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4 **Epstein-Barr virus nuclear protein EBNA3C is required for cell cycle progression**
5 **and growth maintenance of lymphoblastoid cells**

6

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19 Abbreviations: EBV, Epstein-Barr virus; EBNA, EBV nuclear antigen; E3C-HT,

20 conditionally active EBNA3C ; LCL, lymphoblastoid cell line; 4HT, 4-hydroxytamoxifen;

21 HTER, 4HT-dependent mutant estrogen receptor hormone-binding domain; pRb,

22 retinoblastoma protein; Cdk, cyclin-dependent kinase

23

Abstract

1
2 Epstein-Barr virus (EBV) infection converts primary human B cells into continuously
3 proliferating lymphoblastoid cell lines (LCLs). To examine the role of EBV nuclear
4 antigen 3C (EBNA3C) in the proliferation of LCLs, we established LCLs infected with an
5 EBV recombinant that expresses EBNA3C with a carboxy-terminal fusion to a 4-
6 hydroxytamoxifen (4HT)-dependent mutant estrogen receptor (E3C-HT). In the presence
7 of 4HT, LCLs expressed E3C-HT protein and grew like wild-type LCLs. When E3C-HT
8 EBV-infected LCLs were transferred to medium without 4HT, E3C-HT protein slowly
9 disappeared and the LCLs gradually ceased growing. Wild-type EBNA3C expression
10 from an oriP plasmid transfected into E3C-HT LCLs protected the LCLs from growth
11 arrest in medium without 4HT, whereas expression of EBNA3A or EBNA3B did not. The
12 expression of other EBNA proteins, and of LMP1, CD21, CD23, and c-myc, was
13 unaffected by EBNA3C inactivation. However, EBNA3C inactivation resulted in the
14 accumulation of p16^{INK4A}, a decrease in the hyperphosphorylated form of the
15 retinoblastoma protein, and a decrease in the proportion of cells in S or G₂/M phase.
16 These results indicate that EBNA3C has an essential role in cell cycle progression and
17 growth maintenance of LCLs.

18

Introduction

1
2 Epstein-Barr virus (EBV) causes lymphocyte-proliferative diseases in immune-deficient
3 patients, and is associated with Burkitt's lymphoma, Hodgkin's lymphoma, other B- and
4 T-cell lymphomas, anaplastic nasopharyngeal carcinoma, and some gastric carcinomas
5 (for review, see ref. 1). EBV infection *in vitro* converts primary human B cells into
6 continuously proliferating lymphoblastoid cell lines (LCLs) (2). In LCLs, EBV expresses
7 six nuclear proteins (EBV nuclear antigens EBNA1, -2, -3A, -3B, -3C, and -LP), three
8 integral membrane proteins (LMP1, -2A, and -2B), two small non-polyadenylated RNAs
9 (EBER1 and EBER2), and Bam A rightward transcripts (for review, see ref. 3). Six of
10 these viral latency proteins, EBNA1, -2, -3A, -3C, -LP, and LMP1, are absolutely or
11 critically required for the transformation phenotype (3).

12 EBNA3A, EBNA3B, and EBNA3C, which are arranged in tandem in the EBV genome,
13 are encoded by genes that are similar in structure (4-8), leading to the proposal that the
14 EBNA3 genes may have arisen from a tandem triplication of an ancestral gene (9-11).
15 The N-terminal amino acids of EBNA3A, EBNA3B, and EBNA3C mediate interaction
16 with a sequence-specific DNA-binding protein, RBP-J κ (12-16). Reverse genetic
17 experiments with recombinant EBVs indicate that EBNA3A and EBNA3C are essential
18 for the EBV-mediated conversion of primary B cells into LCLs, whereas EBNA3B is
19 dispensable (17-20). The role of EBNA3C in LCL outgrowth and continuous proliferation
20 has been only partially delineated, but, using transient transfection reporter assay,
21 EBNA3C has been shown to play a complex regulatory role in the transcription of viral
22 and cellular genes (21-26). EBNA3C has also been shown to interact with histone
23 deacetylase and with the corepressor CtBP (27-29). In addition to its transcriptional

1 functions, it has been reported that EBNA3C has cell cycle regulatory functions,
2 presumably mediated by direct protein-protein interactions. EBNA3C expression
3 stimulates cyclin A-dependent kinase activity (30, 31). It also recruits the SCF^{SKP2}
4 ubiquitin ligase complex, and regulates the stability of the cell cycle modulatory protein
5 p27 (32). More recently, EBNA3C has been shown to mediate the degradation of the
6 retinoblastoma protein (pRb), with the assistance of the SCF^{SKP2} complex, in transiently
7 or stably transfected cells (33).

8 The experiments reported here were performed to identify the mechanisms by which
9 EBNA3C contributes to LCL growth maintenance. The EBNA3C open reading frame
10 (ORF) was fused in-frame to a 4-hydroxytamoxifen (4HT)-dependent mutant estrogen
11 receptor hormone-binding domain (34) to create an ORF that encodes a conditionally
12 active EBNA3C (E3C-HT) in infected LCLs. We demonstrate that EBNA3C inactivation
13 in LCLs results in growth arrest without affecting the expression of other EBNA proteins
14 or of LMP1. This growth arrest is accompanied by an accumulation of p16^{INK4A} and a
15 decrease in the hyperphosphorylated form of pRb.

