

# Epstein-Barr virus LMP1 blocks p16<sup>INK4a</sup>-RB pathway by promoting nuclear export of E2F4/5

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The p16<sup>INK4a</sup>-RB pathway plays a critical role in preventing inappropriate cell proliferation and is often targeted by viral oncoproteins during immortalization. Latent membrane protein 1 (LMP1) of Epstein-Barr virus (EBV) is often present in EBV-associated proliferative diseases and is critical for the immortalizing and transforming activity of EBV. Unlike other DNA tumor virus oncoproteins, which possess immortalizing activity, LMP1 does not bind to retinoblastoma tumor suppressor protein, but instead blocks the expression of p16<sup>INK4a</sup> tumor suppressor gene. However, it has been unclear how LMP1 represses the p16<sup>INK4a</sup> gene expression. Here, we report that LMP1 promotes the CRM1-dependent nuclear export of Ets2, which is an important

transcription factor for p16<sup>INK4a</sup> gene expression, thereby reducing the level of p16<sup>INK4a</sup> expression. We further demonstrate that LMP1 also blocks the function of E2F4 and E2F5 (E2F4/5) transcription factors through promoting their nuclear export in a CRM1-dependent manner. As E2F4/5 are essential downstream mediators for a p16<sup>INK4a</sup>-induced cell cycle arrest, these results indicate that the action of LMP1 on nuclear export has two effects on the p16<sup>INK4a</sup>-RB pathway: (1) repression of p16<sup>INK4a</sup> expression and (2) blocking the downstream mediator of the p16<sup>INK4a</sup>-RB pathway. These results reveal a novel activity of LMP1 and increase an understanding of how viral oncoproteins perturb the p16<sup>INK4a</sup>-RB pathway.

## Introduction

It is well accepted that immortalization is one of the hallmarks of cancer cells (DePinho, 2000; Lundberg et al., 2000; Campisi, 2001). Cells in primary culture undergo irreversible growth arrest, termed cellular senescence when cultured cells reach the end of their replicative lifespan (Hayflick and Moorhead, 1961). It has recently become evident that a similar phenotype can be induced when primary cells are challenged by an activated Ras oncogene or its downstream mediators, Raf and MAPK/ERK kinase (MEK;\* Serrano et

al., 1997; Lin et al., 1998; Zhu et al., 1998). This phenomenon, termed "Ras-induced senescence," is proposed to be a fail-safe mechanism, which protects normal cells from uncontrolled cell proliferation and tumor formation (Serrano et al., 1997; Weinberg, 1997; Serrano and Blasco, 2001; Sotillo et al., 2001; Drayton and Peters, 2002; Lloyd, 2002). In each case, the arrest is accompanied by induction of p16<sup>INK4a</sup>, an inhibitor of Cdks, and accumulation of the unphosphorylated form of the retinoblastoma tumor suppressor protein (pRB) (Stein et al., 1990; Alcorta et al., 1996; Hara et al., 1996; Serrano et al., 1997; Stein et al., 1999). Moreover, ectopic expression of p16<sup>INK4a</sup> alone is sufficient to induce features of cellular senescence in human fibroblasts (McConnell et al., 1998). Therefore, p16<sup>INK4a</sup> is thought to be a key mediator of cellular senescence at least in human fibroblasts. The p16<sup>INK4a</sup>-RB pathway plays a critical role in preventing inappropriate cell proliferation and is often targeted by viral oncoproteins during immortalization (Jansen-Durr, 1996; Sherr, 1996; Hunter, 1997;

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\*Abbreviations used in this paper: ChIP, chromatin-immunoprecipitation; E2F4/5, E2F4 and E2F5; EBV, Epstein-Barr virus; HDF, human diploid fibroblast; LMB, leptomycin B; LMP1, latent membrane protein 1; MEF, mouse embryonic fibroblast; MEK, MAPK/ERK kinase; NES, nuclear export signal; pRB, retinoblastoma tumor suppressor protein.

Key words: cell cycle; immortalization; Cdk; Ets; senescence

Kiyono et al., 1998; Nevins, 2001; Classon and Harlow, 2002; Ortega et al., 2002).

Epstein-Barr virus (EBV) is a prevalent human  $\gamma$  herpes virus. It is frequently associated with a number of human proliferative and malignant diseases, including Burkitt's lymphoma, nasopharyngeal carcinoma, Hodgkin's lymphoma, and gastric carcinoma (for review see Farrell, 1995; Thorley-Lawson, 2001). Nine viral oncoproteins are expressed in EBV-established lymphoblastoid cell lines, five of which appear to be absolutely required for B cell immortalization (Hammerschmidt and Sugden, 1989, Cohen et al., 1991; Kaye et al., 1993; Tomkinson et al., 1993; Kilger et al., 1998). Among these oncoproteins, latent membrane protein 1 (LMP1) has been shown to transform established rodent fibroblasts and immortalize primary rodent fibroblasts (Wang et al., 1985; Yang et al., 2000a,b; Eliopoulos and Young, 2001). Unlike other DNA tumor virus oncoproteins, which possess immortalizing activity, such as human papillomavirus E7 or adenovirus E1A (Jansen-Durr, 1996; Classon and Harlow, 2002), LMP does not bind to the pRB family proteins. Recently, LMP1 was shown to block the induction of p16<sup>INK4a</sup> and to prevent Ras-induced senescence in human fibroblasts, suggesting that the p16<sup>INK4a</sup> could be an important target of LMP1 in fibroblasts (Yang et al., 2000b). However, until now, it has been unclear how LMP1 blocks induction of p16<sup>INK4a</sup> expression in primary human fibroblasts.

To obtain mechanistic insight into how LMP1 inhibits p16<sup>INK4a</sup> expression, we examined the effect of LMP1 on Ets2, which is an important transcription factor inducing p16<sup>INK4a</sup> expression in Ras-induced senescence (Ohtani et al., 2001). Here, we report that LMP1 inactivates Ets2 by promoting the intracellular redistribution of Ets2 from the nucleus to the cytoplasm in a CRM1-dependent manner. Furthermore, we find here that LMP1 also inactivates E2F4 and E2F5 (E2F4/5), which are essential downstream mediators of the p16<sup>INK4a</sup>-RB growth arrest pathway (Gaubatz et al., 2000), also through promoting the CRM1-dependent intracellular redistribution of E2F4/5. These findings reveal a novel activity of the LMP1 oncoprotein and would facilitate understanding of how LMP1 oncoprotein of EBV perturbs p16<sup>INK4a</sup>-RB pathway.

## Results

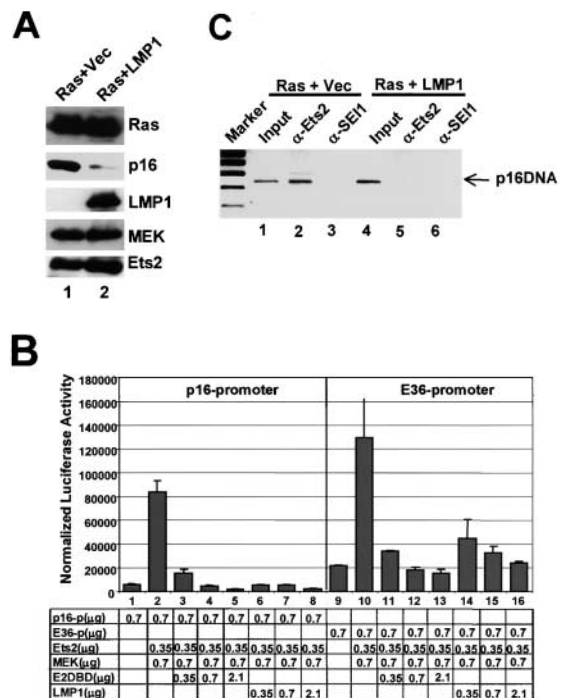
### Inhibition of Ets2 transcriptional activity by LMP1

