MYC overexpression and *BAG1* inhibition establishes a new pathway that might be exploited to reactivate the latent apoptotic potential of MYC as a cancer therapy.

The MYC oncoprotein is among the most commonly overexpressed proteins in human cancer. However, efforts to target MYC therapeutically have been unsuccessful. Many transforming proteins, including MYC, E2F, Ela, etc., can induce robust apoptosis when expressed in specific cellular contexts. This ability of potent oncoproteins to induce cell death under certain conditions has been termed “intrinsic tumor suppression” (25). This property provides complex multicellular organisms with a mechanism for the selective elimination of malignant cells, thereby protecting the organism as a whole. In principle, successful cancer therapies could be based on reactivation of this apoptotic potential in human tumors in which MYC is overexpressed. To date, these efforts have been hampered by our limited understanding of the biochemical distinction between cells in which MYC drives malignant transformation and those in which it induces apoptosis. Recent evidence that MYC levels in normal cells are well below those required for apoptosis suggests the possibility that clinical ac-

tivation of MYC-induced apoptosis in cancer patients might have a viable therapeutic index (29).

MYC functions in large part as a transcriptional regulator capable of modulating the expression of several thousand downstream genes (reviewed in reference 31). Current models suggest that deciphering the functional relevance of individual downstream targets of MYC will allow advances in our understanding of this previously intractable pathway (15). In an effort to clarify the identities of essential nodes within this large set of targets, we conducted an unbiased screen focused on the selective identification of the subset of downstream MYC targets that transmit critical effector functions. Via this screen, we identified MTA1 as a novel MYC target that is essential for invasive growth of tumor cells (44). We report here the identification of the *BAG1* gene in the same screen for critical MYC effectors. MYC directly binds the *BAG1* locus, and transcription of the *BAG1* gene is regulated by MYC in all human and mouse cell backgrounds examined, including a murine tumor model of MYC-induced lymphoma.

The *BAG1* locus encodes a family of cochaperone proteins important for stabilizing a number of prosurvival client proteins in a wide variety of cell types (41). *BAG1* is frequently overexpressed in human cancer, and high levels of expression have been associated with a poor prognosis (4, 8, 24, 26). Evidence from genetic studies in mice has shown that a 50% reduction in *BAG1* gene dosage results in increased apoptosis in tumor cells and a nearly complete inhibition of lung adenoma progression, without any effect on cell cycle progression

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(17). Given the important role of BAG1 in controlling cell survival pathways, we assessed whether induction of BAG1 by MYC might participate in controlling the decision between transformation and apoptosis. Remarkably, blocking of BAG1 induction by MYC was sufficient to convert cells from MYC-driven proliferation to MYC-induced apoptosis. The identification of a single node regulating the apoptosis-survival balance in MYC-expressing cells may provide a novel point of attack for targeted therapies of cancer.

MATERIALS AND METHODS

Cell culture. Normal human fibroblasts, MCF7 human breast cancer cells, and H1299 human lung cancer cells were from the ATCC. U2OS MYC/estrogen receptor (ER) cells were generously provided by M. Eilers (University of Würzburg). Cell lines were maintained in 10% fetal bovine serum (FBS)–Dulbecco's modified Eagle's medium (DMEM) with 100 μ g/ml penicillin and 100 μ g/ml streptomycin. For fibroblasts, quiescence was achieved by incubation in 0.1% FBS-DMEM for 48 h, and serum stimulation was accomplished by replacing the medium with 10% FBS-DMEM for the indicated time. MYC/ER was activated by adding 4-hydroxytamoxifen (4-OHT; Sigma) to the medium at 200 nM.

Plasmids, transfection, small interfering RNA (siRNA), and viral packaging. MIGR1-based retroviral expression cassettes were generated by insertion of the human *c-myc*/estrogen receptor ligand binding domain fusion cassette (43). The MYC Δ MbII/ER mutant was generated by deletion of amino acids 110 to 150 by use of the QuikChange mutagenesis protocol (Stratagene). For retroviral production, the Phoenix packaging cell line was transfected using calcium phosphate. Viral supernatant was harvested at 48 to 60 h posttransfection and used to infect fibroblasts in the presence of 8 μ g/ml Polybrene. After 2 to 3 days, infected cells were sorted for the presence of green fluorescent protein (GFP). GFP-positive cells were expanded for a limited number of population doublings prior to use in individual experiments.

