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Role of c-myc Regulation in Zta-Mediated Induction of the Cyclin-Dependent Kinase Inhibitors p21 and p27 and Cell Growth Arrest

Antonio Rodriguez,[1] Eun Joo Jung, Qinyan Yin, Corinne Cayrol,[2] and Erik K. Flemington[3]

Department of Pathology, Tulane Cancer Center, Tulane University Health Sciences Center, New Orleans, Louisiana 70112

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Latency-associated Epstein–Barr virus (EBV) gene expression induces cell proliferation. Unlike the latency associated genes, lytic gene expression in EBV, as well as other herpesviruses, elicits cell cycle arrest. Previous studies have shown that the EBV immediate early lytic transactivator, Zta, induces a G_0/G_1 cell cycle arrest through induction of the cyclin-dependent kinase inhibitors, p21 and p27. Here we show that while EBV latency is intimately linked to activation of the protooncogene, c-myc, Zta represses c-myc expression. We also show that inhibition of c-myc expression is required for Zta-mediated growth arrest and for maximal induction of p21 and p27. Nevertheless, induction of p21 and p27 is also influenced by a c-myc-independent mechanism. A detailed genetic analysis of Zta's basic/DNA binding region identified two distinct subregions that contribute to full induction of p21 and p27. One subdomain influences p21 and p27 expression through the c-myc-dependent mechanism and the other subdomain influences p21 and p27 induction through the c-myc-independent pathway. Together, these studies further our understanding of the complex nature of Zta-induced growth arrest. © 2001 Academic Press

Key Words: EBV; Zta; BZLF1; ZEBRA; myc; lytic; cell cycle; p21; p27; p53.

INTRODUCTION

Herpesviruses are important pathogens in humans. Both the human cytomegalovirus (CMV, a beta herpesvirus) and the Epstein–Barr virus (EBV, a gamma herpesvirus) are oncogenic viruses (Britt and Alford, 1996; Rickinson and Kieff, 1996). While oncogenic transformation by CMV may occur through an abortive replication mechanism, the oncogenic potential of EBV lies largely in a set of cell cycle promoting genes that are expressed during latency (Kieff, 1996; Rickinson and Kieff, 1996). Expression of the latency-associated EBV immortalizing genes likely plays a role in the viral life cycle by causing amplification and distribution of the infected B-cell population during the early stages of host infection. Following the development of an immune response to several of these latency associated proteins, a significantly more restricted pattern of latency gene expression arises and persists in the host (Babcock *et al.,* 1998, 1999; Miyashita *et al.,* 1997). In combination with other genetic events in the host cell, however, this restricted form of latency likely contributes to a variety of human cancers in immunocompetent individuals including nasopharyngeal carcinoma, African Burkitt's lymphoma, high-grade breast cancer (Bonnet *et al.,* 1999; Labrecque *et al.,* 1995; Luqmani and Shousha, 1995), and some cases of gastric carcinoma (Burke *et al.,* 1990; Kieff, 1996; Rickinson and Kieff, 1996; Shibata *et al.,* 1991) .

Induction of the cellular oncogene, c-myc, is a crucial step in the activation of cell cycle progression in both epithelial and B-lymphocytes and c-myc is either induced, amplified, and/or translocated in most epithelial and B-lymphocyte tumors (Nesbit *et al.,* 1999). c-myc is a key downstream target of EBV latency-associated genes in infected B-lymphocytes (Alfieri *et al.,* 1991) and induction of c-myc expression by latency-associated genes likely plays a crucial role in promoting cell cycle progression (Kaiser *et al.,* 1999; Polack *et al.,* 1996). Although some latency-associated EBV genes can induce c-myc expression, these genes are not expressed in EBV-associated tumors in immunocompetent individuals (Kaiser *et al.,* 1999). Instead, c-myc is translocated [Burkitt's lymphoma (Nesbit *et al.,* 1999)], amplified [in some cases of breast cancer (Nesbit *et al.,* 1999)], or overexpressed through some other mechanism [nasopharyngeal carcinoma (Lin *et al.,* 1993) and some cases of breast cancer (Nesbit *et al.,* 1999)].

Despite the clear association between herpesviruses and human cancers, some early studies suggested that productive infection/replication occurs in growth arrested cells (de Bruyn and Knipe, 1988; Mocarski, 1996; Roizman and Sears, 1996). Moreover, these studies, as

[1] Present address: Centro de Biologia Molecular, Facultad de Ciencias–Edif. Biologicas, Universidad Autonoma de Madrid, Madrid, Spain.
[2] Present address: Institut de Pharmacologie et de Biologie Structurale, 205, route de Narbonne, 31077 Tolouse, France.
[3] To whom correspondence and reprint requests should be addressed at Department of Pathology, SL79, Tulane University Health Sciences Center, 1430 Tulane Avenue, New Orleans, LA 70112. Fax: 504-588-5516. E-mail: eflemin@tulane.edu.

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well as more recent studies (Bresnahan *et al.,* 1996; de Bruyn and Knipe, 1988; Dittmer and Mocarski, 1997; Ehmann *et al.,* 2000; Hobbs and DeLuca, 1999; Lomonte and Everett, 1999; Lu and Shenk, 1996, 1999; Olgiate *et al.,* 1999; Salvant *et al.,* 1998; Song *et al.,* 2000; Wiebusch and Hagemeier, 1999), have demonstrated that during productive replication, herpes simplex virus (HSV, an alpha herpesvirus) and CMV can actively block cell cycle progression. We have previously shown that the EBV-encoded immediate early transactivator, Zta, can elicit a G_0/G_1 cell cycle block in EBV positive and EBV negative cells (Cayrol and Flemington, 1996a,b; Rodriguez *et al.,* 1999) and HSV and CMV encoded genes that block cell cycle progression have recently been identified (Hobbs and DeLuca, 1999; Lomonte and Everett, 1999; Lu and Shenk, 1999). Evidence that blocking cell cycle progression is important for replication of EBV was shown by Takase *et al.* (1996), who demonstrated that forcing cells to progress into the S phase of the cell cycle inhibits EBV replication.