16

Results

1
2 **Establishment of LCLs by infection with recombinant EBV expressing**
3 **conditionally active EBNA3C.** AK-BAC-GFP, which has a BAC sequence and a Neo^R
4 marker for drug selection in mammalian cells (35), was used to construct a recombinant
5 EBV that expresses a conditionally active form of EBNA3C fused to a 4-
6 hydroxytamoxifen (4HT)-responsive modified estrogen receptor hormone-binding
7 domain (HTER). A DNA fragment containing HTER and the zeocin-resistance gene (a
8 bacterial selection marker) was inserted downstream of the last codon of the EBNA3C
9 ORF of AK-BAC-GFP by GET recombination in *E. coli* (Fig. 1A). A successfully
10 recombined BACmid that expressed EBNA3C with its carboxy-terminus fused in-frame
11 to HTER (E3C-HT-zeo BAC) was purified from *E. coli*, and the zeocin marker was
12 removed *in vitro* by treatment with Cre recombinase to make E3C-HT BAC (Fig. 1A).
13 Restriction analysis of E3C-HT BACmid demonstrated the expected digestion patterns
14 using several different enzymes (data not shown). P3HR-1 cells were transfected with
15 E3C-HT BACmid, followed by selection with G418. A P3HR-1 cell clone containing an
16 intact E3C-HT BAC was induced to produce virus. The resulting virus was used to infect
17 primary human B cells, and LCLs were established in microwells in medium
18 supplemented with 4HT. The LCLs were screened by PCR using primers that distinguish
19 between P3HR-1 DNA (type 2 EBV) and AK-BAC-GFP DNA (type 1 EBV). Thirty
20 percent (9 of 30) of the LCLs were positive for type 1 EBNA3C and negative for type 2
21 EBNA3C (data not shown).

22 Four independent LCLs infected with type 1 EBNA3C-containing EBV alone were
23 expanded and reanalyzed by PCR for the presence of type 1 E3C-HT and flanking DNA
24 (Fig. 1B). These LCLs contained type 1 EBNA3B and EBNA3C DNA, and lacked type 2

1 EBNA3B and EBNA3C DNA. Thus, the LCLs were infected with type 1 E3C-HT EBV,
2 but not type 2 P3HR-1 EBV. These LCLs contained the E3C-HT fusion construct, as
3 revealed by PCR analysis with a primer set that amplified the junction between the
4 EBNA3C and HTER sequences (Fig. 1B). Western blot analysis using EBV-immune
5 human serum confirmed that the E3C-HT LCLs did not express wild-type EBNA3C but
6 instead expressed E3C-HT, which is larger than EBNA3C (Fig. 1C).

7 **EBNA3C is essential for LCL growth maintenance.** Kinetic experiments showed
8 that the expression level of E3C-HT protein was stable in the presence of 4HT. When
9 4HT was removed from the culture medium, however, E3C-HT protein slowly
10 disappeared and was barely detectable after 2 weeks of cultivation, indicating that it is
11 regulated by 4HT (Fig. 2A). The growth of four independent E3C-HT LCLs was
12 examined in the absence or presence of 4HT. All E3C-HT LCLs grew continuously in
13 medium with 4HT, but the same cells gradually ceased growing in medium without 4HT
14 (Fig. 2B). The growth of control LCLs that expressed wild-type EBNA3C was unaffected
15 by 4HT (Fig. 2B). To evaluate whether heterologous EBNA3C expression could
16 complement E3C-HT inactivation, E3C-HT LCLs were transfected with an oriP plasmid
17 expressing wild-type EBNA3C, followed by cultivation in medium with or without 4HT.
18 The LCL transfection efficiency with an EGFP-expressing oriP plasmid was 20-40%, as
19 estimated from the number of EGFP-positive cells. Wild-type EBNA3C-transfected cells
20 grew almost as well in the absence of 4HT as in its presence (Fig. 2D). In contrast,
21 transfection with EBNA3A, EBNA3B or the control oriP plasmid did not rescue the LCLs
22 from the growth arrest induced by E3C-HT inactivation (Fig. 2D). Importantly, wild-type
23 EBNA3C expression was stabilized at a higher level in cells growing in the absence of
24 4HT than in its presence (Fig. 2E). This result demonstrates that wild-type EBNA3C

1 expression specifically enhances the proliferation of E3C-HT LCLs in the absence of
2 4HT. These results indicate that EBNA3C is essential for the maintenance of LCL
3 growth.

4 **E3C-HT inactivation does not affect the expression of other EBNA proteins, or**
5 **LMP1, c-myc, CD21, or CD23.** Since EBNA3C inactivation could affect the growth of
6 LCLs by affecting the expression of other critical EBV latent proteins, we investigated
7 the effect of E3C-HT inactivation on the expression of other EBNA proteins and LMP1.
8 The expression of EBNA1, EBNA2, EBNA3A, EBNA3B and LMP1 in E3C-HT LCLs did
9 not clearly change over 20 days of E3C-HT inactivation (Fig. 3A). Similar results were
10 repeatedly obtained and were reproduced with four independent E3C-HT LCLs (Figs. 2C
11 and 2F). We also evaluated the effect of E3C-HT inactivation on the expression of c-myc,
12 CD21, and CD23, which are regulated by EBNA2 (36-39). The levels of c-myc
13 expression were similar in the absence or presence of 4HT (Fig. 3A). The levels of
14 CD21 and CD23 expression on the surfaces of LCLs were also indistinguishable under
15 both conditions (Fig. 3B). Therefore, E3C-HT inactivation seems to affect the growth of
16 LCLs without changing the expression of other critical viral latent proteins, or c-myc.