Lentiviral short hairpin RNA (shRNA) plasmids corresponding to MYC, BAG1, and control luciferase shRNAs were obtained from TRC (Sigma). Lentiviral packaging plasmids (pCMV- Δ R8.2 and pCMV-VSV-G [23]) were cotransfected with shRNA vectors into 293T cells. Viral supernatants were collected, filtered, and added directly to target cells in the presence of 8 μ g/ml Polybrene. Cells were selected with puromycin for 48 h. siRNAs for human MYC (Dharmacon) were transfected at 200 nM in 6-well plates by use of Lipofectamine 2000 (Invitrogen).

mRNA analysis and Western blotting. mRNA was harvested from cells by use of TRIzol (Invitrogen), converted to cDNA by using a high-capacity reverse transcription kit (Applied Biosystems), and subjected to quantitative PCR (qPCR) by using a model 7000 sequence detection system (ABI Prism) and a SYBR green PCR master mix kit (Applied Biosystems). For qPCR of cDNA, primer pairs were designed to generate products of 55 to 75 bp. Primer sequences are available upon request. mRNA levels of samples were normalized to actin levels. qPCR assays were performed in triplicate, and data are expressed as means $\pm$ standard errors.

Western blots were performed as previously described (27). Antibodies used to detect MYC (Santa Cruz) and tubulin (Sigma) were obtained from commercial sources.

Tumor induction. Female bistransgenic MMTV-rtTA; TetO-MYC mice (10, 19) were administered 2 mg/ml doxycycline in drinking water beginning at 6 weeks of age to induce c-MYC expression in mammary tissue, and mice were palpated weekly to identify mammary tumors. Mammary tumors were harvested from mice on doxycycline as well as from tumor-bearing mice following 96 h of doxycycline withdrawal to induce c-MYC downregulation. Tumor tissue was snap-frozen, and RNA was extracted and purified as described above.

ChIP assay. For chromatin immunoprecipitation (ChIP) assays, human fibroblasts were plated on 15-cm dishes, incubated for 24 h, and then deprived of growth factors. After 2 h of serum stimulation, cells were fixed in 1% formaldehyde. Chromatin was sheared to an average size of 500 to 1,000 bp by sonication (6 to 8 times with 10-s pulses at 30% output; Branson model 250 sonifier). Lysates corresponding to 5×10^6 to 10×10^6 cells were rotated at 4°C overnight with 2 μ g of polyclonal antibody specific for MYC (sc-764; Santa Cruz Biotechnology), anti-acetyl histone H3, anti-acetyl histone H4 (Upstate), or normal rabbit IgG. Precipitated DNA fragments were quantified using qPCR as described above.

Apoptosis assays. To quantitate apoptotic cell death, cells were collected by trypsinization, washed with phosphate-buffered saline, and stained using an an-

nexin V-fluorescein isothiocyanate (annexin V-FITC) or annexin V-phycoerythrin-7-amino-actinomycin (annexin V-PE-7AAD) apoptosis detection kit (BD Pharmingen) as described previously (33). Propidium iodide (PI) staining for DNA content was also described previously (40).