Previous studies showed that Zta induces growth arrest in part through induction of the cyclin-dependent kinase inhibitors, p21 and p27 (Cayrol and Flemington, 1995, 1996a,b; Rodriguez *et al.,* 1999). Induction of p21 and p27 was shown to occur at the RNA and posttranscriptional level, respectively (Cayrol and Flemington, 1996b). Reports from other groups have shown that in certain settings, c-myc expression decreases the stability of p27 (Muller *et al.,* 1997; Nass and Dickson, 1998; Perez-Roger *et al.,* 1997). Further, it has been shown that c-myc can down regulate p21 expression (Mitchell and El-Diery, 1999). Based on these studies, we reasoned that the continued presence of c-myc following Zta induction would preclude up regulation of p21 and p27, thus antagonizing Zta-mediated cell cycle arrest. We therefore explored whether Zta has a means of regulating c-myc function and/or expression. We show here that Zta down regulates c-myc function by inhibiting c-myc RNA expression and that down regulation of c-myc is required for Zta-mediated growth arrest (and for efficient induction of p21 and p27). Genetic studies revealed that an intact basic region is required for c-myc down regulation. A genetic analysis of Zta's basic/DNA binding region identified two domains that mediate distinct c-myc-dependent, and c-myc-independent, growth arrest signaling. These studies indicate a multifaceted role of Zta's basic region in facilitating full growth arrest function.

RESULTS

Zta down regulates c-myc protein expression

Activation of the lytic cycle *in vitro*, through treatment of EBV-infected cell lines with a wide variety of inducing agents (including Zta) results in a G_0/G_1 growth arrest

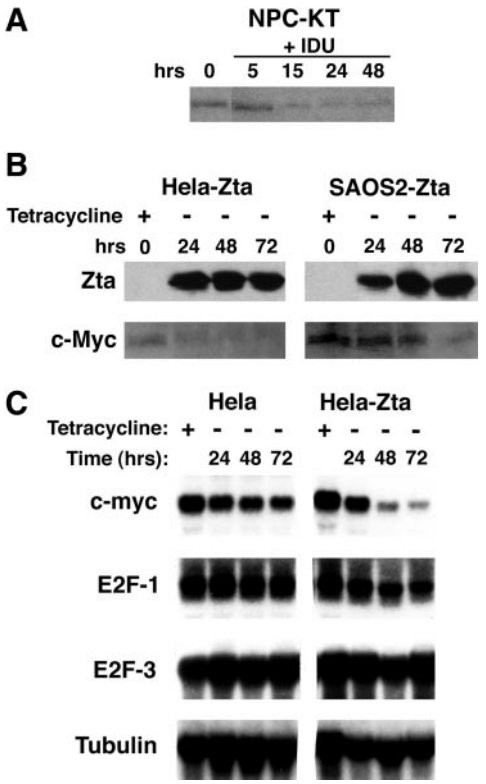

FIG. 1. (A) Down regulation of c-myc expression following treatment of NPC-KT cells with the lytic cycle inducing agent, iodo deoxyuridine (IDU). c-Myc protein levels were assessed utilizing the anti-c-Myc antibody, C33 (Santa Cruz Biotech). (B) Expression of Zta in two tetracycline inducible Zta cell lines, HeLa and SAOS2, elicits down regulation of c-Myc. c-Myc protein levels were assessed as in A, and Zta was detected using the M47 anti-Zta antibody (Rodriguez *et al.,* 1999). (C) Zta down regulates c-Myc at the RNA level. Hela–Zta and control cells (Hela) were incubated in the presence or absence of tetracycline and RNA levels of c-myc, E2F1, E2F3, and tubulin were assessed by consecutive probings of the indicated Northern blot.

(Cayrol and Flemington, 1996a; Rodriguez *et al.,* 2001). Although EBV latency is associated with activated expression of the protooncogene, c-myc, induction of the lytic cycle in the EBV positive nasopharyngeal cell line, NPC-KT, results in decreased amounts of c-myc (Fig. 1A). We have previously shown that the EBV immediate early gene product, Zta, induces growth arrest, in part, through induction of the cyclin-dependent kinase inhibitors, p21 and p27 (Cayrol and Flemington, 1996a) and others have shown that p21 and p27 are regulated by c-myc (Mitchell and El-Diery, 1999; Muller *et al.,* 1997; Nass and Dickson, 1998; Perez-Roger *et al.,* 1997). We therefore tested whether Zta influences c-myc expression. As shown in Fig. 1B, induction of Zta in two tetracycline-inducible EBV negative cell lines, Hela and SAOS2, resulted in decreased amounts of c-myc protein. Northern blot analysis revealed that Zta regulates c-myc expression at the RNA level (Fig. 1C). Zta also down regulates expression of the another cell cycle regulated transcription factor,