We have demonstrated previously that the activation of Ets2, which is a downstream mediator of the MAPK cascade, is responsible for the up-regulation of p16<sup>INK4a</sup> in Ras-induced senescence, whereas Ets1 seems to play a role in replicative senescence (Ohtani et al., 2001; Huot et al., 2002). Thus, we first tested whether LMP1 prevents p16<sup>INK4a</sup> expression by blocking Ets2 activity. As shown previously (Yang et al., 2000b), coexpression of LMP1 significantly blocked the induction of p16<sup>INK4a</sup> by oncogenic Ras in human diploid fibroblasts (HDFs; Fig. 1 A, lane 2). Like a dominant negative form of Ets2 (E2DBD; Foos et al., 1998), expression of LMP1 inhibited transcriptional activity of Ets2 on the human p16<sup>INK4a</sup> promoter and on an artificial promoter (E36) containing tandem repeats of the Ets-bind-

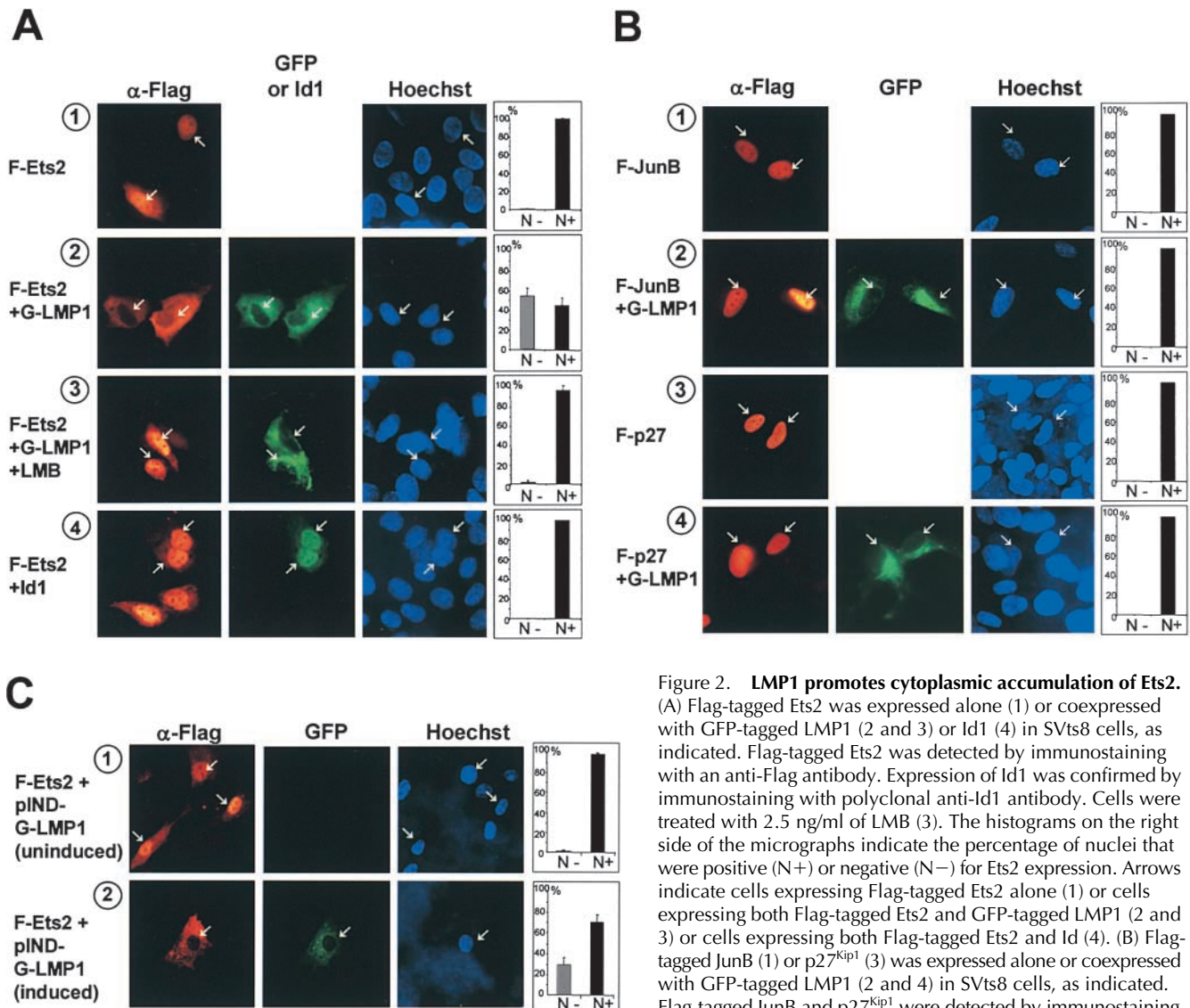
ing sequence (Fig. 1 B). Moreover, chromatin-immunoprecipitation (ChIP) analysis indicated that Ets2 was not bound to the p16<sup>INK4a</sup> promoter in HDFs expressing LMP1 (Fig. 1 C, lanes 2 and 5). However, the level of Ets2 protein was unaffected by LMP1 expression in HDFs (Fig. 1 A). These results strongly suggest that LMP1 blocks Ets2 binding to DNA without affecting the expression level of Ets2.

### LMP1 induces the CRM1-dependent nuclear export of Ets2

Because the LMP1 protein localizes to the cytoplasm (Eliopoulos and Young, 2001), we next asked how LMP1 blocks Ets2 binding to the p16<sup>INK4a</sup> promoter. We examined the subcellular localization of ectopically expressed Ets2 protein in the presence or absence of LMP1 in the human fibroblast cell line, SVts8 cells. In most of the cells, flag-tagged Ets2 is expressed either in the nucleus or in the nucleus and cytoplasm (Fig. 2 A, 1). Coexpression with GFP-tagged LMP1, however, resulted in the accumulation of Flag-tagged Ets2 in the cytoplasm (Fig. 2 A, 2). Similar results were obtained using nontagged Ets2 and nontagged LMP1 expres-



**Figure 1. LMP1 blocks Ets2 binding to DNA.** (A) Early passage (38 PDLs) HDFs expressing ecotropic receptor were infected with a retrovirus encoding H-RasV12 and sequentially infected with a retrovirus encoding LMP1 (lane 2) or control vector (lane 1). 4 d after superinfection, levels of a series of endogenous proteins were examined by immunoblotting using antibodies shown right. MEK was used here as a loading control. (B) Dose-dependent ability of LMP1 and E2DBD to block activation of the p16<sup>INK4a</sup> promoter (left) or E36 promoter (right) by Ets2 and activated MEK in SVts8 cells. Expression plasmids encoding proteins shown bottom were introduced into SVts8 cells along with 0.2  $\mu$ g of MMLV-lacZ plasmid. Luciferase activities were normalized by lac-Z activities. Error bars indicate SD. (C) ChIP assays were performed using cells described in A and antibody against Ets2 or SEI-1 (control). The p16<sup>INK4a</sup>-promoter was recovered by PCR using primers flanking the Ets binding sites in the human p16<sup>INK4a</sup> promoter.