RESULTS

Identification of BAG1 gene as a transcriptional target of MYC. The sequence-specific transcription factor encoded by the MYC oncogene has several thousand downstream targets (reviewed in reference 31). Among the important unresolved issues regarding the MYC pathway is which of these targets are essential for specific biological functions. In order to identify the subset of MYC's transcriptional targets that are functionally important, a conditional allele of the MYC gene was expressed in primary diploid human fibroblasts. This allele comprises the sequence for a human c-MYC–estrogen receptor ligand binding domain fusion protein. When this protein is expressed in cells, it is activated posttranslationally by the addition of the synthetic estrogen 4-OHT. This conditional allele of MYC has been used extensively over the past 2 decades (11), allowing investigators to mimic the elevated levels of MYC activity observed in human tumors. In parallel, wild-type and mutant (Δ MbII) versions of this conditional MYC allele were expressed. The Δ MbII mutant is defective for biological functions, including transformation and apoptosis (36, 39), and lacks the ability to recruit acetyltransferase complexes that facilitate the transcription of some target genes (27, 28). The rationale for this strategy was that MYC targets specifically required for biological function would not be induced by the defective mutant, while the majority of nonessential targets would be induced by both wild-type and mutant MYC proteins. This premise is supported by previous empirical studies of MYC targets (6). After assessing the expression of approximately 10,000 human transcripts, 20 were identified and validated as being induced by the conditional allele of wild-type MYC but not by the Δ MbII mutant. As expected, the identification of this small number of differentially expressed targets contrasts with the results of other studies designed to identify all targets of wild-type MYC. These studies have consistently identified several thousand loci as being MYC responsive (31). The overall strategy for the screen and initial validation of MTA1 as an important target have been described previously (43, 44). Expression profiling demonstrated that activation of the wild-type MYC (MYC/ER) allele in primary human fibroblasts caused a consistent 2- to 3-fold induction of BAG1 transcript levels (Fig. 1A). In contrast, no induction of BAG1 was observed upon activation of the biologically defective mutant Δ MbII, despite equal expression of both MYC alleles (44). Quantitative reverse transcription-PCR (qRT-PCR) validated the results observed by expression profiling (Fig. 1B). MYC is a weak transcription factor, and the 2- to 3-fold induction of BAG1 is comparable to that observed for known transcriptional targets of MYC in this system and others. Consistent with this, the MYC target cyclin D2 was induced to levels similar to those of BAG1 upon MYC activation (Fig. 1A). The control of BAG1 by MYC is consistent with information available in public gene expression databases (35). For example, when a panel of 27 human Burkitt's lymphomas, where transformation is the result of MYC translocation, were compared

FIG. 1. Identification of BAG1 as a transcriptional target of the MYC oncoprotein. (A) BAG1 is regulated by MYC in primary human fibroblasts. Expression profiling was performed as described previously (43, 44), using mRNA harvested from primary human fibroblasts expressing the conditional MYC/ER allele (either wild type [wt] or a mutant lacking the MbII domain). Control cells carried the empty vector. MYC was activated by the addition of 4-OHT 4 h prior to harvesting mRNA. Array signals for BAG1 and cyclin D2 are shown for 4-OHT-treated groups. (B) Validation of expression profiling by qRT-PCR. qRT-PCR was performed to assess the levels of BAG1 transcript before and after MYC activation. Actin levels were used to normalize expression patterns between samples. (C) Endogenous MYC regulates BAG1 protein and mRNA levels in H1299 human lung cancer cells and MCF7 human breast cancer cells. For protein analysis, MYC was depleted by use of a lentiviral shRNA targeting MYC. An shRNA targeting luciferase (shLUC) was used as a control. Protein lysates were analyzed by Western blotting for BAG1, MYC, and tubulin, as indicated. In an independent experiment, mRNA levels were quantified in H1299 and MCF7 cells after transfection with siRNA oligonucleotides targeting either MYC or GFP (as a control). Three days after transfection, MYC, BAG1, and ELF1a mRNA levels were determined by qRT-PCR. Transcript levels of these genes in GFP and MYC siRNA-treated cells are shown. Error bars represent standard errors.

to normal B lymphocytes, BAG1 expression was significantly elevated (~ 2 -fold increase; P value, 9.9×10^{-6}) (3). Correlations between increased BAG1 expression and MYC translocation are evident in other data sets as well (20, 22).

To assess whether levels of the endogenous MYC protein are responsible for regulating BAG1 expression, a pool of 4 siRNA oligonucleotides was used to deplete endogenous MYC from the human small cell lung cancer line H1299. BAG1 mRNA and protein levels were then assessed. MYC levels were depleted at both the mRNA and protein levels (Fig. 1C).

qRT-PCR analysis showed that BAG1 mRNA levels were decreased by depletion of MYC, while those of a control gene not regulated by MYC, the ELF1 gene, were not. Accompanying the decrease in BAG1 mRNA was a decrease in the BAG1 protein level (Fig. 1C). BAG1 protein is expressed as three well-characterized isoforms in most human cells, termed BAG1L, BAG1M, and BAG1S (16). All three isoforms were decreased upon loss of MYC (Fig. 1C), consistent with the isoforms being generated by alternative translation start sites rather than by differential promoter utilization. This correlation between MYC expression and BAG1 levels extended to the human breast cancer line MCF7 as well (Fig. 1C). Since a 50% decrease in BAG1 gene dosage is sufficient to block tumorigenesis (17), the effect of MYC on BAG1 levels observed here is likely sufficient to trigger biological defects.