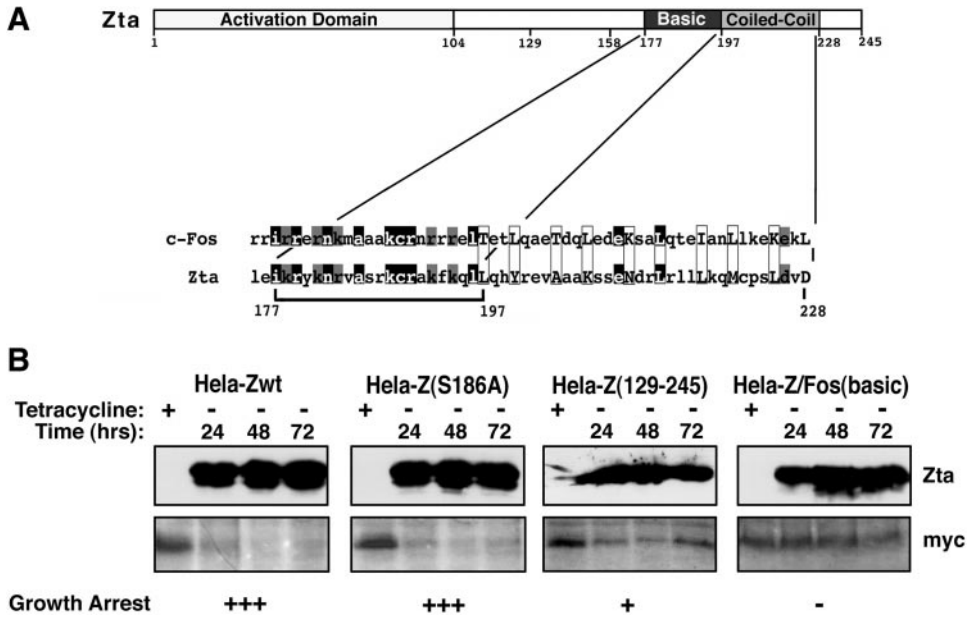

FIG. 2. Genetic analysis of Zta-mediated c-myc down regulation. (A) Structure of functional domains of Zta and alignment of the basic domain with the cellular homolog, c-Fos. (B) Initial genetic study of Zta-mediated growth arrest and c-Myc down regulation. The indicated stable inducible Hela cell lines were generated previously (Rodriguez *et al.,* 1999). Cell cycle analysis following tetracycline withdrawal was previously determined (Rodriguez *et al.,* 1999) and the results are summarized [+++ = 86–87%, + = 70%, and − = 44% of cells in G_0/G_1 at 48 h postinduction (Rodriguez *et al.,* 1999)]. Zta expression was determined using the anti-Zta polyclonal antibody, M47 (Rodriguez *et al.,* 1999) and c-Myc was analyzed using the anti-c-Myc monoclonal antibody, C33 (Santa Cruz Biotech). Results shown in B have been repeated in transient transfection studies with similar results.

E2F-1, although not as significantly as that observed with c-myc. Zta had little effect on the expression of two other cellular genes, E2F-3 and Tubulin (Fig. 1C).

Initial genetic analysis of Zta-mediated down regulation of c-myc expression

We previously identified a series of Zta mutants that defined important Zta-mediated growth arrest functions (Rodriguez *et al.,* 1999). A Zta point mutant that is defective for its ability to trigger the lytic cycle, Z(S186A) (Francis *et al.,* 1997), induces growth arrest and induces p21, p27, and p53 as effectively as wild-type Zta (Rodriguez *et al.,* 1999). As shown in Fig. 2, this mutant efficiently down regulates c-myc expression. Deletion of Zta's activation domain completely abrogates its ability to activate reporter constructs but is only partially defective for its growth arrest functions (Rodriguez *et al.,* 1999) [this partial defect is likely due to a compromised ability to induce p27 (but not p21 or p53) (Rodriguez *et al.,* 1999)]. As shown in Fig. 2B, this mutant modulates the levels of c-myc but is not as effective as wild-type Zta. Finally, although we have shown previously that DNA binding is not required for Zta-mediated growth arrest, replacement of Zta's basic/DNA binding domain with the analogous region of the Zta cellular homolog, c-fos, completely abrogates Zta's ability to carry out this function (Rodriguez *et al.,* 1999). This mutant, Z/Fos(basic) (Kolman *et al.,* 1996), is expressed well (Fig. 2), is localized to the nucleus (data not shown), and activates reporter plasmids containing AP1 promoter elements (Francis *et al.,* 1997). In line with its inability to induce growth arrest, Z/Fos(basic) is unable to down regulate c-myc expression (Fig. 2). Together, this limited genetic analysis suggested a preliminary link between Zta-mediated growth arrest and Zta-mediated down regulation of c-myc expression.

Enforced expression of c-myc overrides Zta-mediated growth arrest and inhibits full induction of p21 and p27

To directly test whether Zta-mediated growth arrest requires down regulation of c-myc, a Zta expression vector was cotransfected with either a control vector or different amounts of a c-myc expression vector. As shown in Fig. 3, expression of Zta alone elicits a G_0/G_1 growth arrest in HeLa and NPC-KT cells. In contrast, cotransfection of a c-myc expression vector with a Zta expression vector significantly inhibits Zta's ability to induce growth arrest in both cell lines. Interestingly, while constitutive expression of c-myc inhibited the induction of p21 and p27, it had no effect on Zta's ability to induce p53 expression. This indicates that while down regulation of c-myc likely plays a role in the induction of p21 and p27, c-myc is either downstream or is indepen-