**Figure 2. LMP1 promotes cytoplasmic accumulation of Ets2.**

(A) Flag-tagged Ets2 was expressed alone (1) or coexpressed with GFP-tagged LMP1 (2 and 3) or Id1 (4) in SVts8 cells, as indicated. Flag-tagged Ets2 was detected by immunostaining with an anti-Flag antibody. Expression of Id1 was confirmed by immunostaining with polyclonal anti-Id1 antibody. Cells were treated with 2.5 ng/ml of LMB (3). The histograms on the right side of the micrographs indicate the percentage of nuclei that were positive (N+) or negative (N-) for Ets2 expression. Arrows indicate cells expressing Flag-tagged Ets2 alone (1) or cells expressing both Flag-tagged Ets2 and GFP-tagged LMP1 (2 and 3) or cells expressing both Flag-tagged Ets2 and Id1 (4). (B) Flag-tagged JunB (1) or p27<sup>Kip1</sup> (3) was expressed alone or coexpressed with GFP-tagged LMP1 (2 and 4) in SVts8 cells, as indicated. Flag-tagged JunB and p27<sup>Kip1</sup> were detected by immunostaining with an anti-Flag antibody. Histograms on the right side of the

micrographs indicate the percentage of nuclei that were positive (N+) or negative (N-) for Flag-tagged protein expression. Arrows indicate cells expressing Flag-tagged protein alone (1 and 3) or cells expressing both Flag-tagged protein and GFP-tagged LMP1 (2 and 4). (C) Flag-tagged Ets2 expression vector (pRc/RSV; Invitrogen) was cotransfected with an Ecdyson-inducible vector encoding GFP-tagged LMP1 (pIND; Invitrogen) into early passage (38 PDLs) TIG-3 cells. Expression of GFP-tagged LMP1 was induced by addition of 0.5 μg/ml of Ecdyson homologue, Muristeron A (panel 2). Flag-tagged Ets2 was detected with an anti-Flag antibody 2 d after addition of Muristeron A. Histograms on the right side of the micrographs indicate the percentage of nuclei that were positive (N+) or negative (N-) for Flag-tagged protein expression. Arrows indicate cells expressing Flag-tagged Ets2 alone (1) or cells expressing both Flag-tagged Ets2 and GFP-tagged LMP1 (2). For all panels, error bars indicate SD.

sion vectors (unpublished data). This effect was blocked by treatment with leptomycin B (LMB; Fornerod et al., 1997; Stade et al., 1997; Kudo et al., 1998), a specific inhibitor of CRM1-dependent nuclear export (Fig. 2 A, 3). In contrast, overexpression of GFP-tagged CRM1 resulted in the accumulation of Ets2 in the cytoplasm (unpublished data), suggesting that these effects were mediated through a CRM1-dependent nuclear export mechanism. These effects were specific to Ets2, because LMP1 expression did not have any significant impact on the subcellular localization of other transcription factor, such as JunB (Fig. 2 B, 1 and 2), Elk1, or p53 (not depicted). Moreover, LMP1 failed to promote nuclear export of p27<sup>Kip1</sup>, which is known as a nuclear shuttling protein (Tomoda et al., 1999; Rodier et al., 2001;

Ishida et al., 2002; Fig. 2 B, 3 and 4). Furthermore, another p16<sup>INK4a</sup> repressor, Id1 (Lyden et al., 1999; Ohtani et al., 2001), did not have any impact on the subcellular localization of Ets2 (Fig. 2 A, 4). To confirm that these effects were not due to transfection artifacts, nor limited to this cell line, the subcellular localization of Ets2 was examined using an Ecdyson-inducible vector encoding GFP-tagged LMP1 in the TIG-3 strain of primary HDFs. Although nuclear staining of Ets2 was predominantly observed in the absence of the Ecdyson analogue, Muristeron A (Fig. 2 C, 1), cytoplasmic staining of Ets2 was predominantly observed in the significant percentage of the cells when the expression of GFP-tagged LMP1 was induced by the addition of the Muristeron A (Fig. 2 C, 2). These results strongly suggest that LMP1 re-

presses p16<sup>INK4a</sup> expression through, at least partly, blocking the nuclear localization of Ets2 transcription factor.

### LMP1 targets downstream mediators of the p16<sup>INK4a</sup>-induced growth arrest pathway

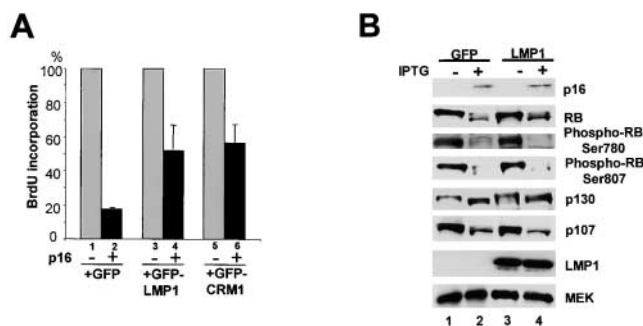
If repression of p16<sup>INK4a</sup> expression is the major function of LMP1 in blocking the p16<sup>INK4a</sup>-RB pathway, ectopic expression of p16<sup>INK4a</sup> should be dominant over the LMP1 function. To test this idea, we used the U2OS cells that have been engineered to induce p16<sup>INK4a</sup> expression by addition of IPTG (EH1 cells; McConnell et al., 1999). As shown previously (McConnell et al., 1999), IPTG treatment significantly blocks entry into S-phase (Fig. 3 A, lanes 1 and 2). However, surprisingly, the ability of p16<sup>INK4a</sup> to induce a G1 arrest was significantly attenuated when LMP1 was coexpressed as seen in CRM1-expressing cells (Fig. 3 A, lanes 3–6). Induction of p16<sup>INK4a</sup> is similarly observed in both LMP1-expressing cells and in control cells expressing GFP (Fig. 3 B, lanes 2 and 4). Moreover, phosphorylation of the pRB family proteins was blocked by the induction of the p16<sup>INK4a</sup> in both cases (Fig. 3 B, lanes 2 and 4), showing that p16<sup>INK4a</sup> is effectively functioning as a Cdk inhibitor in LMP1 expressing cells. These results led us to hypothesize that LMP1 also targets downstream mediator(s) of the p16<sup>INK4a</sup>-induced growth arrest pathway.

### LMP1 induces the CRM1-dependent nuclear export of E2F4/5

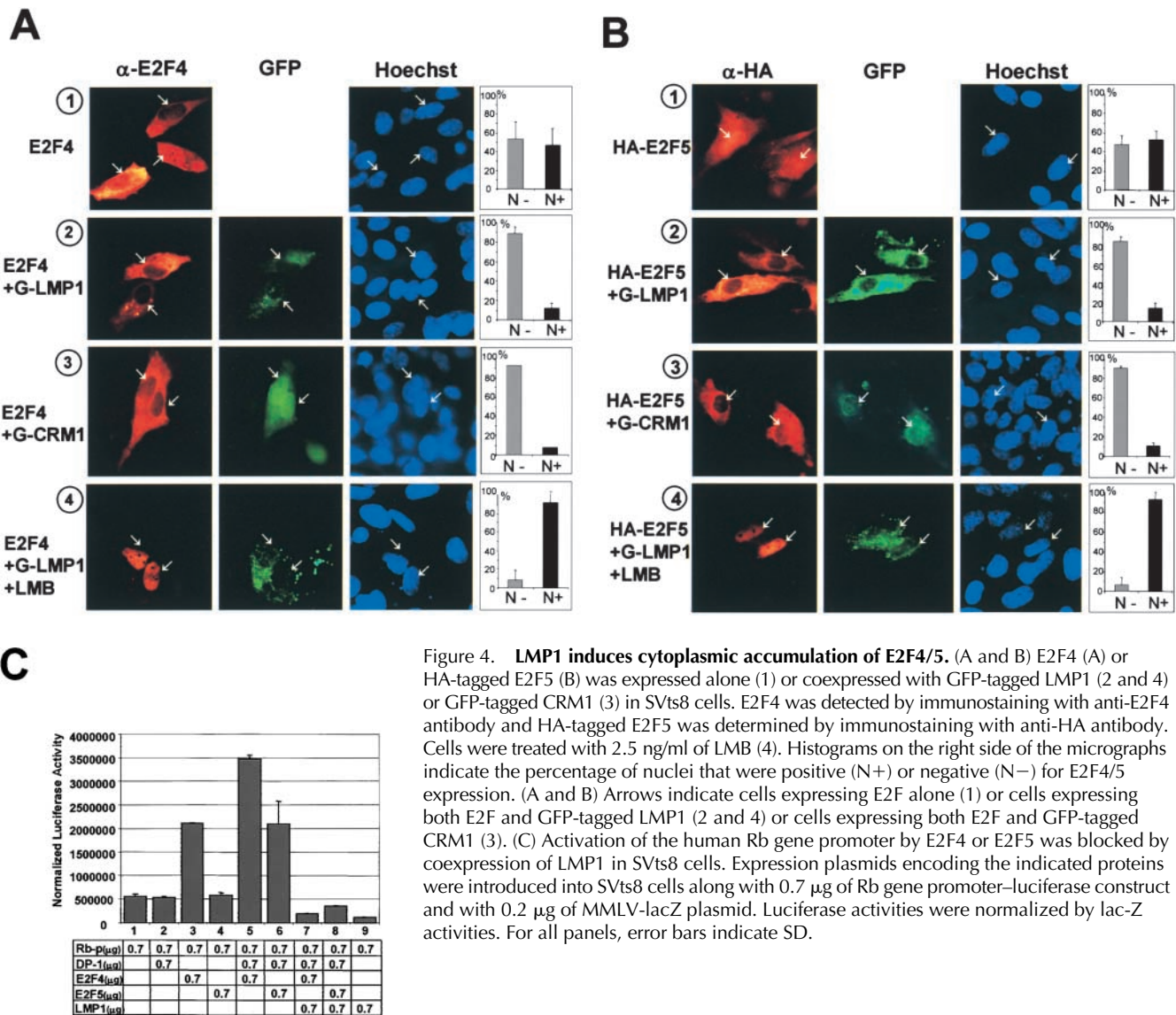
Recent reports suggest that E2F4/5 mainly act as “repressor” E2Fs, which have opposing functions against “the activator” E2Fs, E2F1–3 (Trimarchi and Lees, 2002). Mouse embryonic fibroblasts (MEFs) lacking both repressor E2Fs, E2F4/5, grow normally but are insensitive to a p16<sup>INK4a</sup>-induced