To assess whether the level of MYC present in nontransformed cells is also sufficient to affect BAG1 transcription, MYC was depleted from normal diploid human fibroblasts (Fig. 2A). These studies showed that BAG1 mRNA levels are controlled by MYC, even when the proto-oncogene is expressed at nonpathological levels. Furthermore, activation of MYC/ER with very low doses of 4-OHT was also sufficient to induce BAG1 transcription to a level similar to that of cyclin D2 (Fig. 2B). Thus, different degrees of BAG1 induction do not explain the different outcomes that a cell has in response to low versus high levels of MYC.

Regulation of BAG1 by MYC *in vivo*. While the relevance of our findings with H1299 cells and MCF7 cells is supported by studies implicating MYC expression as important in both lung and breast cancers in humans, a more direct assessment of the correlation between MYC and BAG1 expression *in vivo* was warranted. For this purpose, BAG1 mRNA expression was quantitated for tumors from a well-established mouse model of MYC-driven mammary cancer (10, 19). In these animals, MYC expression is mammary gland specific and is also regulated by a doxycycline-responsive promoter. Tumors were induced in 8 mice, beginning at 6 weeks of age, by activating MYC expression with doxycycline treatment. After the appearance of palpable mammary tumors, 4 mice were withdrawn from doxycycline for 96 h, while MYC activation was maintained in the other 4 mice. Tumors were then harvested, and mRNA levels for BAG1 were determined by qRT-PCR (Fig. 3A), with protein levels determined by Western blotting (Fig. 3B). This analysis demonstrated that BAG1 transcript and protein levels tightly correlate with MYC activity in an *in vivo* setting, with a significant decrease in BAG1 mRNA and protein observed in mice in which MYC activity was inhibited for even a few days. A tight correlation between MYC activity and BAG1 transcript levels was also observed in a mouse model of MYC-driven lymphomagenesis (Fig. 3C). Thus, data from independent *in vivo* models are consistent with the *in vitro* data demonstrating that BAG1 mRNA is regulated by MYC. Furthermore, the conservation of BAG1 as a MYC-regulated gene in both humans and mice suggests that the link may be of functional importance.

MYC binds directly to the BAG1 locus. To assess whether BAG1 is bound directly by the MYC transcription factor, the BAG1 locus was analyzed for sites with similarity to the high-affinity MYC binding site CACGTG. This analysis revealed several potential MYC binding sites dispersed throughout the

FIG. 2. BAG1 transcription is regulated by low levels of endogenous MYC in nontransformed cells. (A) H1299 and 2091 cells were infected with a recombinant lentivirus containing either a luciferase or Myc shRNA construct, followed by puromycin selection at 5 μ g/ml and 2 μ g/ml, respectively. Cells were harvested at 36 h postinfection, and quantitative PCR was used to determine the relative expression of mRNA for the indicated transcripts. Transcript levels were normalized to DR5. Error bars represent the standard deviations for three qRT-PCR replicates. (B) Induction of BAG1 transcription occurs with a dose-response curve similar to that for the established MYC target gene encoding cyclin D2. In order to assess whether the activation of BAG1 transcription has a different sensitivity to MYC levels than other MYC targets, the MYC/ER allele in 2091 fibroblasts was activated with a range of 4-OHT concentrations (0 to 2,000 nM). Four hours after 4-OHT treatment, mRNA was harvested and analyzed for BAG1 and cyclin D2 levels by qRT-PCR, as indicated. Data are displayed as fold inductions of mRNA after normalization to actin levels.

locus. Site A, like many MYC binding sites, is within the first intron of the gene and is the closest match to the MYC consensus binding sequence (Fig. 4A). For both H1299 and 2091 cells, ChIP revealed specific binding to site A (Fig. 4B and C). In contrast, relatively little MYC binding was observed at other potential sites located more 3' in the gene. MYC binding to the BAG1 locus is conserved across species, since analysis of genomewide MYC binding revealed significant peaks at intronic sequences in the mouse BAG1 gene (7). Coupled with data showing that posttranslational activation of the MYC/ER allele can induce BAG1 mRNA levels even in the absence of protein synthesis and the production of intermediates (Fig. 4D), these findings support BAG1 as a direct transcriptional target of MYC.