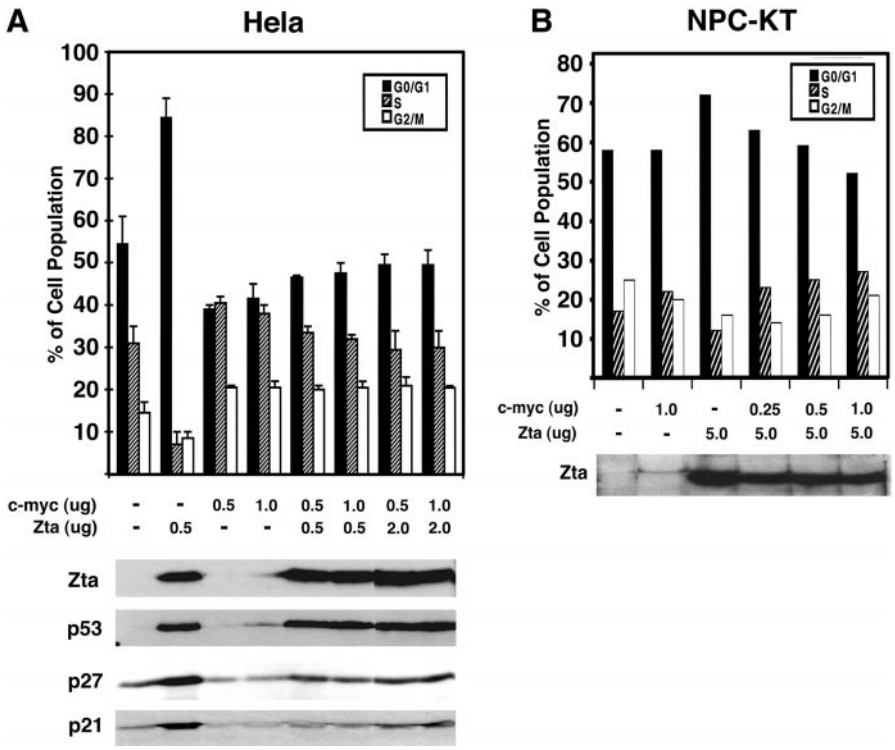

FIG. 3. Forced expression of c-myc overrides Zta-mediated growth arrest. Hela and NPC-KT cells were transfected in 100 mm dishes with 200 ng of pGFP-Sp and the indicated amounts of c-myc and Zta-expression plasmids [plus 28 μg of the carrier plasmid, pGL3Basic (Promega)]. Forty-eight hours later, cells were harvested and subjected to FACS analysis to determine the cell cycle distribution of the GFP positive cells. Western blot analysis was performed using the following antibodies: anti-Zta (M47), anti-p53 (D01, Santa Cruz Biotech), anti-p21(C19G, Santa Cruz Biotech), and anti-p27 (Transduction Laboratories).

dent of p53 in Zta-mediated growth arrest. Notably, low-level induction of p21 and p27 is observed at the higher input amount of the Zta expression vector. This could result from incomplete c-myc override of these Zta functions. Alternatively, it is possible that other factors (pathways) also contribute to Zta-mediated induction of p21 and p27. Additional evidence shown below supports this second model.

Genetic analysis of Zta's basic/DNA-binding domain in Zta-mediated growth arrest

To date, our studies addressing the mechanisms of Zta-mediated growth arrest indicate that Zta affects growth arrest through more than one independent pathway (Rodriguez *et al.,* 1999 and see above). These studies have also indicated that the basic/DNA binding region of Zta is essential for eliciting these growth arrest activities. Despite the dependence on Zta's basic/DNA-binding domain, Zta-mediated growth arrest is largely independent of Zta's transactivation function (Cayrol and Flemington, 1996b; Rodriguez *et al.,* 1999). To further explore the role of Zta's basic/DNA-binding domain in facilitating growth arrest, we generated and analyzed a panel of basic region point mutants [note that wild-type and mutant Zta constructs contain an in frame nuclear localization signal at the carboxyl terminal end of the Zta reading frame (Flemington *et al.,* 1994)]. As shown in Figs. 4A and 4B, two previously described DNA binding mutants, Zdbm1 and Zdbm2 (Flemington *et al.,* 1994), induce growth arrest, although not as efficiently as wild-type Zta. As shown in Fig. 4C, a wider panel of more subtle mutants were also tested. While most mutations had no apparent effect on growth arrest, two mutants, Z(I177D) and Z(A185K), had a moderate effect, and one mutant, Z(S186E), was almost completely defective for induction of growth arrest. Since Z(A185K) and Z(S186E) are proximal, these mutations may have related functional effects. Interestingly, although mutation of serine 186 to glutamic acid [i.e., Z(S186E)] has a significant impact on Zta-induced growth arrest, mutation of this residue to an alanine has little affect on Zta-mediated growth arrest (Rodriguez *et al.,* 1999 and Fig. 4). This suggests the possibility that phosphorylation of this site may abrogate Zta's growth arrest function. While this site is a previously described protein kinase C (PKC) phosphorylation site (Baumann *et al.,* 1998), we have been unable to address whether PKC-mediated phosphorylation of S186 alters Zta's growth arrest function because activation of the PKC pathway alone elicits growth arrest (data not shown).

