

## Short Communication

# Upregulation of special AT-rich-binding protein 1 by Epstein–Barr virus latent membrane protein 1 in human nasopharyngeal cells and nasopharyngeal cancer

Kazuhira Endo,<sup>1,2</sup> Julia Shackelford,<sup>1</sup> Mitsuharu Aga,<sup>1,2</sup> Tomokazu Yoshizaki<sup>2</sup> and Joseph S. Pagano<sup>1</sup>

Correspondence  
Joseph S. Pagano  
joseph\_pagano@med.unc.edu

<sup>1</sup>Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, NC 27599-7295, USA

<sup>2</sup>Division of Otolaryngology, Graduate School of Medicine, Kanazawa University, Kanazawa, 920-8641, Japan

A global regulator of chromatin remodelling and gene expression, special AT-rich-binding protein 1 (SATB1) has been implicated in promotion of growth and metastasis of a number of cancers. Here, we demonstrate that the principal oncogene of Epstein–Barr virus (EBV), latent membrane protein 1 (LMP1) upregulates SATB1 RNA and protein expression in human nasopharyngeal cell lines. Silencing of endogenously expressed SATB1 with specific short hairpin RNA decreases cell proliferation and resistance to apoptosis induced by growth factor withdrawal. Additionally, we provide evidence that LMP1-mediated expression of Survivin, a multifunctional protein involved in promoting cell growth and survival, is mediated at least in part by SATB1 in human nasopharyngeal cells. Finally, we show that SATB1 protein levels are elevated in tissue samples from patients with nasopharyngeal carcinoma (NPC), and are directly correlated with the expression of LMP1. Taken together, our results suggest that SATB1 functions as a pro-metastatic effector of LMP1 signalling in EBV-positive NPC.

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Special AT-rich sequence-binding protein 1 (SATB1) was originally identified as protein binding to matrix attachment regions (MARs) of unpaired DNA (Dickinson *et al.*, 1992; Sun *et al.*, 2006). SATB1 was shown to regulate distant genes by selectively tethering MARs to the nuclear matrix, resulting in the formation of a characteristic ‘cage-like’ network that circumscribes heterochromatin (Cai *et al.*, 2003). Furthermore, SATB1 acts as a ‘docking site’ for several chromatin modifiers (Kumar *et al.*, 2005; Yasui *et al.*, 2002), and these chromatin modifiers were suggested to suppress gene expression through histone deacetylation and nucleosome remodelling at SATB1-bound MARs (Yasui *et al.*, 2002). Phosphorylation of SATB1 might act as a molecular switch in determining whether the protein acts as a transcriptional activator or repressor (Pavan Kumar *et al.*, 2006). Recently, SATB1 has attracted considerable attention due to its elevated expression in advanced stages of various types of malignant tumours, such as breast, gastric, colorectal and laryngeal carcinomas, which suggests a crucial role in promoting tumour invasion and metastasis (Cheng *et al.*, 2010; Han *et al.*, 2008; Iorns *et al.*, 2010; Lu *et al.*, 2011; Meng *et al.*, 2012; Patani *et al.*, 2009; Shushan *et al.*, 2009; Zhao *et al.*, 2010; Zheng, 2008). However, although there are reports that

SATB1 regulates transcription of genes that have direct impact on cellular proliferation and apoptosis (Alvarez *et al.*, 2000; Kumar *et al.*, 2007), the mechanism by which SATB1 regulates these phenomena is still unclear and even controversial (Iorns *et al.*, 2010; Li *et al.*, 2007).