17 **EBNA3C inactivation results in a decrease of cells in S and G₂/M, which is**
18 **accompanied by a decrease in hyperphosphorylated pRb.** The effect of E3C-HT
19 inactivation on the cell cycle distribution of LCLs was assessed (Fig. 4A). In 4HT-
20 containing medium, 54% of E3C-HT LCLs were in G₀/G₁, 25% in S, 13% in G₂/M, and
21 8% in SubG₁. Cell cycle profile of E3C-HT LCLs in the presence of 4HT was unchanged
22 with time (data not shown). When LCLs were cultured in medium without 4HT, the
23 proportions of cells in S and G₂/M progressively fell to 5% and below 3%, respectively,
24 and the proportion in SubG₁ progressively increased to 31% (Fig. 4A). Growth and cell

1 cycle distribution of wild-type LCLs did not change in the absence or presence of 4HT,
2 indicating that 4HT itself has no effect on cell cycle entry and cell survival (Fig. 2B and
3 data not shown). Thus, EBNA3C inactivation inhibits cell cycle progression and results in
4 an increase in the percent of hypodiploid cells. Western blot analysis showed that a
5 fraction of pRb was consistently hyperphosphorylated in the presence of 4HT. By
6 contrast, in the absence of 4HT, hyperphosphorylated pRb progressively diminished (Fig.
7 4B). The expression levels of hypophosphorylated pRb were similar in the absence and
8 presence of 4HT (Fig. 4B, short exposure). Therefore, EBNA3C inactivation decreases
9 cell cycle progression, which is accompanied by a decrease in hyperphosphorylated
10 forms of pRb.

11 **EBNA3C inactivation results in the accumulation of p16^{INK4A}.** Since EBNA3C
12 inactivation caused a decrease in pRb phosphorylation, we examined the effect of
13 EBNA3C inactivation on the expression of cyclin-dependent kinase (Cdk) inhibitors.
14 Western blot analysis showed that the p16^{INK4A} protein accumulated in E3C-HT LCLs
15 that were cultured in medium without 4HT (Fig. 5A). The expression of cyclin A gradually
16 decreased after EBNA3C inactivation (Fig. 5A). In contrast, we did not see any clear
17 differences in the expression of p21 and p27 in the absence or presence of 4HT (Fig.
18 5A). p16^{INK4A} did not accumulate in the E3C-HT LCLs that were transfected with an oriP
19 plasmid expressing wild-type EBNA3C and cultured in medium without 4HT, indicating
20 that heterologous expression of EBNA3C prevents the accumulation of p16^{INK4A} in LCLs
21 (Fig. 5B). Real-time RT-PCR analysis revealed that the level of p16^{INK4A} transcript was
22 higher in the absence of 4HT than in its presence and that it progressively increased
23 after EBNA3C inactivation. Thus, the accumulation of p16^{INK4A} occurs at the mRNA level
24 (Fig. 5C). Since p16^{INK4A} was previously shown to disrupt cyclin D/Cdk4, 6 kinase

1 complexes (40, 41), Cdk4 complexes in E3C-HT LCLs were analyzed in the absence
2 and presence of 4HT. Lysates prepared from LCLs cultured with or without 4HT were
3 immunoprecipitated with an anti-Cdk4 antibody. The immunoprecipitates were then
4 subjected to Western blot analysis with anti-p16 and anti-cyclin D2 antibodies. We
5 examined cyclin D2, but not cyclin D1, because it had been reported that cyclin D1 is not
6 detected in LCLs (42). The expression levels of Cdk4, Cdk6, and cyclin D2 in the LCL
7 lysates were similar in the absence or presence of 4HT (Fig. 5D). Almost identical
8 amounts of Cdk4 were immunoprecipitated from LCLs cultured with or without 4HT.
9 Cdk4 complexes from LCLs cultured without 4HT contained p16^{INK4A}, but Cdk4
10 complexes from LCLs cultured with 4HT did not (Fig. 5D). In contrast, Cdk4 complexes
11 from LCLs cultured with 4HT contained significantly more cyclin D2 than did LCLs
12 cultured without 4HT (Fig. 5D). These results indicate that the p16^{INK4A} that accumulates
13 in LCLs after EBNA3C inactivation physically associates with Cdk4, and that EBNA3C
14 inactivation results in an increase of the p16^{INK4A}/Cdk4 complex and a decrease of the
15 cyclinD2/Cdk4 complex in LCLs.

16

Discussion

We established LCLs expressing a conditionally active form of EBNA3C (E3C-HT), a system that allowed us to clarify the physiological role of EBNA3C in LCL growth maintenance. The level of E3C-HT protein gradually diminished in the absence of 4HT, and it was almost undetectable after two weeks of culture without 4HT. Concomitant with the disappearance of E3C-HT, E3C-HT EBV-infected LCLs ceased growing in the absence of 4HT. Moreover, expression of wild-type EBNA3C from a transfected oriP plasmid enabled E3C-HT LCLs to grow in the absence of 4HT. These experiments clearly show that EBNA3C has an essential role in maintaining LCL growth.