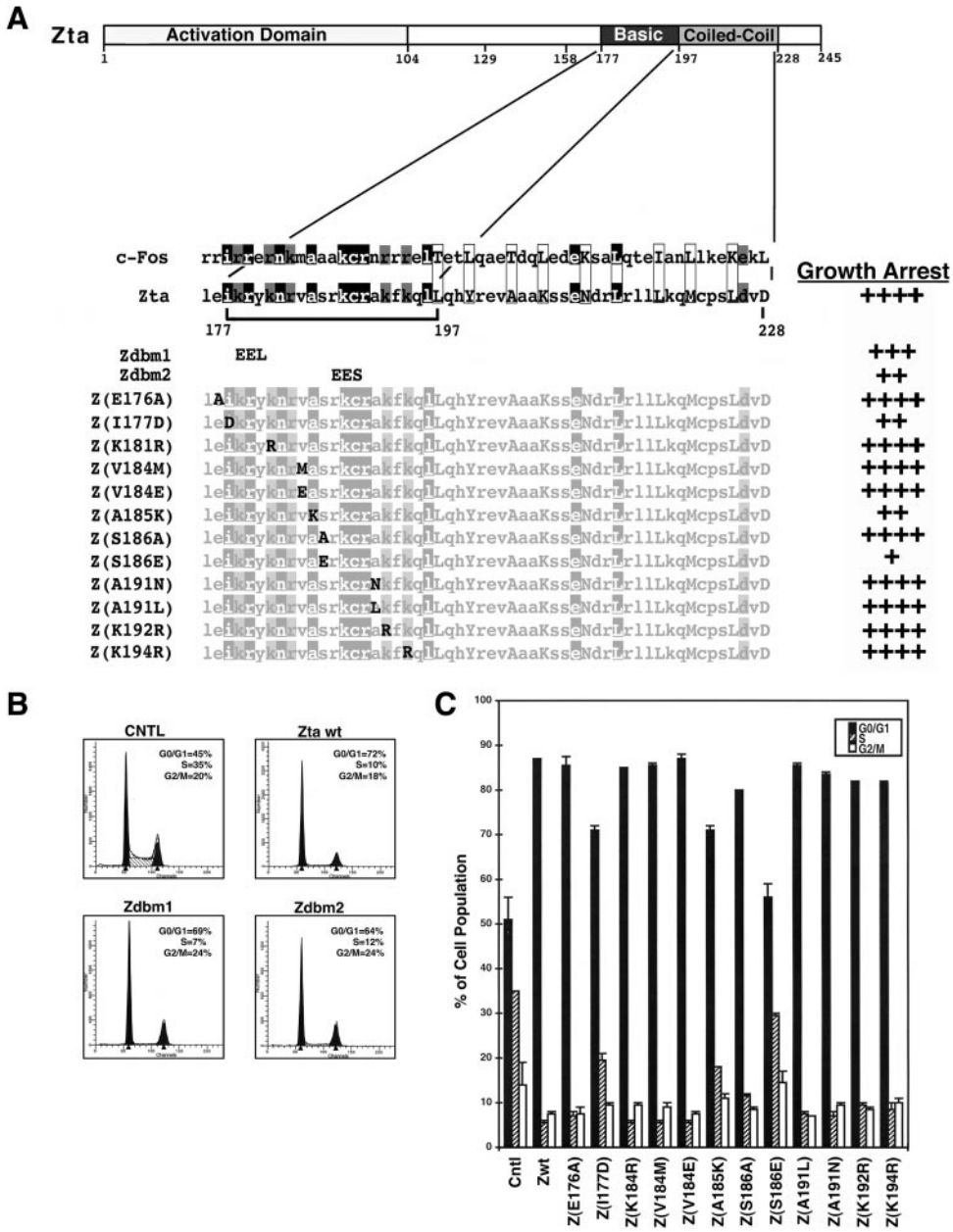

FIG. 4. Genetic analysis of Zta basic DNA binding domain. (A) Schematic representation of basic region mutants and summary of growth arrest analysis. (B) Analysis of two previously characterized Zta DNA binding mutants, Zdbm1 and Zdbm2 (Flemington *et al.,* 1994). Hela cells were cotransfected with 2 μg of the indicated control, or Zta expression plasmid and 200 ng of the GFP expression plasmid, pGFP-Sp [plus 28 μg of the carrier plasmid, pGL3Basic (Promega)]. GFP positive (i.e., transfected cell population) was specifically analyzed by FACS analysis. (C) Cell cycle analysis of basic region mutants. Experiments were carried out in HeLa cells as described in B.

The mutation series shown in Fig. 4 contained a two amino acid gap of positions N182 and R183. Analysis of the solved structure of the Zta homolog, c-Fos (Glover and Harrison, 1995), suggests that these two residues are in close proximity to S186 and that they would be on the same side of the alpha helix. In contrast, V184 would be predicted to be on the other side of the alpha helix. Accordingly, a nonconservative mutation of V184 to glutamic acid [i.e., Z(V184E)] has no significant impact on Zta-mediated growth arrest (Fig. 4). We therefore generated mutations specifically at residues N182 and R183 and assessed the ability of the resulting mutants to induce growth arrest. As shown in Fig. 5, nonconservative mutation of each of these residues significantly inhibits Zta's ability to induce growth arrest. Together these data identify a short interaction surface which likely contacts a factor that is crucial for transducing a Zta-mediated growth arrest signal.

We next assayed the ability of these key mutants to affect the expression of c-myc, p53, p27, and p21. As

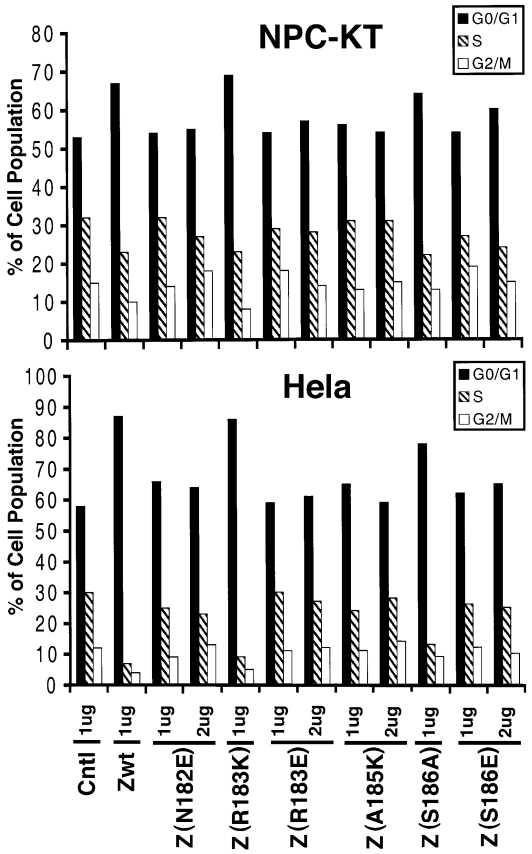

FIG. 5. Analysis of residues N182 and R183 in growth arrest function. HeLa and NPC-KT cells were transfected with pGFP-Sp and the indicated Zta expression vector as described in Fig. 4 legend. Forty-eight hours later, cells were harvested and FACS analysis was carried out on the GFP positive population to assess the cell cycle distribution.

shown in Fig. 6, Z(S186E), Z(N182E), and Z(R183E) are impaired for their ability to suppress c-myc expression. Consistent with a compromised ability to suppress c-myc expression, Z(S186E), Z(N182E), and Z(R183E) do not fully induce p21 and p27. Nevertheless, these mutants induced p21 and p27 expression significantly above background (Cntl), suggesting the possibility that these factors are also influenced by a c-myc-independent pathway. Using stable inducible Zta expressing cell lines, previous studies (Rodriguez *et al.,* 1999), and experiments discussed above (Fig. 2), have shown that the Zta basic region mutant, Z/Fos(basic), is defective for induction of p21, p27, and down regulation of c-myc. In addition, we have observed a complete failure of Z/Fos(basic) to regulate these factors in transient transfection experiments (data not shown). This suggested that another region of Zta's basic domain may also influence these pathways. Notably, the initial panel of basic region mutants shown in Fig. 4 contained a number of conservative mutations in the carboxyl terminal region of this domain. We therefore generated a new series of mutants containing nonconservative mutations at amino acids K192, F193, K194, and Q195. All four of these mutants are fully capable for repressing c-myc expression (Fig. 7). Nevertheless, Z(K192E) and Z(K194E) showed impaired growth arrest function and both of these mutants are compromised for induction of p21 and p27. This further supports the notion that although Zta-mediated down regulation of c-myc influences the induction of p21 and p27, these factors are also regulated by Zta through a c-myc-independent pathway. In addition, these data identify a second domain of Zta's basic region that elicits growth arrest signaling through a distinct pathway.