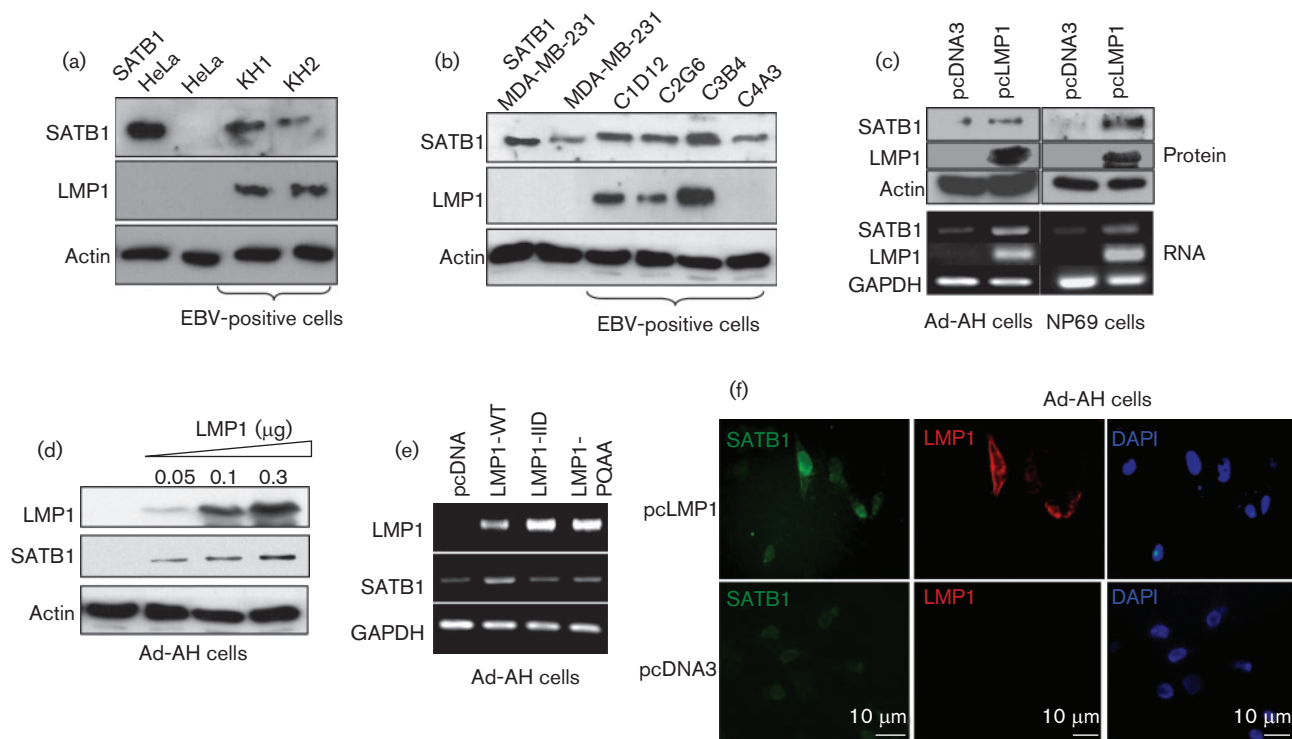
Nasopharyngeal carcinoma (NPC) is the most common Epstein–Barr virus (EBV)-associated tumour, and it is noteworthy for its proclivity to early invasion and metastasis (Burgos, 2005; Pagano, 2009; Wakisaka & Pagano, 2003; Zheng *et al.*, 2007). The principal EBV oncoprotein LMP1, a member of the tumour necrosis factor receptor superfamily, is detected in at least 70 % of NPC cases, and is considered to be the main mediator of the oncogenic properties of EBV in the development of NPC and its metastasis (Marquitz & Raab-Traub, 2012; Tsao *et al.*, 2002b; Wakisaka & Pagano, 2003; Yoshizaki *et al.*, 2005). In a series of reports we have shown that LMP1 induces expression of a variety of cell-invasiveness and angiogenic factors and explored their effects in nasopharyngeal (NP) cells in culture (Yoshizaki *et al.*, 1998; Kondo *et al.*, 2005; Wakisaka *et al.*, 2002; Muroto *et al.*, 2001; Wakisaka *et al.*, 2004; Kondo *et al.*, 2006, 2007; Horikawa *et al.*, 2007). Since recent studies demonstrate that SATB1 promotes tumour growth and metastasis in several carcinomas (Lu *et al.*, 2011; Meng *et al.*,

2012; Patani *et al.*, 2009; Tu *et al.*, 2012; Zhao *et al.*, 2010), we hypothesized that LMP1 upregulates expression of SATB1 in EBV-positive NPC cells.