G1 arrest (Gaubatz et al., 2000). Moreover, enforced nuclear export of E2F4/5 by overexpression of CRM1 prevents the ability of p16<sup>INK4a</sup> to induce a G1 arrest in U2OS cells (Gaubatz et al., 2001). This evidence strongly suggests that E2F4/5 are essential downstream mediators of the p16<sup>INK4a</sup>-induced growth arrest pathway (Gaubatz et al., 2000). Therefore, we tested if LMP1 has any effect on the subcellular localization of E2F4 and/or E2F5, which have previously been shown to be regulated by CRM1-dependent nuclear export machinery (Gaubatz et al., 2001; Trimarchi and Lees, 2002). Although 50–60% of cells expressed transfected E2F4/5 in both cytoplasm and nucleus under normal proliferating conditions, coexpression of LMP1 significantly abolished the nuclear localization of E2F4/5 in Svts8 cells (Fig. 4, A and B, 1 and 2). As reported previously (Gaubatz et al., 2001), similar effects were seen by ectopic expression of CRM-1 (Fig. 4, A and B, 3). This cytoplasmic accumulation was blocked by the addition of LMB (Fig. 4, A and B, 4), indicating that LMP1 also promotes intracellular redistribution of E2F4/E2F5 from the nucleus to the cytoplasm in a CRM1-dependent manner. To obtain further proof of the inactivation of E2F4/5 by LMP1, we monitored the transcriptional activity of E2F4/5 using the human Rb gene promoter, which is known to be a target of E2F4 (Ren et al., 2002). Although E2F4/E2F5 induced Rb gene promoter activity, this was abolished when LMP1 was coexpressed, suggesting that the transcriptional activity of E2F4/5 is indeed blocked by LMP1 (Fig. 4 C, 5–8).

To examine whether this is also the case for the endogenous proteins, we have established an LMP1-inducible cell line using an Ecdyson-inducible vector. The level of LMP1 induced in this cell line was similar to the levels expressing in EBV-positive human B cells (Fig. 5 A, lanes 2 and 4), suggesting that the levels of LMP1 in this cell line is likely to be a physiological level. Because the levels of both endogenous Ets2 and endogenous E2F4 were under detectable level by immunofluorescence in this cell line, we examined the levels of both proteins in isolated nuclear and cytoplasmic fractions in the presence or absence of LMP1 expression by immunoblotting. As shown in Fig. 5 B, the levels of endogenous Ets2 and E2F4 in the nuclear fraction were significantly reduced in cells expressing LMP1 (Fig. 5 B, lanes 3 and 4). Moreover, similar results were obtained using TIG-3 cells expressing LMP1 using a retroviral vector (Fig. 5 C, lanes 3 and 4). Although we were unable to examine endogenous E2F5 due to lack of an antibody, these results strongly suggest that LMP1 induces intracellular redistribution of endogenous Ets2 and E2F4/5 from the nucleus to the cytoplasm in human fibroblasts. To test whether this is also the case under the physiological condition of EBV infection, we examined the subcellular localization of endogenous E2F4 in Burkitt lymphoma cells that are positive or negative for EBV infection. As shown in Fig. 5 D, significant levels of E2F4 were observed in both nuclear and cytoplasmic fractions of EBV-negative Burkitt lymphoma cell line, BL41 cells. However, we were unable to detect E2F4 in the nuclear fraction of the BL41 + B95 cells, which are experimentally infected with EBV (Fig. 5 D, lane 4). Ets2 levels were under detectable levels in these cell lines (unpublished data). These results further support the idea that



**Figure 3. LMP1 blocks downstream mediators of the p16<sup>INK4a</sup> pathway.** (A) The p16<sup>INK4a</sup> inducible cell line, EH1 cells (McConnell et al., 1999), were transfected with an expression plasmid encoding GFP-tagged LMP1 or GFP-tagged CRM1 or a control vector expressing GFP alone. 12 h later, cells were treated with (+) or without (–) 1 mM IPTG for p16<sup>INK4a</sup> induction. 48 h after IPTG treatment, cells were labeled with BrdU for 1 h, fixed, and stained with an anti-BrdU antibody. Transfected cells were identified by their green fluorescence, and the percentages of cells that incorporated BrdU compared with the cells without induction of p16<sup>INK4a</sup> were determined. (B) EH1 cells were transfected with an expression plasmid encoding GFP-tagged LMP1 or a control vector expressing GFP alone. 12 h later, cells were treated with (+) or without (–) 1 mM IPTG for p16<sup>INK4a</sup> induction. 48 h after IPTG treatment, GFP-positive cells were isolated by FACS<sup>®</sup> and cell lysates were immunoblotted with antibodies shown right. Error bars indicate SD.



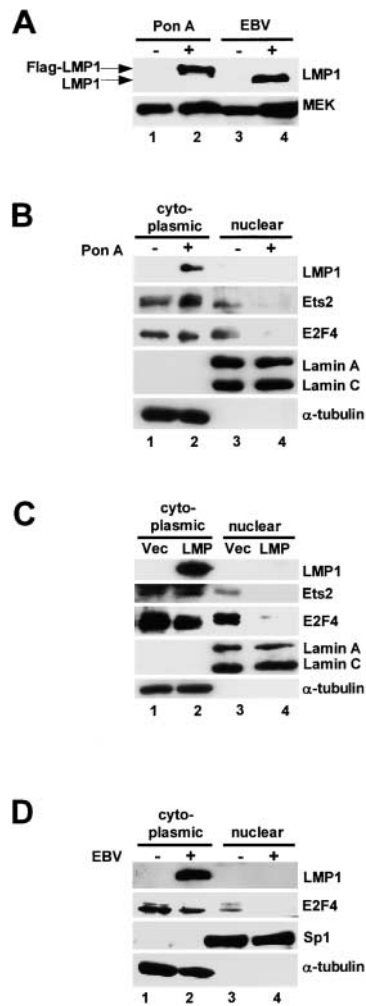
**Figure 4. LMP1 induces cytoplasmic accumulation of E2F4/5.** (A and B) E2F4 (A) or HA-tagged E2F5 (B) was expressed alone (1) or coexpressed with GFP-tagged LMP1 (2 and 4) or GFP-tagged CRM1 (3) in SVts8 cells. E2F4 was detected by immunostaining with anti-E2F4 antibody and HA-tagged E2F5 was determined by immunostaining with anti-HA antibody. Cells were treated with 2.5 ng/ml of LMB (4). Histograms on the right side of the micrographs indicate the percentage of nuclei that were positive (N+) or negative (N-) for E2F4/5 expression. (A and B) Arrows indicate cells expressing E2F alone (1) or cells expressing both E2F and GFP-tagged LMP1 (2 and 4) or cells expressing both E2F and GFP-tagged CRM1 (3). (C) Activation of the human Rb gene promoter by E2F4 or E2F5 was blocked by coexpression of LMP1 in SVts8 cells. Expression plasmids encoding the indicated proteins were introduced into SVts8 cells along with 0.7  $\mu$ g of Rb gene promoter-luciferase construct and with 0.2  $\mu$ g of MMLV-lacZ plasmid. Luciferase activities were normalized by lac-Z activities. For all panels, error bars indicate SD.