DISCUSSION

Accumulating evidence clearly indicates that in direct contrast to small DNA tumor viruses, herpesviruses have an evolutionarily conserved preference to replicate in a

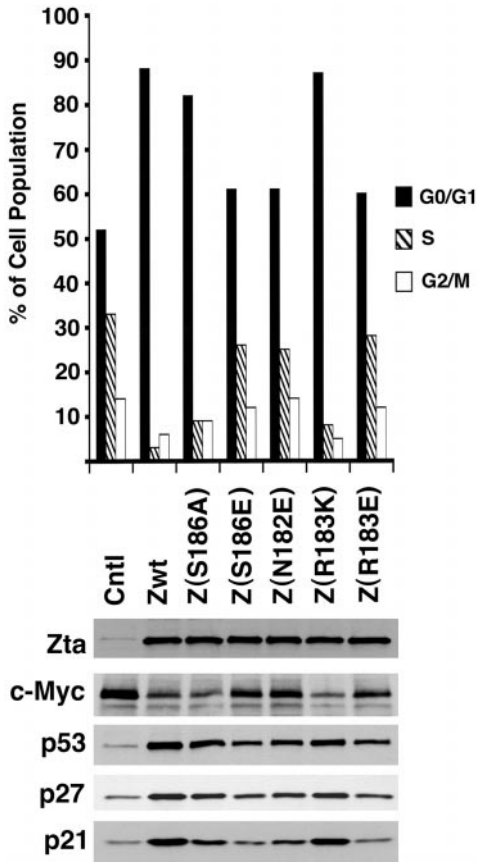

FIG. 6. Regulation of c-Myc, p53, p27, and p21 by Zta basic region point mutants. HeLa cells were transfected with pGFP-Sp and the indicated Zta expression vector as described in Fig. 4 legend. Forty-eight hours later, cells were harvested and FACS analysis was carried out on the GFP positive population to assess the cell cycle distribution. A fraction of the cells were lysed directly in SDS–PAGE loading dye, boiled for 20 min, and analyzed for expression of Zta (M47), c-Myc (C33, Santa Cruz), p53 (D01, Santa Cruz), p27 (anti-p27/Kip1, Transduction Laboratories), and p21 (anti-p21/Cip1, Transduction Laboratories). This experiment was carried out independently of the experiment shown in Fig. 5.

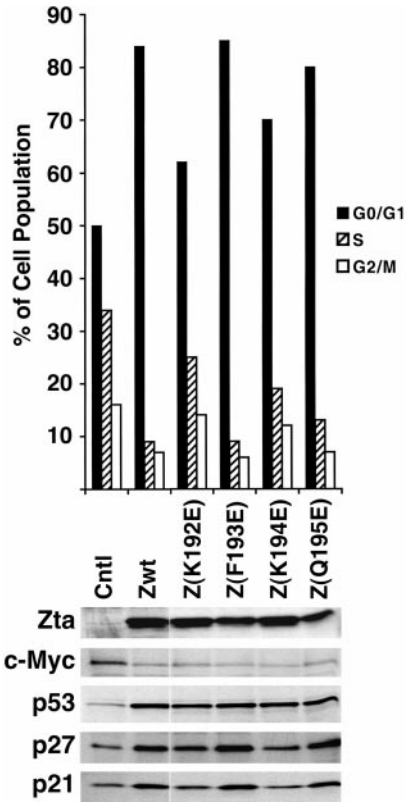

FIG. 7. Regulation of c-Myc, p53, p27, and p21 by Zta basic region point mutants. HeLa cells were transfected with pGFP-Sp and the indicated Zta expression vector as described in Fig. 4 legend. Forty-eight hours later, cells were harvested and FACS analysis was carried out on the GFP positive population to assess the cell cycle distribution. A fraction of the cells were lysed directly in SDS–PAGE loading dye, boiled for 20 min, and analyzed for expression of Zta (M47), c-Myc (C33, Santa Cruz), p53 (D01, Santa Cruz), p27 (anti-p27/Kip1, Transduction Laboratories), and p21 (anti-p21/Cip1, Transduction Laboratories). This experiment has been repeated with similar results.

growth arrested environment. Having the genetic capacity to encode much of the machinery that is required for efficiently replicating their own genomes, these viruses have evolved specific mechanisms to help ensure that viral replication occurs in the absence of competitive cellular genomic replication. Our previous studies have shown that in some settings, the EBV-encoded immediate early gene product, Zta, plays a role in facilitating a cell growth arrested environment to help enhance viral genomic replication. These studies have begun to reveal some of the mechanistic characteristics of Zta-mediated growth arrest as well as some of the key cellular cell cycle control factors that are involved in this process.

While viral proteins that induce cell cycle progression have been extensively studied for a number of years, the discovery and analysis of viral proteins with cell growth inhibitory activity is relatively new. Studies from transforming proteins such as adenovirus E1A or SV40 T antigen have revealed that they directly target multiple distinct cellular cell cycle control proteins (e.g., p300, CBP, p53, pRB, etc.) and together these interactions help facilitate activation of cell proliferation (Ludlow and Skuse, 1995). Although the effects of Zta on cell cycle regulatory functions are the opposite of E1A and T antigen, Zta similarly carries out its functions through a complex series of interactions with multiple cell cycle control proteins (Fig. 8). Specifically, our previous studies have shown that induction of p21 occurs in part through a p53-dependent and in part through a p53-independent pathway. Here we have shown that Zta down regulates c-myc expression and that although this activity is crucial for mediating growth arrest (and for fully inducing p21 and p27), another distinct Zta-mediated signaling event also plays a crucial role in inducing growth arrest and facilitating maximal p21 and p27 induction (Fig. 8). Finally, we have also shown here that the Zta-mediated induction of p53 expression occurs through a c-myc-independent mechanism.