First, we examined the endogenous levels of SATB1 and LMP1 in two sets of EBV-infected cell lines: one set, epithelial-like cell lines with type II EBV latency obtained by fusion of a human type III EBV latency lymphoblastoid cell line with epithelial HeLa cells (Contreras-Brodin *et al.*, 1991); and another, EBV-infected breast cancer cell lines that express different levels of LMP1 (Arbach *et al.*, 2006; Horikawa *et al.*, 2007). Western blot analysis shows that the levels of SATB1 protein are significantly higher in the fused LMP1-expressing cells KH-1 and KH-2, than in the EBV-negative parental HeLa cells (Fig. 1a), and that the expression of SATB1 is also upregulated in the LMP1-positive breast cancer clones C1D12, C2G6 and C3B4, compared with either the EBV-infected, but LMP1-negative, C4A3 cells or the uninfected parental line, MDA-MB-231 (Fig. 1b). These data illustrate the direct correlation between endogenous LMP1 and SATB1 protein levels in different EBV-positive carcinoma-derived cell lines.

To investigate whether LMP1 alone can induce SATB1 expression, we transiently transfected LMP1 into two EBV-negative human NP cell lines, Ad-AH (Takimoto *et al.*, 1988) and NP69 (Tsao *et al.*, 2002b) and observed significant increases in levels of SATB1 protein and RNA in both sets of cell lines (Fig. 1c). To confirm these observations further we showed that transient overexpression of LMP1 into Ad-AH cells induces SATB1 in a dose-dependent manner (Fig. 1d). Moreover, fluorescence immunostaining revealed much higher levels of SATB1 protein in LMP1-expressing cells (Fig. 1e) (we did not observe any effects of the empty vector on SATB1 endogenous levels). Taken together these results demonstrate that LMP1 upregulates SATB1 RNA and protein expression in NP cells.

Two LMP1 intracellular signalling domains, the C-terminal regulatory regions of the protein, CTAR1 and CTAR2, are well established as major transmitters of LMP1-induced signalling pathways involved in initiation and maintenance of malignant potential in EBV-associated cancers including NPC (Brinkmann & Schulz, 2006; Hatzivassiliou & Mosialos,



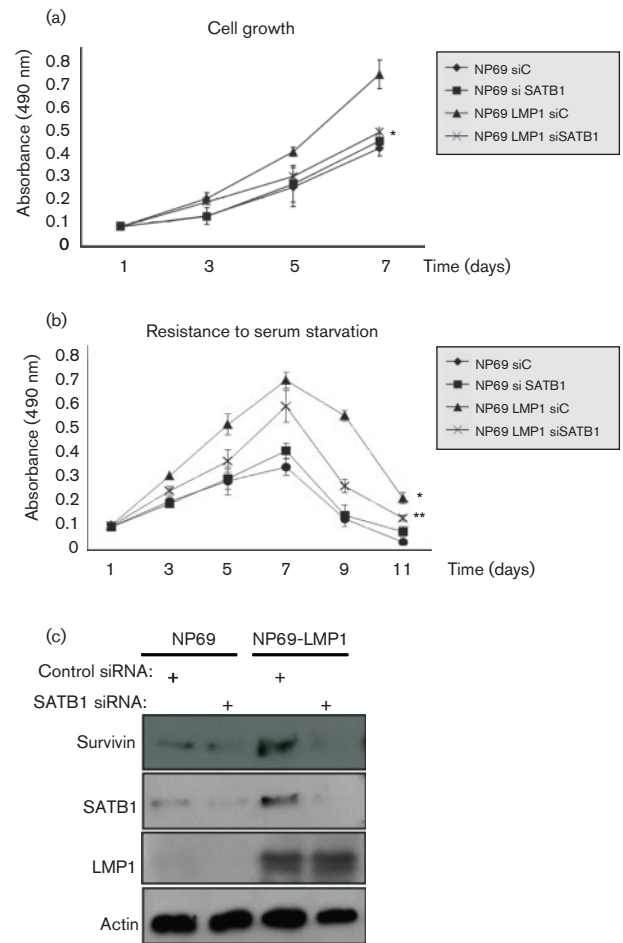
**Fig. 1.** Expression of SATB1 is increased in EBV latently infected cells. Increase in endogenous SATB1 protein levels correlates with the levels of the EBV primary oncogene LMP1 in EBV-positive fusion cervical carcinoma cells (a) and in EBV-infected breast cancer cell lines (b). Transient transfection of SATB1 served as a positive control. EBV primary oncogene LMP1 induces expression of endogenous SATB1 in human NP epithelial cell lines. Stable exogenous expression of LMP1 in Ad-AH and NP69 cells increases endogenous levels of SATB1 protein (c, top panel) and RNA (c, bottom panel). Dose-dependent upregulation of endogenous SATB1 by LMP1 overexpression in Ad-AH cells (d). Both CTAR1 and CTAR2 regions of LMP1 are involved in LMP1-mediated induction of SATB1 gene expression (e). Immunofluorescence analysis showing elevated levels of endogenous SATB1 protein in LMP1-transfected Ad-AH cells (f).