In LCLs, EBNA3C extensively associates with a cellular transcription factor, RBP-J κ , and it is presumed to modulate EBNA2-RBP-J κ -mediated transcription (12-15). In transient transfection assays, EBNA3C expression strongly represses the EBNA2-mediated activation of the EBV Cp EBNA promoter, and it also activates the EBV LMP1 promoter in cooperation with EBNA2 (21-26). However, the effects of EBNA3C on cellular and viral gene expression have never been investigated using a system in which EBNA3C is expressed at physiological levels. The data presented here indicate that EBNA3C inactivation does not clearly affect viral EBNAs or LMP1 expression. c-myc is a well-known EBNA2-regulated gene that has a critical role in LCL growth (37-39). Our data shows that the expression of c-myc is not affected by EBNA3C inactivation in LCLs. Thus, EBNA3C inactivation seems to inhibit LCL growth without affecting the expression of other EBNAs, LMP1, or c-myc.

It has been reported that EBNA3C has cell cycle regulatory functions. It was shown that transfection of cells with EBNA3C results in the accumulation of cells in G₂/M (30).

1 Consistently, our data shows that cells in G₂/M decreased after EBNA3C inactivation.
2 EBNA3C has also been shown to stimulate cyclin A-dependent kinase activity without
3 dramatically changing the level of cyclin A (30, 31). In our data, however, EBNA3C
4 inactivation caused a decrease in the level of cyclin A. This may be due to the decrease
5 of cells in S and G₂/M phase after EBNA3C inactivation, because cyclin A is expressed
6 mainly in S and G₂/M phases (43). Recently, it was reported that EBNA3C recruits the
7 SCF^{SKP2} ubiquitin ligase complex and mediates the degradation of p27 and pRb (32,
8 33). Both hyperphosphorylated and hypophosphorylated forms of pRb are reduced in
9 EBNA3C-transfected cells (33). However, in our data, there was no obvious increase in
10 p27 or pRb mediated by EBNA3C inactivation in LCLs, whereas hyperphosphorylated
11 pRb was decreased by EBNA3C inactivation. Differences in the expression levels of
12 EBNA3C and/or differences in cell type may be possible explanations for these
13 discrepancies. Alternatively, the increase of hypodiploid cells after EBNA3C inactivation
14 might mask the increase in pRb, because it has been reported that pRb is a substrate of
15 caspases and is degraded in apoptotic cells (44). This may also explain why EBNA3C
16 inactivation in LCLs did not cause the accumulation of hypophosphorylated pRb, even
17 though hyperphosphorylated pRb decreased.

18 In the studies presented here, a dominant effect of EBNA3C inactivation in LCLs was
19 the induction of p16^{INK4A} transcription and the accumulation of p16^{INK4A} protein. p16^{INK4A}
20 has been implicated in G₁ cell cycle arrest in various cell types, and it specifically
21 associates with Cdk4 and Cdk6 and prevents them from binding D-type cyclins (40, 41).
22 The association of p16 with Cdk4 and Cdk6 thus results in the inactivation of Cdk
23 proteins, accumulation of hypophosphorylated pRb, and eventual G₁ arrest (40, 41).
24 Immunoprecipitation experiments showed that p16^{INK4A}, which was induced by EBNA3C

1 inactivation, associated with Cdk4 in LCLs. Moreover, the amount of cyclin D2 that co-
2 precipitated with Cdk4 was less in EBNA3C-inactive LCLs than in EBNA3C-active LCLs.
3 These results indicate that EBNA3C inactivation indeed causes an increase in the level
4 of the p16^{INK4A}/Cdk4 complex and a decrease in the level of the cyclin D2/Cdk4 complex
5 in LCLs. Consistently, a decreased level of hyperphosphorylated pRb and a reduced
6 frequency of cells in S and G₂/M were observed after EBNA3C inactivation. Since the
7 level of p16^{INK4A} mRNA increased after EBNA3C inactivation, EBNA3C may repress
8 p16^{INK4A} expression at the transcriptional level. Nonetheless, it is still unclear whether
9 EBNA3C directly regulates the expression of p16^{INK4A}, and the mechanism by which
10 p16^{INK4A} is induced after EBNA3C inactivation remains to be elucidated. It has been
11 reported that EBNA3C enhances the transformation of rat embryo fibroblasts and
12 relieves p16's suppression of transformation (45). EBNA3C may regulate the p16^{INK4A}-
13 Rb pathway by various mechanisms to maintain the growth of LCLs.

14 Our data also showed that EBNA3C inactivation in LCLs resulted in the increase of
15 hypodiploid cells. This result suggests that EBNA3C contributes to the survival of LCLs.
16 Further investigations are needed to clarify the link between EBNA3C and cell survival.

17 One important finding of our experiments is that EBNA3C has a role in LCL growth that
18 cannot be replaced by EBNA3A or EBNA3B. Transduction of EBNA3C into E3C-HT
19 LCLs prevented growth arrest that was caused by E3C-HT inactivation, whereas the
20 expression of EBNA3A or EBNA3B had no effect. Previous reports of LCLs expressing a
21 conditionally active EBNA3A indicated that EBNA3A, but not EBNA3B or EBNA3C,
22 rescues growth arrest caused by EBNA3A inactivation (16, 19). These results suggest
23 that EBNA3C and EBNA3A have unequivocally distinct functions in supporting LCL
24 growth.