The results presented here have defined a small region of the basic/DNA binding domain of Zta that plays a crucial role in inducing growth arrest (residues NRVAS). Interestingly, the previously published structure c-Fos/c-Jun heterodimer (Glover and Harrison, 1995) predicts that the Zta residues identified here to be crucial for

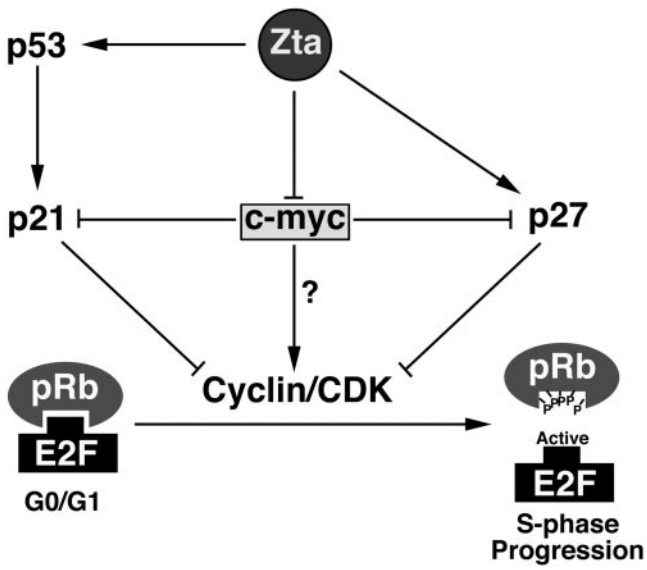

FIG. 8. Model for Zta-mediated growth arrest. Previous studies (Cayrol and Flemington, 1996a) showed that Zta causes hypophosphorylation of the tumor suppressor protein, pRb, a key target of cyclin/cdks in cell cycle regulation through induction of the cyclin-dependent kinase inhibitors, p21 and p27. We show here that Zta mediates down regulation of c-myc expression and that this is essential for high-level p21 and p27 expression. In addition, however, we provide evidence for an additional independent mechanism that is essential for efficient growth arrest and plays a role in modulating p27 and p21 levels. Both the c-Myc-dependent pathway and the c-Myc-independent pathway require an intact basic domain of Zta but these two signaling pathways require distinct subregions (and functions) of this domain.

inducing growth arrest, N182, R183, and S186, may be predominantly positioned away from the DNA helix when Zta is bound to DNA. This raises the possibility that the growth arrest function (and the c-myc down regulatory function) of this region may be elicited through interactions with key cell cycle regulatory proteins and not through binding to DNA. Although we cannot, at this time, discount the possible role of DNA binding in influencing c-myc repression and inducing growth arrest, the Zta mutant, Zdbm1, which is defective for its ability to bind DNA, can repress c-myc expression (data not shown). Interestingly, Baumann *et al.* have provided evidence that a cellular factor interacts with this region of Zta when S186 is phosphorylated, but not when it is not phosphorylated (Baumann *et al.,* 1998). It was also shown that this interaction likely plays a role in facilitating Zta-mediated activation of genomic viral gene expression (Baumann *et al.,* 1998; Francis *et al.,* 1997). In contrast, our studies suggest that a growth arrest mediator interacts with Zta's basic region specifically when serine 186 is *not* phosphorylated. Importantly, such a factor(s) would be predicted to have overlapping binding specificity with the factor identified by Baumann *et al.* (1998). Whether these factors are related or distinct, it is likely that these factors would compete for binding to this region. Such competition would then be part of a regulatory mechanism that elicits switching between different Zta functions (i.e., transcriptional activation vs growth arrest).

It is clear that Zta's basic region is a complex functional domain that not only plays a role in direct DNA binding but also in multiple protein–protein interactions. Besides the unknown factor identified by Baumann *et al.* (1998) that interacts specifically with Zta's basic region when S186 is phosphorylated, several other studies have either implicated, or directly demonstrated, the binding of cellular proteins to this region of Zta. Using Zta as a probe to screen an expression library, Aho *et al.* (2000) identified a ubiquitously expressed factor, referred to as ubinuclein, that interacts directly with the basic region of Zta. Although this factor is of unknown function, Aho *et al.* (2000) found that overexpression of ubinuclein in keratinocytes caused morphological changes resembling terminal differentiation. A limited genetic analysis of Zta's interaction with ubinuclein indicated that the second half of Zta's basic region is crucial. Moreover, a mutant in which K192 and K194 are substituted for alanine residues was found to be defective for binding to ubinuclein. We show here that nonconservative mutations in either K192 or K194 decreases Zta-mediated growth arrest and compromises Zta's ability to induce p21 and p27 through a c-myc-independent mechanism. Therefore, it is possible that the interaction between Zta and ubinuclein might play a role in influencing p21 and p27 induction and Zta-mediated growth arrest. Further studies will be required to rigorously address this issue.

Another study has shown that Zta's basic region interacts directly with the TATA box binding factor, TBP (Mikaelian *et al.,* 1993). This study showed that a number of different Zta basic region mutations inhibited, but did not abrogate, the binding of TBP. One of these Zta mutants was the K192 and K194 alanine mutant used in the ubinuclein study mentioned above. Therefore, it will also be important to determine whether Zta's interaction with TBP might influence Zta-mediated growth arrest.