2002; Izumi, 2004; Lam & Sugden, 2003; Li & Chang, 2003; Morris *et al.*, 2009; Soni *et al.*, 2007; Tsao *et al.*, 2002a; Zheng *et al.*, 2007). Fig. 1(e) shows that transfections of LMP1 inactive mutants with point mutations in CTAR1 (LMP1-PQAA) and CTAR2 (LMP-IID) (Mainou *et al.*, 2007) reduced SATB1 RNA levels, indicating that LMP1-dependent expression of SATB1 most probably depends on activation of multiple signalling pathways from both CTAR1 and CTAR2 domains of LMP1.

Many studies, including ours, demonstrate that LMP1 expression induces an invasive phenotype in NP cells (Pagano, 2009; Tsao *et al.*, 2002a; Zheng *et al.*, 2007). To determine whether SATB1 is required for oncogenic properties of LMP1-expressing NP cells we generated a stable NP cell line in which SATB1 expression was suppressed with short hairpin RNA (shRNA) targeting SATB1 [pSuper-puro vector with SATB1 shRNA was the kind gift of Dr Hye-Jung Han (Han *et al.*, 2008)]. First, we analysed cell growth with the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt (MTS) assay. Growth curves are presented with the standard deviations (SD) as error bars. As shown in Fig. 2(a), silencing of the *SATB1* gene with specific shRNA significantly reduced growth of LMP1-expressing NP69 cells, but had minimal effect on the parental NP69 cell line.

Deprivation of growth factors leads to cell-cycle arrest following the induction of apoptosis (Lo *et al.*, 2003; Zhang *et al.*, 2008a). We tested the ability of SATB1 to protect the cells from effects induced by growth factor withdrawal by measuring cell viability of control and NP69 cells expressing SATB1 shRNA, cultivated in medium without growth-factor supplements (Fig. 2b). For the first 7 days all cell lines demonstrated some increase in cell growth, but eventually underwent starvation-induced cell-cycle arrest. As expected, LMP1-expressing cells were more resistant to growth factor withdrawal than parental NP69 cells, which is consistent with a previous report (Lo *et al.*, 2003). While the difference in survival between parental NP69 cells expressing control and SATB1 shRNAs was minimal, resistance to growth factor withdrawal was significantly decreased by SATB1 shRNA in NP69-LMP1 cells (Fig. 2b). The differences in cell growth and apoptosis between NP69 cell clones were analysed by the paired Student's *t*-test. Error bars indicate SD of the mean and significance was defined at the level of  $P < 0.05$ .