Materials and Methods

Cell lines. P3HR-1 (clone 16) is a Burkitt's lymphoma cell line that contains a type 2 EBV genome. IB4 is an LCL transformed with B95-8 strain EBV. LCLs were maintained in RPMI 1640 medium supplemented with 15% FBS, L-glutamine, streptomycin, penicillin, and with or without 400 nM 4-hydroxytamoxifen (4HT) (Sigma).

Plasmids. Two *loxP* sites of pBS246 (Life Technologies) were replaced with mutated *loxP* sites (2272 loxP) with synthetic oligonucleotides to construct pBS246-mloxp2272. The blunted FokI-BclI fragment of pcDNA4/HisMax (Invitrogen) containing the zeocin resistance marker gene (*zeo*) was cloned into the EcoRV site of pBS246-mloxp2272 to make pBS246-mloxp-*zeo*. A SpeI fragment containing 4-hydroxy-tamoxifen-responsive modified estrogen receptor hormone-binding domain (HTER) from pBSKS+ERTM (a gift of T. Littlewood) was cloned into SpeI site of pBS246-mloxp-*zeo* to construct pBS246-mloxp-*zeo*-HTER (34). OriP plasmids expressing flag, EBNA3A, EBNA3B, or EBNA3C under the control of SV40 promoter have been described (19). Lytic EBV replication was induced with pSVNael Z (19).

Construction of E3C-HT EBV BACmid. AK-BAC-GFP derived from a type 1 EBV Akata strain has been described (35). Miniprep, maxiprep, and analysis of BACmid DNA have also been described (35). Construction of E3C-HT EBV BACmid was performed in *E. coli* containing AK-BAC-GFP by Red γ , RecE, RecT (GET) recombination as described previously (35, 46). Plasmid pBS246-mloxp-*zeo*-HTER was PCR amplified with primers 3CHT-FW (CGTGTTCCCAAAGGACGTGAAGCAGACTGACTACGATGCATCCACTGAAAGTGAG CTGGATCCACGAAATGAAATGG) and 3CHT-RV

1 (CCATGTTTATGTGTCAGTCAAAGATCAAATAGCTAACAGGGGGGCACCTTGGATCCC
2 CCCTTTCGTCTTCAAGAATTC) to obtain a linear targeting construct required for GET
3 recombination. The resultant PCR product (1768 bp long) containing HTER, zeocin
4 marker surrounded by *mloxP* sites, and 57-bp sequences homologous to the target
5 regions of AK-BAC-GFP at 5' and 3' ends was purified, *DpnI*-digested, and
6 electroporated into recombinase-induced DH10B electrocompetent *E. coli* (harboring
7 AK-BAC-GFP and pGETrec). The successfully recombined BACmid (E3C-HT-zeo BAC)
8 was purified from zeocin-resistant *E. coli*, and treated with Cre recombinase (Novagen)
9 *in vitro* to remove zeocin marker. The resultant BACmid (E3C-HT BAC) was purified
10 from re-transformed *E. coli*.

11 **Transfection of BACmids into P3HR-1 cells.** P3HR-1 c16 cells (5×10^6) were
12 transfected with 5 μ g of BACmid DNA via electroporation (Bio-Rad Gene Pulser II; 190
13 V, 950 μ F). Cells were selected in medium containing 1 mg of G418 (Sigma) per ml in
14 96-well tissue culture plates. G418-resistant clones were screened for the presence of
15 intact BACmids. BAC episomes were isolated from P3HR-1 cells by an alkaline lysis
16 procedure and analyzed as described previously (35).

17 **Virus production and primary B-lymphocyte infections.** Lytic EBV infection was
18 induced in P3HR-1 cell clones (5×10^6) by transfection with 20 μ g of pSVNael Z via
19 electroporation. Cell-free virus was prepared by filtration (0.45- μ m-pore-size filter) of the
20 culture supernatant. Primary human mononuclear cells were infected with diluted virus
21 and aliquots were distributed into each well of 96-microwell plates in medium with
22 400nM 4HT. LCLs were macroscopically visible 3 to 5 weeks after plating.

1 **PCR analyses.** Oligonucleotide primers for amplification of distinctive fragments from
2 type 1 versus type 2 EBNA3B, or EBNA3C have been described (19). Oligonucleotides
3 E3C-FW (AAGCTACTGCTGAAGCACAG) and HT-RV (CTGAAGGGTCTAGAAGGATC)
4 were used to amplify the junction between EBNA3C- and HTER-encoding DNA. Cell
5 DNA was prepared for polymerase chain reaction (PCR), amplified for 40 cycles with
6 annealing at 58°C, and analyzed by electrophoresis. For Real-Time RT-PCR, SYBR
7 Premix Ex Taq kit (Takara) and Light Cycler (Roche) was used. p16-FW primer
8 (CCCGCTTTCGTAGTTTTTCAT) and p16-RV primer (TTATTTGAGCTTTGGTTCTG)
9 were used to quantify p16 transcript. Reactions were done in triplicate. The results were
10 normalized against GAPDH. The calculation for the Real-Time RT-PCR data has been
11 described (47)