LMP1 affects the intracellular location of Ets2 and E2F4/5 under the physiological condition.

### LMP1 induces dissociation of E2F4 from pRB family proteins

To seek mechanistic insight into how LMP1 promotes intracellular redistribution of Ets2 and E2F4/5 from the nucleus to the cytoplasm, we decided to focus our attention on E2F4, because E2F4 contains typical nuclear export signal (NES) sequences and is well established as a nuclear shuttling protein (Gaubatz et al., 2001; Trimarchi and Lees, 2002). Because E2F4/5 lack an NLS, it has been suggested that association with an NLS-containing protein, such as pRB family proteins or with DP2, plays important roles in the nuclear localization of E2F4 (Muller et al., 1997; Verona et al., 1997). Furthermore, a recent report from Rayman and co-workers has shown that E2F4 is localized only in the cytoplasm of MEFs lacking both p107 and p130 (Rayman et al., 2002), suggesting that the association with p107 or p130 are required for the nuclear localization of E2F4. Therefore, we tested if LMP1 blocks the interaction

between endogenous E2F4 and endogenous pRB family proteins. As shown in Fig. 6 A, the interaction between endogenous E2F4 and endogenous p107 was significantly reduced if LMP1 expression was induced in the LMP1-inducible cell line. Similarly, the interaction between endogenous E2F4 and endogenous pRB was also inhibited by LMP1 expression (Fig. 6 A, lanes 1 and 2). These effects were not observed in the control cells, which do not induce LMP1 expression by the addition of Ecdysone homologue, Ponasteron A (Fig. 6 B, lanes 1 and 2), precluding the possibility that these effects were caused by the Ponasteron A treatment. We were unable to see interaction between E2F4 and p130 in this cell line (unpublished data). These effects were not due to the phosphorylation of pRB family proteins by Cdk, because we were unable to see any difference of the phosphorylation pattern of pRB and p107 in the presence or absence of LMP1 expression (Fig. 6 A, lanes 1 and 2). To examine whether or not dissociation of E2F4 from the NLS-containing protein is required for intracellular redistribution of E2F4 from the nucleus to the cytoplasm, E2F4 was fused to the NLS sequence and coexpressed with



**Figure 5. LMP1 induces intracellular redistribution of endogenous Ets2 and E2F4 proteins.** (A) LMP1-inducible cell line was established using SVts8 cells and Ecdyson inducible system (lanes 1 and 2). LMP1 expression was induced by the incubation with 0.5  $\mu$ g/ml of Ecdyson homologue, Ponasteron A for 2 d, and levels were compared with that of EBV-positive (lane 4) and -negative (lane 3) human B cells. MEK was used here as a loading control. (B) LMP1-inducible cell line described in A, was treated with (lanes 2 and 4) or without (lanes 1 and 3) 0.5  $\mu$ g/ml of Ponasteron A for 2 d. Next, both cytoplasmic (lanes 1 and 2) and nuclear (lanes 3 and 4) fractions were prepared and the levels of endogenous E2F4 and Ets2 were analyzed.  $\alpha$ -Tubulin and LaminA/C were used here as cytoplasmic or nuclei marker, respectively. (C) Early passage (38 PDLs) HDFs expressing ecotropic receptor were infected with a retrovirus encoding LMP1 (lanes 2 and 4) or control vector (lanes 1 and 3). 4 d after infection, both cytoplasmic (lanes 1 and 2) and nuclear (lanes 3 and 4) fractions were prepared and the levels of endogenous E2F4 and Ets2 were analyzed.  $\alpha$ -Tubulin and LaminA/C were used here as cytoplasmic or nuclei marker, respectively. (D) Both cytoplasmic and nuclear fractions were prepared from BL41 cells (EBV negative) and BL41 + B95 cells (EBV positive). The levels of endogenous E2F4 were analyzed by Western blotting.  $\alpha$ -Tubulin and Sp1 were used here as cytoplasmic or nuclei marker, respectively. LMP1 expression was confirmed by anti-LMP1 antibody.

LMP1. As shown in Fig. 6 C, the NLS-E2F4 fusion protein is predominantly expressed in the nucleus and is resistant to LMP1-induced cytoplasmic accumulation. This is suggesting that dissociation of E2F4 from the NLS-con-

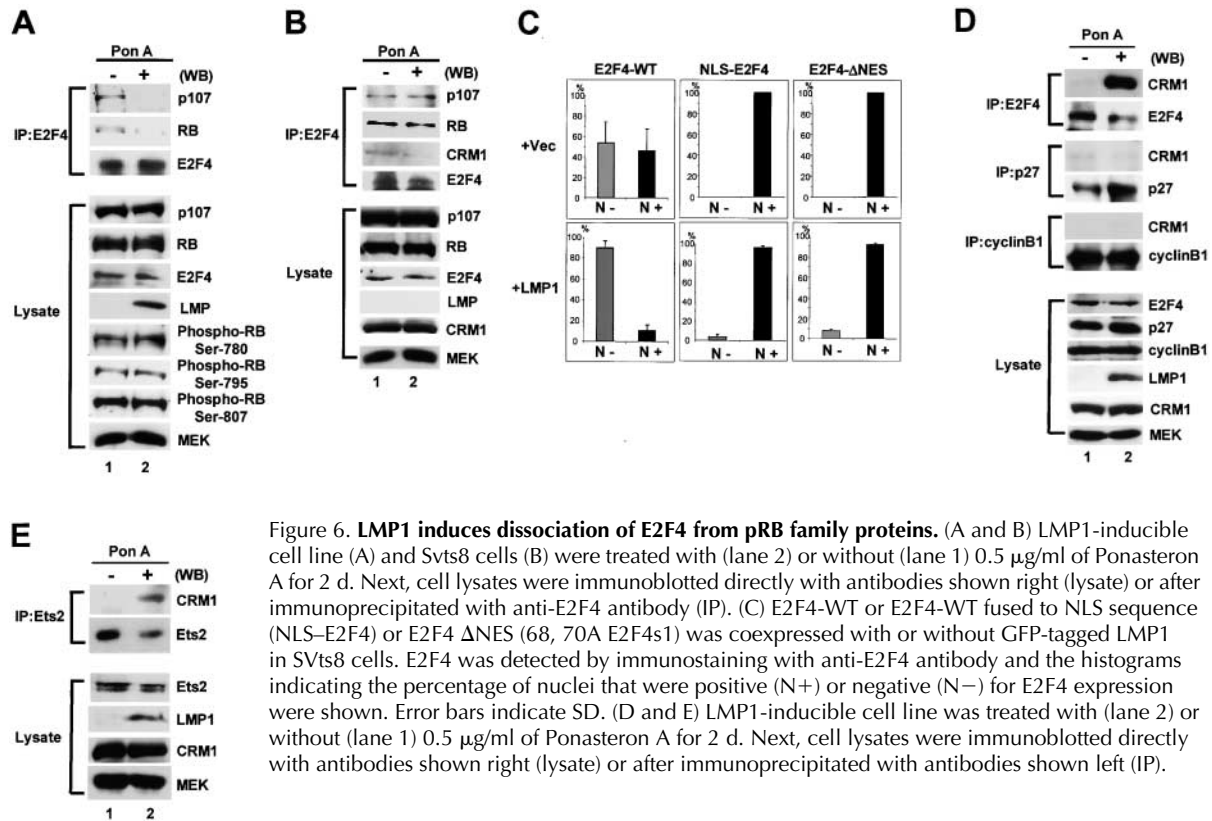
taining protein is required for the LMP1-induced intracellular redistribution of E2F4. However, it is still possible that dissociation of E2F4 from pRB family proteins is a consequence of the cytoplasmic accumulation of E2F4 and does not have a causal role in promoting nuclear export of E2F4. Indeed, LMB treatment abolished LMP1-induced cytoplasmic accumulation of E2F4 (Fig. 4 A). Moreover, the mutation of NES sequences (Gaubatz et al., 2001) accumulated E2F4 in the nucleus and made E2F4 less sensitive to LMP1-induced intracellular redistribution (Fig. 6 C). These results suggest the possibility that activation of nuclear export machinery could be involved in the LMP1-induced intracellular redistribution of E2F4 from the nucleus to the cytoplasm.