While it remains unclear how MYC distinguishes between different potential binding sites within the same gene, one consistent observation has been that MYC preferentially binds to E boxes that have preexisting acetylation of histone H3 (18). Our ChIP analysis showed that site A did indeed have high levels of H3 acetylation even prior to MYC occupancy (Fig. 4C), in contrast to the relatively low H3 acetylation levels at the other site. MYC activates many of its transcriptional targets in part through the recruitment of cofactors that have histone acetyltransferase activity (5, 14). As mentioned, the Δ MbII mutant is defective for recruiting these acetyltransferase complexes. ChIP analysis revealed that MYC binding to site A was coincident with a substantial increase in histone H4 acetylation, consistent with BAG1 transcription depending on the MbII/acetyltransferase recruitment domain.

BAG1 is essential for protecting cells from MYC-mediated apoptosis. Little is known about how direct transcriptional targets of MYC contribute to the decision between cell cycle progression and apoptosis. In order to assess whether activation of the prosurvival *BAG1* gene inhibits the apoptotic response that might otherwise accompany high MYC levels, a well-defined system for the analysis of MYC-mediated apoptosis was utilized. This system, developed by the Eilers group, is based on expression of the conditional MYC/ER allele in the human osteosarcoma cell line U2OS (34). U2OS cells retain wild-type p53 and undergo robust apoptosis in response to a variety of stimuli (1, 13). Induction of MYC-driven apoptosis in these cells requires the withdrawal of growth factors for several days, followed by the subsequent activation of the conditional MYC allele for another several days. As in other systems, activation of the conditional MYC/ER allele in U2OS cells under normal growth factor conditions leads to cell cycle progression rather than apoptosis. To test whether BAG1 induction plays a role in blocking apoptosis under normal growth factor conditions, BAG1 levels were depleted using RNA interference (RNAi). In the presence of growth factors, activation of the MYC/ER allele increased the levels of all three isoforms of the BAG1 protein (Fig. 5A). Introduction of two distinct shRNAs targeting BAG1 resulted in a dramatic reduction in BAG1 protein levels compared to those in the presence of an irrelevant shRNA. To test whether the shRNA-mediated block of BAG1 induction results in altered survival, apoptosis was measured in these cells by staining for annexin V. As expected, induction of MYC in control shRNA-treated cells

FIG. 3. BAG1 levels are regulated by MYC *in vivo*. Mammary tumors were induced in 6-week-old female bitransgenic MMTV-rtTA;TetO-MYC mice (10, 19) via administration of 2 mg/ml doxycycline in drinking water. After mammary tumor formation, doxycycline was withdrawn from the water of 4 mice (MYC off), while the other mice were maintained on doxycycline (MYC on). Ninety-six hours after doxycycline withdrawal, tumors were harvested. (A) mRNAs from tumors were converted to cDNAs and then analyzed by quantitative RT-PCR. BAG1 levels were normalized to actin. Error bars represent standard deviations for quadruplicates of each sample. The *P* value was calculated using Student's *t* test (two-sided; $t = -25.67$). (B) One representative tumor from each group (MYC on and MYC off) was analyzed by Western blotting for protein levels of MYC, BAG1, and actin, which served as a loading control. (C) Tumors were induced via inoculation of cells from a transplantable B cell lymphoma originally developed by infection of bone marrow from a p53^{-/-} mouse with the MYC/ER retrovirus. In total, these cells were transplanted into the flanks of 6 naïve mice. After 10 days of 4-OHT treatment, mice developed aggressively growing lymphomas. At this point, two mice were continued on 4-OHT treatment (mice A1 and A2), while 2 mice were withdrawn from treatment for 2 days (mice B1 and B2) and the remaining 2 mice were withdrawn for another 10 days (mice C and D). Subsequently, one of the remaining mice was treated with 4-OHT (mouse D), while the final mouse was not (mouse C). Tumors were harvested and mRNAs analyzed for BAG1 levels by quantitative RT-PCR, at the indicated times.

did not result in increased apoptosis when growth factors were present. Remarkably, when BAG1 induction was blunted by shRNA, activation of MYC/ER caused substantial apoptosis (Fig. 5A). This occurred even though the cells were exposed to normal levels of serum growth factors. Depletion of BAG1 did not increase apoptosis in cells lacking MYC/ER activation, suggesting that apoptosis induced by depletion of BAG1 is dependent on MYC activity in this system. Parallel experiments in growth factor-deprived cells showed that MYC/ER induction under these conditions resulted in the predicted increase in apoptosis. The strict requirement for BAG1 expression in blocking MYC-dependent apoptosis was observed by using shRNA molecules targeting two distinct regions of the BAG1 mRNA, with the shRNA displaying weaker BAG1 depletion resulting in less apoptosis (Fig. 5A). The prosurvival effect of BAG1 was consistently observed in multiple independent experiments (Fig. 6A). When apoptosis was measured using other methods, such as quantitation of cells with sub-2N

DNA content, the same requirement for BAG1 was observed (Fig. 5B). No overt defect in cell proliferation was observed upon BAG1 depletion (data not shown). Considered together, these data support a model in which induction of BAG1 by MYC is important for conferring on cells the ability to avoid MYC-mediated apoptosis.