12 **Western blotting, immunoprecipitation.** Sodium dodecyl sulfate-polyacrylamide gel
13 electrophoresis (SDS-PAGE) and Western blotting procedures were described previously
14 (19). Anti-c-myc, anti-p16, anti-p21, anti-p27, anti-Cdk4, anti-Cdk6, and anti-cyclin D2
15 antibodies were purchased from Santa Cruz Biotechnology, mouse anti-human Rb
16 monoclonal antibody G3-245 was purchased from Pharmingen. For
17 immunoprecipitation, cells were lysed in immunoprecipitation buffer (1% NP40, 50 mM
18 Tris [pH 7.5], 2 mM EDTA, 150 mM NaCl, supplemented with protease inhibitors).
19 Lysates were precleared and rotated with anti-cdk4 antibody and immune complexes
20 were precipitated with protein A-Sepharose beads. Samples were washed and analyzed
21 by SDS-PAGE and Western blotting.

22 **FACS analyses for cell DNA and surface expression of CD21 and CD23.**

23 Approximately 10⁶ cells were fixed and stained with propidium iodide (Sigma) or
24 incubated live with CD21 or CD23 antibodies (DAKO), followed by incubation with

1 biotinylated anti-mouse IgG and Streptavidin-PE (DAKO). Cells were analyzed using
2 FACSCalibur and Cell Quest software (Becton Dickinson). The percentages of cells in
3 G₀/G₁, S, G₂/M, and SubG₁ were calculated by FLOWJO software (Tree Star).

4 **LCL growth.** 1 x 10⁶ LCL cells were cultured in 25cm² culture flasks in 10 ml complete
5 medium (1 x 10⁵/ml) with or without 4HT. Every 3 to 7 days, the viable cell number was
6 determined by hemocytometer based on trypan blue exclusion, and cultures were
7 diluted to 1 x 10⁵ cells/ml with fresh medium to maintain optimum growth. Total viable
8 cell numbers are calculated based on the expansion from the initial 1 x 10⁶ cells.

9 **Complementation experiments.** 1 x 10⁷ E3C-HT LCLs were transfected with 30 µg of
10 oriP plasmid expressing wild-type EBNA3C, EBNA3A, EBNA3B, or control oriP plasmid
11 via electroporation (Bio-Rad Gene Pulser II; 210 V, 950 µF). Transfected LCLs were
12 cultured in medium with 4HT for 5 days. Cells were then washed and 1 x 10⁶ cells were
13 cultured in 10 ml of complete medium with or without 4HT in a 25cm²-culture flask.
14 Every 4 to 8 days, viable cell numbers were counted, cultures were split and total viable
15 cell numbers were calculated relative to the initial culture.

16

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1
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- 11

Figure Legends

1
2
3 **Figure 1.** Establishment of LCLs infected with recombinant EBV expressing E3C-HT.
4 (A) Construction of the E3C-HT EBV BACmid. A schematic representation of the EBV
5 genomic EBNA3 region of AK-BAC-GFP is shown. A DNA construct, containing the 4-
6 hydroxytamoxifen-responsive mutant estrogen receptor hormone-binding domain
7 (HTER) and a zeocin marker (zeo) flanked by mutant loxP sites, was inserted
8 downstream of the EBNA3C ORF in AK-BAC-GFP by GET recombination. The zeocin
9 marker was removed *in vitro* by treatment with Cre recombinase. The positions of PCR
10 primers are indicated (arrows). (B) PCR analysis of the EBNA3B and EBNA3C
11 genotypes of E3C-HT LCLs. The specific primer pairs distinguish between EBV type 1
12 and type 2 EBNA3B (E3B) and EBNA3C (E3C), and primers flanking the junction
13 between the EBNA3C and HTER genes (E3C-HT) were used for amplification of
14 fragments from type 1 wild-type (WT) LCLs, type 2 P3HR-1 cells, and four independent
15 E3C-HT LCLs. Control amplifications without template are also shown (Primers). The
16 E3B primers amplify 125- or 149-bp fragments from type 1 or type 2 DNA, respectively.
17 The E3C primers amplify 153- or 246-bp fragments from type 1 or type 2 DNA,
18 respectively. (C) E3C-HT LCLs express a large E3C-HT fusion protein and do not
19 express WT-EBNA3C. Total cell lysates of two independent WT LCLs and four
20 independent E3C-HT LCLs were tested for the expression of EBNA3s by Western
21 analysis with EBV-immune human serum.

22

1 **Figure 2.** EBNA3C is essential for LCL growth maintenance. (A) The E3C-HT protein
2 slowly disappears after LCLs are transferred to medium without 4HT. Total cell lysates
3 of E3C-HT LCLs, cultured in the absence (-) or presence (+) of 4HT for the indicated
4 number of days, were immunoblotted with EBV-immune human serum or with an actin
5 antibody. (B) E3C-HT LCLs depend on 4HT for growth. WT LCLs and four independent
6 E3C-HT LCLs were cultured with (+) or without (-) 4HT. Viable cell numbers were
7 determined by trypan blue exclusion, and total viable cell numbers derived from initial
8 cell cultures are plotted. (C) Four independent E3C-HT LCLs were cultured in the
9 absence (-) or presence (+) of 4HT for 17 days. Total cell lysates were prepared and
10 subjected to Western blot analysis. (D) WT-EBNA3C expression sustains the growth of
11 E3C-HT LCLs in the absence of 4HT. E3C-HT LCLs were transfected with an oriP
12 plasmid expressing EBNA3A (E3A), EBNA3B (E3B), EBNA3C (E3C), or a control oriP
13 plasmid (Cont.). Five days after transfection (day 0), cells were washed and transferred
14 to medium with (+) or without (-) 4HT, and cell growth was determined as described in
15 (B). (E) E3C-HT LCLs express higher levels of transfected WT-EBNA3C in the absence
16 of 4HT than in its presence. Total cell lysates were prepared from the cultures in (D) at
17 the indicated time points and tested for EBNA3 expression by Western blot analysis with
18 EBV-immune human serum. Note that transfected WT-EBNA3C is derived from the EBV
19 strain B95-8 and therefore migrates between EBNA3A and EBNA3B on a SDS-
20 polyacrylamide gel. (F) Total cell lysates were prepared from the cultures in (D) at day
21 16 and subjected to Western blot analysis.