### LMP1 facilitates binding between E2F4 and CRM1

To seek mechanistic evidence that LMP1 promotes nuclear export machinery, we next examined the binding between E2F4 and CRM1 in the presence or absence of LMP1 using the LMP1-inducible cell lines. As shown in Fig. 6 D, the expression of LMP1 significantly increased the binding between endogenous E2F4 and endogenous CRM1 proteins (Fig. 6 D, lanes 1 and 2). This effect was specific to LMP1, because we were unable to see increased interaction between CRM1 and E2F4 in the control cells (Fig. 6 B, lanes 1 and 2). We were also able to see increased interaction between endogenous CRM1 and endogenous Ets2 in the LMP1 expressing cells (Fig. 6 E, lanes 1 and 2). In contrast, we were unable to see any increased interaction between CRM1 and other nuclear shuttling proteins, such as p27<sup>Kip1</sup> or cyclinB1, in the same cell lysates (Fig. 6 D, lanes 1 and 2). Because LMP1 does not increase the level of CRM1 (Fig. 6, D and E, lanes 1 and 2), it is likely that LMP1 modifies CRM1/E2F4 and Ets2 through the signaling activated by LMP1.

### CTAR1 and CTAR2 domains are required for LMP1-induced intracellular redistribution of E2F4 from the nucleus to the cytoplasm

LMP1 is composed of six transmembrane domains and a long carboxy-terminal cytoplasmic segment. The region containing the six transmembrane domains mediates its oligomerization in the cytoplasmic membrane, resulting in the constitutive activation of the downstream signals (Eliopoulos and Young, 2001). There are at least two functional domains (CTAR1 and CTAR2) in the cytoplasmic tail of LMP1, which activate multiple signal transduction pathways (Brown et al., 2001; Schultheiss et al., 2001; Thorley-Lawson, 2001). Therefore, we examined the effect of a series of LMP1 mutants lacking CTAR1 and/or CTAR2 domain on the subcellular localization of E2F4 (Fig. 7 A). As shown in Fig. 7 B, LMP1 mutants lacking CTAR1 and/or CTAR2 domain failed to induce cytoplasmic accumulation of E2F4, suggesting that the signaling from both CTAR1 and CTAR2 domains of LMP1 are required for intracellular redistribution of E2F4. This is consistent with a previous observations that the mutant LMP1 lacking CTAR2 failed to immortalize MEFs (Xin et al., 2001), and both CTAR1 and CTAR2 domains are necessary for efficient B cell immortalization (Eliopoulos and Young, 2001).



**Figure 6. LMP1 induces dissociation of E2F4 from pRB family proteins.** (A and B) LMP1-inducible cell line (A) and Svts8 cells (B) were treated with (lane 2) or without (lane 1) 0.5  $\mu$ g/ml of Ponasteron A for 2 d. Next, cell lysates were immunoblotted directly with antibodies shown right (lysate) or after immunoprecipitated with anti-E2F4 antibody (IP). (C) E2F4-WT or E2F4-WT fused to NLS sequence (NLS-E2F4) or E2F4  $\Delta$ NES (68, 70A E2F4s1) was coexpressed with or without GFP-tagged LMP1 in SVts8 cells. E2F4 was detected by immunostaining with anti-E2F4 antibody and the histograms indicating the percentage of nuclei that were positive (N+) or negative (N-) for E2F4 expression were shown. Error bars indicate SD. (D and E) LMP1-inducible cell line was treated with (lane 2) or without (lane 1) 0.5  $\mu$ g/ml of Ponasteron A for 2 d. Next, cell lysates were immunoblotted directly with antibodies shown right (lysate) or after immunoprecipitated with antibodies shown left (IP).

It has been shown that CTAR1 and CTAR2 domains have ability to activate multiple signal transduction pathways, such as p38 MAPK-, JNK-, MEK-, AKT-, or NF- $\kappa$ B-pathway (Roberts and Cooper, 1998; Eliopoulos and Young, 2001; Thorley-Lawson, 2001; Fukuda et al., 2002; Dowson et al., 2003). To narrow down the signaling pathways that required for LMP1-induced intracellular redistribution of transcription factor, we tested whether specific inhibitors of these signaling pathways have any impact on the subcellular localization of E2F4. As shown in Fig. 7 C, treatment with U0126, a specific inhibitor of MEK1/2, significantly reduced the LMP1 activity on the redistribution of E2F4. Similar effects were seen using another MEK1/2 inhibitor, U0125 (unpublished data). However, other pharmacological inhibitors such as, rapamycin (AKT inhibitor), SB203580 (p38MAPK inhibitor), or LY294002 (PI3K inhibitor), did not have significant impact on the LMP1 activity. Moreover, a recent report demonstrated that inhibition of NF- $\kappa$ B signaling override Ras-induced senescence (Dajee et al., 2003). Thus, it is unlikely that LMP1 blocks p16<sup>INK4a</sup>-RB pathway through activating the NF- $\kappa$ B signaling pathway. Together, these results suggest that MEK1/2 pathway may be, at least partly, involved in the LMP1-induced intracellular redistribution of E2F4.

To evaluate the impact of the LMP1-induced intracellular redistribution of E2F4 on cell growth, we next tested whether or not ectopic expression of NLS-E2F4 can counteract LMP1-induced cell proliferation. Because E2F4 acts as a repressor complex through interacting with pRB family proteins, we coexpressed unphosphorylated form of pRB with NLS-E2F4 in early passage TIG-3 cells. LMP1 ex-