Blocking of BAG1 induction by MYC increases sensitivity to DNA-damaging agents. Blocking BAG1 function with shRNA treatment resulted in heightened sensitivity to apoptosis, as shown above and previously reported by others (42). Furthermore, ectopic MYC expression can also sensitize cells to apoptosis induced by DNA-damaging agents (2). Thus, experiments were conducted to assess whether the simultaneous activation of MYC and depletion of BAG1 can make tumor cells more sensitive to damage-induced apoptosis. Treatment of U2OS cells with low doses of UV irradiation failed to induce significant apoptosis, even when the MYC/ER allele was activated (Fig. 6C). In contrast, when MYC was activated in BAG1-

FIG. 4. Binding of MYC to the endogenous *BAG1* locus in human cells. (A) Within the human *BAG1* genomic locus, several potential matches to the MYC consensus binding site were identified, including the E box sequences labeled A and B. (B) Chromatin immunoprecipitation was performed with antibodies to MYC, using chromatin from lysates of cross-linked H1299 lung cancer cells. In parallel, precipitations were conducted with an IgG control antibody. *BAG1* E boxes A and B were analyzed, along with an E box from chromosome 19 (chr19) that is known to lack MYC occupancy. Values are expressed as relative chromatin binding compared to background levels observed with control antibody. (C) Endogenous MYC was induced in growth factor-deprived primary human fibroblasts by the addition of 10% serum for 2 h. ChIP analysis was performed to detect DNA associated with the MYC protein or with acetylated histone H3 or H4 (AcH3 or AcH4), as indicated. ChIP signals are expressed as relative binding after normalization. Nonimmune rabbit IgG was used as a control in ChIP experiments. (D) *BAG1* mRNA induction by MYC in the absence of protein synthesis. The MYC/ER allele was expressed in primary diploid human fibroblasts. Where specified, cells were exposed to cycloheximide at a concentration of 20 μ g/ml for 30 min before addition of 4-OHT. mRNA was harvested from these cells 4 h after 4-OHT treatment. *BAG1* mRNA levels were determined by qRT-PCR, normalized to actin mRNA levels, and expressed as fold induction levels.

depleted cells, low-dose UV irradiation resulted in substantial cell death. Similar cooperation between *BAG1* inhibition and MYC activation was observed in cells treated with the chemotherapeutic agent camptothecin (Fig. 6D). Consistent with the observations of others (42), *BAG1* depletion cooperated with DNA damage to induce apoptosis in the camptothecin-treated cells, even prior to activation of MYC. These observations support a model in which blocking of *BAG1* induction may increase the efficacy of chemotherapeutic agents and lower the doses required.

In some settings, MYC-mediated apoptosis depends on activation of p53 via the p14ARF pathway or other mechanisms. In order to assess whether p53 induction contributes to the MYC-induced apoptosis observed upon *BAG1* depletion, levels of p53 were assessed by Western blotting. No detectable stabilization of p53 was evident in *BAG1*-depleted cells (Fig. 5C). This lack of p53 induction held true for cells depleted of *BAG1* and simultaneously activated for MYC and/or serum starved. Thus, even under conditions where these cells undergo robust apoptosis, p53 induction does not appear to play a substantial role. The defect in p53 induction in U2OS cells may be due to the fact that the p14ARF/CDKN2A locus is silenced by DNA methylation in this cell line (30).

Since the MYC/ER fusion protein is likely to be regulated via an interaction with HSP70, and perhaps *BAG1*, we assessed whether depletion of *BAG1* had an effect on MYC/ER protein levels. This analysis revealed no appreciable change in MYC/ER levels when *BAG1* was depleted (Fig. 5D). Coupled with serum starvation, MYC activation and *BAG1* depletion led to a slight decrease in MYC/ER protein levels. Since this

modest decrease in MYC/ER protein levels does not provide an obvious explanation for the enhanced MYC-driven apoptosis of these cells, it likely reflects enhanced death and elimination from the cell pool of those cells expressing the highest levels of MYC/ER.