22

1 **Figure 3.** E3C-HT inactivation does not affect the expression of other EBNAs, LMP1, c-
2 myc, CD21, or CD23. (A) E3C-HT LCLs were cultured in the absence (-) or presence (+)
3 of 4HT. Total cell lysates were prepared at the indicated time points and analyzed by
4 Western blot with EBV-immune human serum, or LMP1-, c-myc-, or actin-specific
5 antibodies. (B) E3C-HT LCLs were cultured with (+) or without (-) 4HT for 14 days. The
6 cell surface expression of CD21 and CD23 was monitored by flow cytometry (solid lines).
7 Staining with isotype-matched control antibody is indicated by dotted lines. Each
8 experiment was repeated with two independent E3C-HT LCLs with similar results.
9

1 **Figure 4.** EBNA3C inactivation results in a decrease in the proportion of cells in S and
2 G₂/M, which is accompanied by a decrease in the level of hyperphosphorylated pRb. (A)
3 E3C-HT LCLs were cultured in medium without 4HT for the indicated number of days.
4 Cells were fixed and stained with propidium iodide, and cell cycle analysis was
5 performed with a FACSCalibur. (B) E3C-HT LCLs were cultured in the absence (-) or
6 presence (+) of 4HT for the indicated number of days. Total cell lysates were prepared
7 and Western blot analysis was performed with Rb- or actin-specific antibodies. Each
8 experiment was repeated with two independent E3C-HT LCLs with similar results.
9

1 **Figure 5.** EBNA3C inactivation results in the accumulation of p16^{INK4a}. (A) EBNA3C
2 inactivation results in an increase in the level of p16 protein in LCLs. E3C-HT LCLs were
3 cultured with (+) or without (-) 4HT for the indicated number of days. Western blot
4 analysis with p16-, p21-, p27-, cyclin A (cycA)-, or actin-specific antibodies was
5 performed. (B) Transfection of WT-EBNA3C into E3C-HT LCLs prevents the
6 accumulation of p16 in the absence of 4HT. E3C-HT LCLs were transfected with an oriP
7 plasmid expressing WT-EBNA3C (E3C) or a control oriP plasmid (Cont). Cells were
8 cultured in medium with (+) or without (-) 4HT for 21 days and subjected to Western blot
9 analysis with p16- and actin-specific antibodies. (C) EBNA3C inactivation results in an
10 increase in the level of p16 transcript. Total RNA was extracted from E3C-HT LCLs
11 cultured in medium with (+) or without (-) 4HT for 6 or 11 days. Real-Time RT-PCR for
12 p16 was performed, and the results were normalized against GAPDH. Reactions were
13 done in triplicate. (D) EBNA3C inactivation results in an increase in the level of
14 p16/Cdk4 complex and a decrease in the level of cyclin D2/Cdk4 complex. E3C-HT
15 LCLs cultured with (+) or without (-) 4HT for 20 days were lysed and immunoprecipitated
16 with a Cdk4-specific antibody. Total cell lysates were subjected to Western blot analysis
17 with Cdk4-, Cdk6-, cyclin D2 (cycD2)-, and actin-specific antibodies. Immunoprecipitated
18 samples (Cdk4 IP) were resolved by SDS-PAGE and blotted with Cdk4-, p16-, and
19 cyclin D2- specific antibodies. Each experiment was repeated with two independent
20 E3C-HT LCLs with similar results.