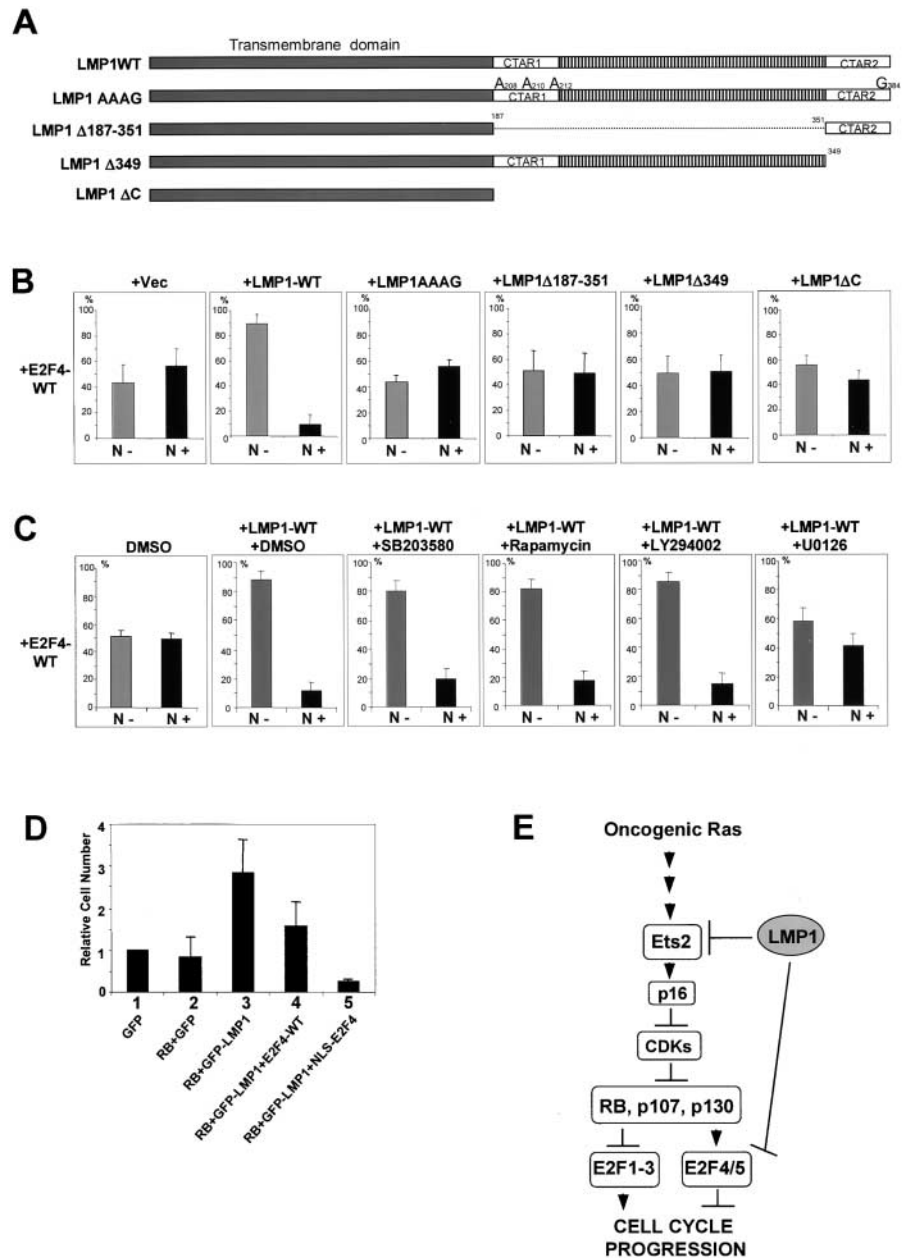
pression significantly increased the cell number even in the presence of unphosphorylated form of pRB (Fig. 7 D, lane 3). This effect was completely blocked by coexpression of NLS-E2F4, whereas coexpression of wild-type E2F4 did not have a significant effect on cell growth (Fig. 7 D, lanes 4 and 5). These results demonstrate the relevance of LMP1-induced intracellular redistribution of E2F4 to LMP1-dependent cell proliferation.

## Discussion

Here, we used human fibroblasts as a model system to understand the signaling pathways that induce Ras-induced senescence and to elucidate how these pathways are blocked in cancer cells. Although p16<sup>INK4a</sup> per se is not a critical player in Ras-induced senescence in mouse fibroblasts (Malumbres et al., 2000; Krimpenfort et al., 2001; Seoane et al., 2001; Sharpless et al., 2001; Stallet et al., 2001), p16<sup>INK4a</sup> seems to be more important in human fibroblasts. For example, primary HDFs from members of melanoma prone family lacking functional p16<sup>INK4a</sup> gene are resistant to Ras-induced senescence, although these cells retain a functional p14<sup>ARF</sup>-p53 pathway (Brookes et al., 2002; Huot et al., 2002). Unlike other DNA tumor virus oncoproteins, which possess immortalizing activity, LMP1 does not bind to pRB but instead blocks the expression of p16<sup>INK4a</sup> gene in human fibroblasts (Yang et al., 2000b). This might correlate with observations that LMP1 is associated with nasopharyngeal carcinoma, where p16<sup>INK4a</sup> expression is frequently decreased without having mutation in p16<sup>INK4a</sup> gene (Sun et al., 1995; Gulley et al., 1998). This

**Figure 7. Both CTAR1/2 domains are required for the nuclear export of E2F4.**

(A) Schematic representation of LMP1 mutants used for assay described in B. (B) Wild-type E2F4 was coexpressed with wild-type and a series of LMP1 mutants in SVts8 cells. E2F4 was detected by immunostaining with anti-E2F4 antibody and the histograms indicating the percentage of nuclei that were positive (N+) or negative (N-) for E2F4 expression were shown. (C) Wild-type E2F4 was coexpressed with LMP1 in the presence or absence of pharmacological inhibitors (SB203580, Rapamycin, LY294002, and U0126) at 10  $\mu$ M, 0.1 nM, 20  $\mu$ M, and 10  $\mu$ M, respectively. 2 d after the transfection, E2F4 was detected by immunostaining with anti-E2F4 antibody, and the histograms indicating the percentage of nuclei that were positive (N+) or negative (N-) for E2F4 expression were shown. (D) Early passage (40 PDLs) TIG-3 cells were transfected with plasmids shown at the bottom by Nucleofector primary cell transfection system (Amara Biosystems). Transfection efficiencies were nearly 100% in all the transfected cells. Relative cell numbers were calculated using the GFP-transfected cells (lane 1) as the reference after 3 d. The experiments were repeated eight times with similar results. (B–D) Error bars indicate SD. (E) Model of LMP1 effects on p16<sup>INK4a</sup>-pRB pathway. Oncogenic Ras/ MAPK signaling activates Ets2 and p16<sup>INK4a</sup> induction, thereby causing growth arrest/ cellular senescence. Expression of the LMP1 oncoprotein blocks this pathway by targeting Ets2 and E2F4/5 for CRM1-dependent intracellular relocation.



evidence prompted us to examine how LMP1 of EBV blocks p16<sup>INK4a</sup> expression in human fibroblasts.

Here, we show that LMP1 blocks Ets2 transcriptional activity through promoting a CRM1-dependent intracellular redistribution of Ets2 from the nucleus to the cytoplasm, thereby reducing the level of p16<sup>INK4a</sup> expression (Figs. 1, 2, 5, and 6). Because p16<sup>INK4a</sup> expression is also regulated by other factors such as bmi-1, JunB, 14-3-3 $\sigma$ , and SNF5 (Jacobs et al., 1999; Dellambra et al., 2000; Passegue and Wagner, 2000; Betz et al., 2002), LMP1 may affect these transcription factors as well. However, we found here that LMP1 also targets downstream mediators of p16<sup>INK4a</sup>-RB pathway. It has been suggested that the p16<sup>INK4a</sup>-induced growth arrest requires a function provided by a complex that contains p107 or p130, and E2F4 or E2F5 (Bruce et al., 2000; Gaubatz et al., 2000). Although inactivation of all three activator E2Fs, E2F1-3, causes a G1 arrest in MEFs

(Wu et al., 2001), MEFs lacking both repressor E2Fs, E2F4/5, grow normally but are insensitive to a p16<sup>INK4a</sup>-induced G1 arrest (Gaubatz et al., 2000). This suggests that E2F4/5 are essential downstream mediators of p16<sup>INK4a</sup>-induced growth arrest pathway.