DISCUSSION

The gene encoding the prosurvival chaperone *BAG1* was identified here as a direct downstream target of MYC in a screen for loci whose expression correlates tightly with MYC function. MYC is among the most potent inducers of cell cycle progression in human cells (11). However, in specific cellular environments, high MYC expression can instead induce robust apoptosis (12). Tumors expressing high levels of MYC avoid apoptosis in part by accumulating mutations in other pathways, such as p53 and BCL2 pathways. However, it has remained less clear how MYC target genes participate directly in the decision between apoptosis and cell cycle progression. This contrasts with our relatively detailed understanding of MYC targets controlling cellular processes such as energy metabolism and protein translation (9). The data presented here demonstrate that induction of *BAG1*, a gene not previously known to be regulated by MYC, protects tumor cells from MYC-induced apoptosis. The observation that blocking a single downstream target of MYC can activate the intrinsic tumor suppressor function raises the possibility that similar strategies might eventually be of therapeutic value.

To date, anticancer therapies targeting MYC have aimed at blocking MYC expression or function (38). However, these

FIG. 5. The MYC target gene *BAG1* encodes a protein required for blocking MYC-mediated apoptosis. (A) Via transduction of either of two *BAG1* lentiviral shRNA molecules (shBAG1-1 and shBAG1-2), *BAG1* was depleted in U2OS human osteosarcoma cells that had previously been engineered to express the MYC/ER allele (34). Cells transduced with shRNA targeting luciferase served as a control (shLUC). MYC was activated in these cells by the addition of 200 nM 4-OHT under growth (10% fetal calf serum [FCS]) or starvation (0.1% FCS) conditions, as indicated. Western blot analysis of *BAG1* protein levels was performed (upper panels). Blotting for actin provided a loading control. Four days after MYC activation, cells were harvested and apoptosis was quantitated by annexin V-PE staining and flow cytometry (bottom panels). Data represent the means for biological triplicates, using at least 10,000 cells per sample. Error bars represent standard errors. (B) Propidium iodide profiles of the cells described for Fig. 5A. Four days after activation of MYC by the addition of 4-OHT, cells were harvested and fixed with 70% ethanol. These cells were stained with propidium iodide and analyzed by flow cytometry. Percentages of sub-G2N cells in the absence and presence of 4-OHT in growth (top) or starvation (bottom) medium for 4 days are shown. (C) U2OS MYC/ER cells were depleted of *BAG1* as indicated. MYC was then activated for 24 h by addition of 200 nM 4-OHT for the indicated groups. These studies were performed in duplicate in cells that were either serum starved or grown in 10% FBS. Western blotting for p53 and actin was performed on whole-cell lysates. As a positive control for p53 stabilization, cells were also treated with the genotoxic chemotherapeutic doxorubicin or vehicle, as indicated. (D) The depletion of *BAG1* fails to alter levels of the MYC/ER fusion protein. U2OS MYC/ER cells were depleted of *BAG1* by using shRNA as described above. MYC was subsequently activated by 4-OHT treatment. These manipulations were repeated in serum-starved and nonstarved cells, as indicated. Protein lysates were generated and analyzed by Western blotting for MYC/ER and *BAG1*. Blotting for ORC1 levels served as a loading control.

strategies fail to capitalize on the robust, latent apoptotic potential that MYC possesses. Rather than blocking MYC expression in order to inhibit its proliferative activity, it may be more efficacious to preserve MYC function and simultaneously activate its apoptotic potential. Of further therapeutic relevance, blocking *BAG1* function without activating MYC fails to induce significant apoptosis. This raises the possibility that activating MYC-dependent apoptosis might preferentially cause the death of tumor cells, which typically harbor MYC levels well above those present in normal cells. This possibility is of even greater interest in light of another recent study that shed light on the decision that MYC-expressing cells make between cell cycle progression and apoptosis. The investigators found that relatively high levels of MYC are required to induce apoptosis *in vivo*, while lower MYC levels drive proliferation without increasing apoptosis (29). Thus, the relatively low level

of MYC present in nontransformed cells is able to drive proliferation without inducing cell death.