Other studies have provided indirect evidence that Zta interacts with chromatin components. Ellwood *et al.* (1999) have previously shown that Zta activates transcription cooperatively with high mobility group (HMG) proteins. This class of proteins generally binds cooperatively to DNA and to transcription factors. It is possible that HMG proteins might make specific interactions with Zta's basic region, although this issue has not been specifically addressed. Interestingly, Zta interacts with the coactivator, CREB binding protein (CBP) (Adamson and Kenney, 1999; Zerby *et al.,* 1999) and Chen *et al.* (2001) have shown that Zta activates the histone acetyl transferase (HAT) activity of CBP specifically in the presence of chromatin. Although the way in which Zta interacts with chromatin in this setting is unclear, Zta might interact directly with chromatin protein components. Such interactions could potentially influence global gene expression patterns which could contribute to gene expression changes that occur during Zta-mediated cell growth arrest.

MATERIALS AND METHODS

Cell culture

The generation of stable inducible HeLa (human cervical carcinoma) cell lines has been described previously (Rodriguez *et al.,* 1999). NPC-KT cells [a hybrid cell line derived from the fusion of AdAH cells and EBV genome-positive NPC epithelial explant (Takimoto *et al.,* 1984)] were a generous gift from Takeshi Sairenji. All cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM; Life Technologies), supplemented with 10% fetal bovine serum (Life Technologies). Media for stable cell lines were also supplemented with 1 μg/ml tetracycline (Sigma) prior to induction.

Transient transfections were performed using a modified calcium phosphate procedure (for a detailed description, go to www.flemingtonlab.com). Approximately 90% confluent cultures were split 1/12 onto 100-mm-diameter tissue culture dishes. The following day, the media were replaced with 8 ml of fresh DMEM (+10% FBS). Four hours later, DNA precipitates were generated by mixing 0.5 ml of 1× HEPES-buffered saline [0.5% HEPES, 0.8% NaCl, 0.1% dextrose, 0.01% anhydrous Na_2HPO_4, 0.37% KCl (pH adjusted to 7.1)] with a total of 30 μg of plasmid DNA (see figure legends for amounts of

plasmid DNA added), followed by the addition of 30 μl of 2.5 M $CaCl_2$ (samples were mixed immediately following the addition of $CaCl_2$). Precipitates were allowed to form at room temperature for 20 min before adding dropwise to cells. Cells were incubated at 37°C with 5% CO_2 for 16 h before the media was replaced with 10 ml of fresh DMEM (+10% FBS).

Plasmids

Plasmid pUHD10 (Gossen and Bujard, 1992) was used to generate tetracycline-inducible (Tet-off) expression plasmids. pUHD10-Zta, -Zta(S186A), and -Zta(129–245) were described previously (Rodriguez *et al.,* 1999). pSV40-based Zta expression plasmids used for transient transfection experiments were previously described (Flemington and Speck, 1990). For transient transfection studies, cells were cotransfected with the green fluorescent protein (GFP) expression vector pGFP-Sp (Kalejta *et al.,* 1997). Site-directed Zta mutant expression vectors were generated using the plasmid, pSV40-Zta. Mutants were generated as previously described (Foss and McClain, 1987) and screened by sequencing.

Northern blot analysis

Northern blot experiments were carried out as previously described (Cayrol and Flemington, 1995). Briefly, RNAs from HeLa and HeLa–Zta cells grown in the presence or absence of tetracycline were isolated by acid guanidium isothiocyanate-phenol-chloroform extraction (Chomczynski and Sacci, 1987). RNAs were denatured, fractionated by electrophoresis through 1% agarose-formaldehyde gels, and transferred to nylon membranes (Hybond-N; Amersham). RNAs were cross-linked to the membranes by UV irradiation. Blots were prehybridized for 2 h and hybridized with the corresponding probes overnight at 42°C in 50% formamide, 6× SSC, 0.5% SDS, 5× Denhardt's solution, 10 mM EDTA (pH 8), 100 μg salmon sperm DNA/ml. Blots were washed with 2× SSC, 0.5% SDS at room temperature for 2 min, then with 2× SSC, 0.1% SDS for 15 min, and with 0.2× SSC, 0.5% SDS for 2 h at 55°C. Autoradiography (Kodak XR) was performed at −70°C for 1–3 days.

Western blot analysis

Following trypsinization and neutralization, a fraction of the harvested cells were washed one time with 1× phosphate buffered saline (PBS). The cell pellet was quickly suspended in 15 vol of sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) loading buffer [0.125 M Tris (pH 6.8), 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, 0.05% (w/v) bromphenol blue] and boiled for 20 min to shear the genomic DNA. Cell lysates were subjected to SDS–PAGE electrophoresis and transferred to nitrocellulose membranes. The blots were blocked for 30 min in 1× Tris-buffered saline plus TWEEN 20 [TBST, 0.25 mM Tris (pH 7.4), 137 mM NaCl, 2.6 mM KCl, 0.1% TWEEN 20] containing 5% low-fat powdered milk and 3% fetal bovine serum and then incubated with the indicated primary antibody (in blocking buffer) for either 1 h at room temperature or overnight at 4°C. The blots were washed three times with 1× TBST (each wash was carried out for approximately 15 min). The blots were then incubated with peroxidase-conjugated secondary antibody in blocking buffer for 1 h at room temperature. Blots were washed as described above and analyzed with an enhanced chemiluminescence detection system (New England Nuclear) according to manufacturer's recommendations, and filters were exposed to Kodak XR film. Antibodies used for each experiment are indicated in their respective figure legends.

Cell cycle analysis

For cell cycle analysis, cells were collected, washed once with 1× PBS, suspended in cold (4°C) 0.5 ml of 1× PBS–0.1% glucose, fixed with 5 ml of 70% cold ethanol (−20°C) for at least 45 min (up to 1 week) at 4°C, washed with 1× PBS, and treated for 30 min at 37°C with RNase A (0.5 mg/ml) in a 69 mM propidium iodide (Sigma), 38 mM sodium citrate solution. Cell cycle analysis was carried out with a fluorescence-activated cell sorter (FACS) (Becton–Dickinson). Detailed protocols for all methods can be found at www.flemingtonlab.com.

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