21

Fig. 1

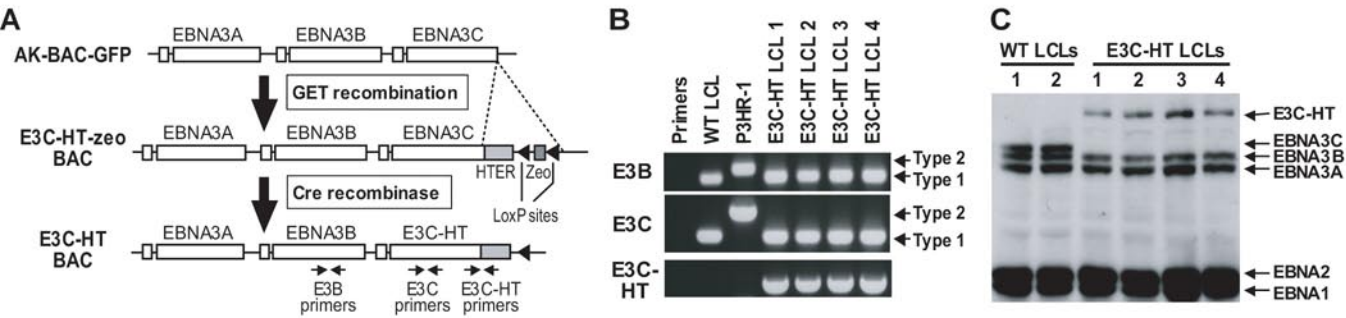


Fig. 2

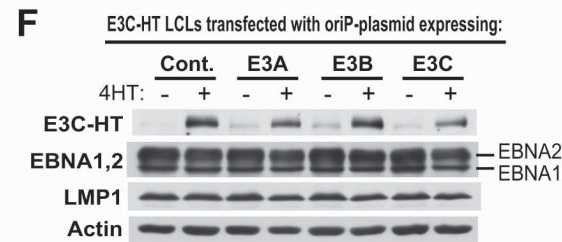
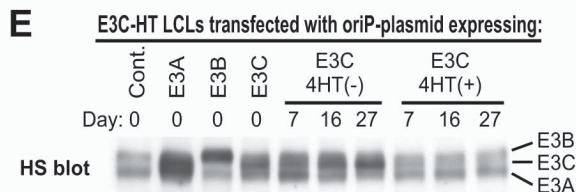
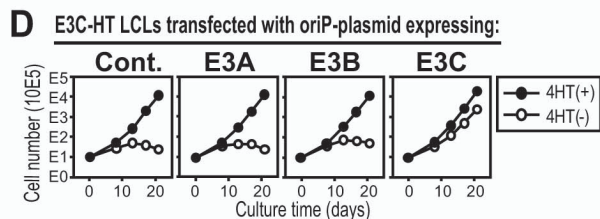
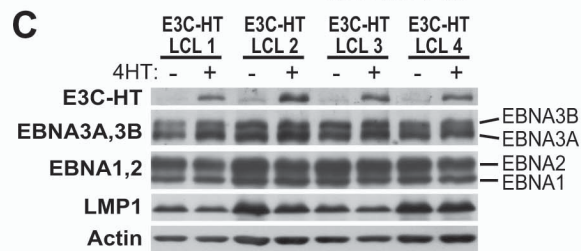
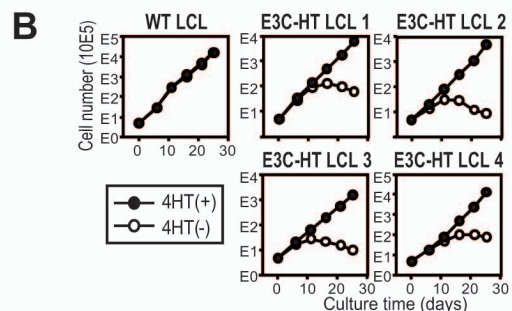
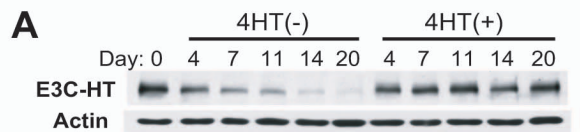
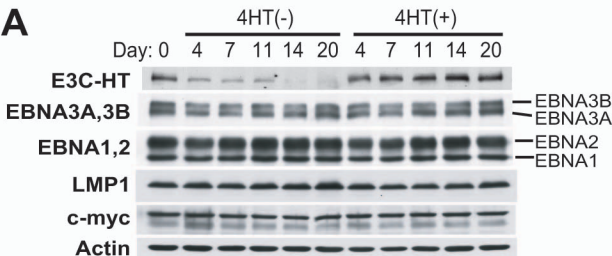


Fig. 3

A



B

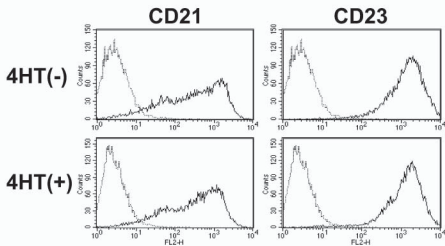


Fig. 4

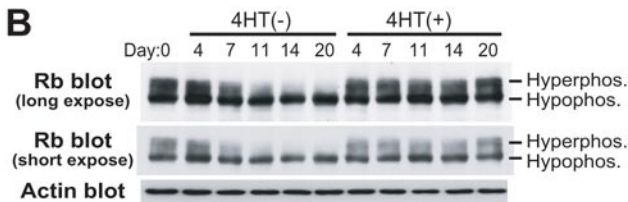
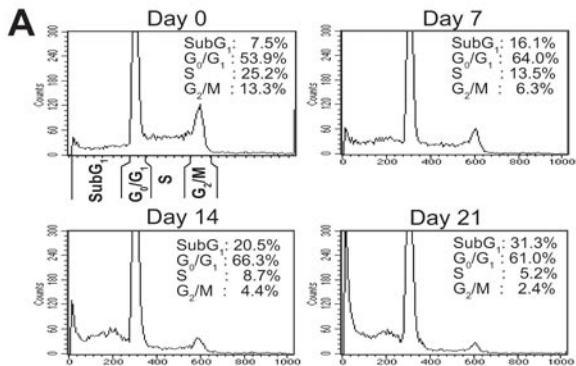


Fig. 5