The strict MYC dependence of the apoptosis induced by *BAG1* shRNA treatment was unexpected. This synthetic lethality suggests that the effect observed here is not related to a general prosurvival requirement for *BAG1*. This distinction heightens the clinical ramifications of these observations, raising the possibility that cells expressing very high levels of MYC will be preferentially sensitive to this type of death. The ability of *BAG1* depletion to sensitize cells to apoptosis induced by a combination of MYC and DNA damage suggests that patients might be treated with lower doses of chemotherapeutics and therefore sustain fewer side effects if current treatments were combined with *BAG1* inhibition. It is worth noting, as evident in Fig. 5, that a simple lack of *BAG1* induction is not the root cause of MYC-induced apoptosis in serum-deprived cells,

FIG. 6. Loss of BAG1 enhances MYC-mediated apoptosis and sensitizes cells to DNA damage when MYC is activated. BAG1 was depleted from U2OS MYC/ER cells by infection with BAG1 lentiviral shRNA or an shRNA targeting luciferase. MYC was activated in these cells by adding 200 nM 4-OHT to either serum deprivation medium (A) or normal growth medium (B) for 4 days. Cells were harvested and either assessed for BAG1 protein levels (top panels) or stained with annexin V-PE and analyzed by flow cytometry (bottom panels). The percentage of apoptotic cells represents the mean and standard deviation (SD) for three independent experiments (10,000 cells/sample). (C) U2OS MYC/ER cells expressing shRNA targeting luciferase or BAG1 were treated with 5 nM 4-OHT for 24 h. Cells were then irradiated with 7.5 J/m² UV for 18 h and harvested for apoptosis quantitation by annexin V staining. (D) U2OS MYC/ER cells expressing shRNA targeting luciferase or BAG1 were treated with 1 nM 4-OHT for 24 h. Cells were then treated with 2 μM camptothecin for 16 h and harvested for apoptosis quantitation by annexin V staining.

since BAG1 was induced to similar degrees by MYC under both apoptotic (serum-starved) and nonapoptotic conditions.

BAG1 functions in part as a cochaperone with HSP70, and the HSP70 gene was among the first transcriptional targets of MYC identified (21). Coupled with the data presented here, these observations strengthen the model that control of the BAG1-HSP70 complex may be of critical importance in the MYC pathway. MYC also stimulates protein biosynthesis on a broad level. Thus, one model for the requirement for BAG1 induction is that it may help to satisfy the increased demand for chaperone activity that is experienced by cells with activated MYC. In the absence of BAG1 induction, MYC's stimulation of translation pathways might initiate an unfolded protein response. However, the lack of p53 induction in this setting suggests that the unfolded protein response is not activated in this setting. An alternative model that is more consistent with our findings is that a small number of BAG1 clients are critical

FIG. 7. Identification of the MYC-BAG1 axis as a critical determinant of the survival-apoptosis decision. As reported here, the MYC oncoprotein binds to and activates expression of the *BAG1* locus. This induction is critical for the survival of MYC-expressing cells. Proof-of-principle studies using shRNA showed that blocking this pathway can convert the proproliferative activity of MYC to a proapoptotic activity, thereby activating MYC's intrinsic tumor suppressor function. Other strategies for blocking this pathway might be more relevant clinically, such as the use of the recently described BAG1 inhibitor NSC71948 (37). In support of the model that inhibition of this pathway may allow reactivation of MYC's tumor suppressor function, we recently reported that inhibiting the major BAG1 client protein, BCL2, can enhance MYC-dependent apoptosis (32).

survival proteins that are essential for blocking the default apoptotic response of cells to elevated MYC (Fig. 7). In the absence of BAG1, these survival proteins are not stable and apoptosis ensues. The loss of this small yet critical set of BAG1 clients is unlikely to trigger the unfolded protein response. Even treatment with high levels of the serum growth factor IGF1 failed to rescue BAG1-depleted cells from apoptosis when MYC was activated (our unpublished data), supporting the model that a limited number of downstream components of the apoptosis pathway comprise the defect responsible for cell death. The role of BAG1 as a chaperone for a small set of client proteins highly enriched for survival function is supported by a number of recent studies (16, 41). BAG1 has nonchaperone functions in transcription and other cellular processes as well (16). Ultimately, understanding which pro-survival clients of the BAG1-HSP70 chaperone complex or which nonchaperone activities are important for blocking MYC-induced apoptosis may help to provide new biomarkers or even therapeutic targets for the treatment of MYC-driven tumors.

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