Our results shown here clearly demonstrate that LMP1 blocks the function of E2F4/5 by promoting a CRM1-dependent intracellular redistribution of E2F4/5 from the nucleus to the cytoplasm. Because E2F4/5 lacks an NLS, E2F4/5 requires binding to NLS-containing proteins for nuclear localization (Trimarchi and Lees, 2002). Interaction between E2F4 and pRB family proteins seems to be a key for its nuclear localization, because E2F4 only localizes in the cytoplasm in MEFs lacking both p107 and p130 (Rayman et al., 2002). Indeed, we observed that LMP1 induces dissociation of E2F4 from pRB family proteins (Fig. 6 A). Moreover, LMP1 failed to promote cytoplasmic accumulation of E2F4



if E2F4 is fused to NLS (Fig. 6 C). This evidence strongly suggests that dissociation of E2F4 from pRB family proteins is essential for LMP1-induced intracellular redistribution of E2F4 from the nucleus to the cytoplasm. However, subcellular localization of cellular proteins is generally dependent on the ratio of nuclear import and export. Thus, nuclear import/export machinery can be affected by LMP1. Indeed, overexpression of CRM1 alone was sufficient to promote cytoplasmic accumulation of E2F4 and mutation of NES sequences in E2F4 or treatment with LMB rendered E2F4 insensitive to LMP1-induced intracellular redistribution (Figs. 4 A and 6 C). Moreover, expression of LMP1 significantly increased the binding between endogenous CRM1 and endogenous E2F4 (Fig. 6 D). Therefore, it is possible that the increased binding between E2F4 and CRM1 is a key for LMP1-induced cytoplasmic accumulation of E2F4, although LMP1 might dissociate E2F4 from pRB family protein in a parallel pathway. Both Ets2 and E2F5 do not contain typical NES sequences (Boulukos et al., 1989; Graves and Petersen, 1998; Ducret et al., 1999; Gaubatz et al., 2001; Sharrocks, 2001). However, it is quite possible that both proteins contain unidentified NES sequences, because NES is not a well-defined sequence (la Cour et al., 2003). Indeed, we were able to see significant interaction between endogenous Ets2 and endogenous CRM1 in LMP1-expressing cells (Fig. 6 E, lanes 1 and 2). This evidence strongly suggests that LMP1 induces intracellular redistribution of Ets2 through, at least partly, increasing the binding between Ets2 and CRM1. It is also important to note that we were unable to see cytoplasmic accumulation of Ets2 in serum-stimulated cells (unpublished data). Moreover, LMP1-induced intracellular redistribution of Ets2 and E2F4 was also seen in the cells arrested in G1 phase (unpublished data), precluding the possibility that these effects may be secondary consequences of cell cycle progression induced by LMP1.

Together, it is evident that LMP1-induced intracellular redistribution has at least two effects on the p16<sup>INK4a</sup>-RB pathway: (1) inhibition of p16<sup>INK4a</sup> expression and (2) blocking the function of downstream mediators of the p16<sup>INK4a</sup>-RB pathway (Fig. 7 E, model). It is interesting to note that other NES-containing proteins, such as p27<sup>Kip1</sup> (Fig. 2 B, 3 and 4), are resistant to LMP1-induced intracellular redistribution. Moreover, we were unable to see any increase of binding between CRM1 and p27<sup>Kip1</sup> (Fig. 6 D). Similar results were seen in interaction between CRM1 and cyclinB1, which is also known as another NES-containing protein (Fig. 6 D), suggesting that there must be some target specificity of LMP1-induced intracellular redistribution. Because both CTAR1 and CTAR2 (CTAR1/2) domains are required for LMP1-induced intracellular redistribution, multiple signal transduction pathways are likely to be involved in LMP1-induced intracellular redistribution of Ets2 and E2F4/5 (Fig. 7, A–C). U0126 and U0125, both are specific inhibitors of MEK1/2 pathway, efficiently attenuated the activity of LMP1 on intracellular redistribution of E2F4, whereas other pharmacological inhibitors did not have significant impact on LMP1 activity (Fig. 7 C). This suggests that LMP1 may induce intracellular redistribution of transcription factors, at least partly, through MEK1/2 pathways. Although further work is required to understand how signal-

ing activated by CTAR1/2 induces Ets2 and E2F4 binding to CRM1 in future studies, our work reveals the novel activity of LMP1 oncoprotein. In conclusion, this paper provides the first evidence that the viral oncoprotein blocks p16<sup>INK4a</sup>-RB pathway through targeting certain transcription factors for CRM1-dependent intracellular redistribution. These findings would provide a new insight into how viral oncoprotein can deregulate cell proliferation leading to cancer.

## Materials and methods

### Cell culture, retrovirus production, and transfection

TIG-3 and Hs68 strains of primary HDFs (Ohtani et al., 2001), human immortalized fibroblast cell line SVts8 cells (Hara et al., 1996), EH1 cells (McConnell et al., 1999), and HEK 293T cells were cultured in DME supplemented with 10% FBS. Human B cells were cultured in RPMI medium supplemented with 10% FBS. Cells were transfected with expression vectors by a modified calcium phosphate method (Chen and Okayama, 1987). Retroviruses were generated by cotransfection of pSIG helper plasmid and H-RasV12pBABE-puro or LMP1pBABE-puro vectors into HEK 293T cells, and the viruses were infected into Hs68 cells expressing an ecotropic retrovirus receptor as described previously (Roussel et al., 1996). Transfections for HDFs were performed using the Nucleofector primary cell transfection system (Amaxa Biosystems) according to the manufacturer's instructions.

### Luciferase reporter assays

Luciferase reporter activities driven by the human p16<sup>INK4a</sup> gene promoter (Ohtani et al., 2001), the human Rb gene promoter, and 36 tandem repeats of Ets binding sites were assayed using SVts8 cells as described previously (Ohtani et al., 2001). Effector plasmids were cotransfected as indicated in the figures, along with a standard amount of the MMLV-lacZ control plasmid. Cells were harvested 48 h after transfection and assayed for luciferase and  $\beta$ -galactosidase. Luciferase activities were normalized to the corresponding  $\beta$ -galactosidase activity.

### ChIPs assay

ChIP assays were performed as reported previously (Ohtani et al., 2001). After immunoprecipitation with a polyclonal antiserum (#57) against Ets2 (Ohtani et al., 2001), the recovered DNA was analyzed by PCR with primers flanking the putative Ets binding site in the p16 promoter: 5'-TGCTCG-GAGTTAATAGCACC-3' and 5'-CTCCATGCTGCTCCCCGCCG-3'.

### Antibodies and protein analysis

Immunoblotting and immunoprecipitation were performed as described previously (Sugimoto et al., 1999) with primary antibodies against p16<sup>INK4a</sup> (Oncogene Research Products), Ras (Calbiochem), MEK1/2 (New England Biolabs, Inc.), phospho-MEK1/2 (New England Biolabs, Inc.), Ets2 (polyclonal antibody; Santa Cruz Biotechnology, Inc.; mAb: 10B3; Sanij et al., 2003), LMP1 (LMP025) Lamin A/C (Santa Cruz Biotechnology, Inc.), E2F4 (Santa Cruz Biotechnology, Inc.),  $\alpha$ -tubulin (Sigma-Aldrich), Flag M2 (Sigma-Aldrich), RB (BD Biosciences), Phospho-RB (Ser780; Cell Signaling), Phospho-RB (Ser795; Cell Signaling), Phospho-RB (Ser807/811; Cell Signaling), p107 (Santa Cruz Biotechnology, Inc.), p130 (Santa Cruz Biotechnology, Inc.), Sp1 (Santa Cruz Biotechnology, Inc.), and CRM1 (Santa Cruz Biotechnology, Inc.). The nuclear and cytoplasmic fractions were prepared using NE-PER nuclear cytoplasmic extraction reagents (Pierce Chemical Co.) as described previously (Chen et al., 2002).

### Immunofluorescence and BrdU incorporation

Immunofluorescence analyses were performed as described previously (Llanos et al., 2001) using primary antibodies against Ets2 (Santa Cruz Biotechnology, Inc.), E2F4 (Santa Cruz Biotechnology, Inc.), Id1 (Santa Cruz Biotechnology, Inc.), LMP1(LMP025), Flag M2 (Sigma-Aldrich), and HA (Roche). Alexa Fluor-546 and -488 (Molecular Probes) and tetramethylrhodamine (DakoCytomation) were used as second antibodies. BrdU incorporation assays were performed as reported previously (Gaubatz et al., 2001).

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