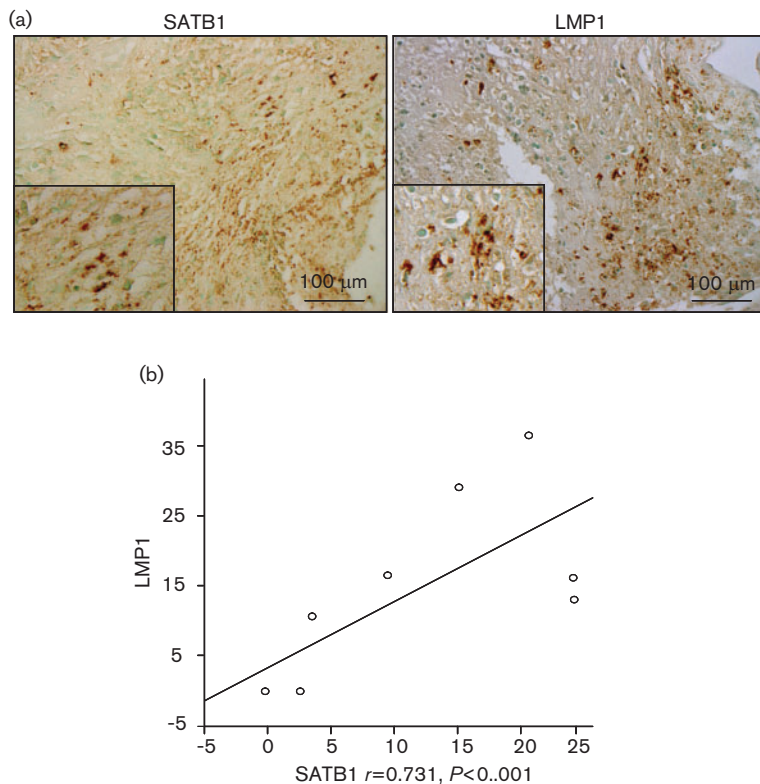
Our results indicate that SATB1 participates in the proliferation and survival of NP epithelial cells, most probably through induction of pro-mitotic and anti-apoptotic factors. One such factor that mediates proliferation, invasion and metastasis of many cancers is Survivin. Originally discovered as an inhibitor of apoptosis, Survivin also promotes cell proliferation and enhances invasion (Cheung *et al.*, 2011; Church & Talbot, 2012; Kanwar *et al.*, 2010, 2011; Lladser *et al.*, 2011). Absent in most adult tissues, Survivin is selectively upregulated in many human carcinomas including NPC and correlates with poor



**Fig. 2.** Expression of SATB1 is required for growth and survival of NP cells. Reduction of SATB1 expression with specific siRNA inhibits proliferation of NP69 and NP69-LMP1 cells (a). Reduction of SATB1 levels results in reduced resistance of NP69 and NP-LMP1 cells to growth factor withdrawal (b). Inhibition of SATB1 expression correlates with the reduction of LMP1-induced levels of anti-apoptotic protein Survivin in NP69 cells (c). Student's *t* test: \*,  $P=0.0019$ , compared with NP69 control siRNA cells. \*\*,  $P=0.0097$ , compared with NP69-LMP1 control siRNA cells.

prognosis (Fukayama *et al.*, 2008; Hino *et al.*, 2008; Lippert *et al.*, 2007; Margulis *et al.*, 2008; McGuire & Fitzpatrick, 2009; Mori *et al.*, 2007; Shi *et al.*, 2006; Zhang *et al.*, 2008b). Survivin is also a well known target of LMP1-mediated activation of cell signalling: LMP1 activates the *survivin* gene through several signalling pathways, and both CTAR1 and CTAR2 domains are required (Brinkmann & Schulz, 2006).

To investigate whether the inhibitory effects on cell growth and survival we observed with suppression of SATB1 (Fig. 2a and b) correlate with the expression of Survivin, we compared endogenous levels of Survivin protein in NP NP69 cells under control conditions and with suppressed levels of SATB1 (Fig. 2c). Western blot analysis shows that inhibition of SATB1 expression with shRNA resulted in



**Fig. 3.** Correlation between SATB1 and LMP1 expression in EBV-positive NPC tissues. Immunohistochemical detection of SATB1 and EBV LMP1 in human NP carcinoma tissues (a). Regression analysis shows the significant correlation between expression of SATB1 and LMP1 in the ten NPC specimens obtained at biopsy (b).

some reduction in Survivin levels, and this effect was much more profound in the presence of LMP1: inhibition of SATB1 expression resulted in a significant reduction of Survivin in LMP1-positive NP69 cells. Different EBV products induce the expression of Survivin through different mechanisms (Bai *et al.*, 2005; Lu *et al.*, 2011; Paydas *et al.*, 2009; Slovin *et al.*, 1981), and our data indicate that SATB1 is directly or indirectly involved in LMP1-mediated upregulation of Survivin. In the future we plan to investigate the mechanisms of such upregulation.

Finally, we tested whether there is a correlation between SATB1 and LMP1 expression in EBV-positive tumour tissue samples from NPC patients. Ten randomly selected NPC paraffin-embedded specimens from Kanazawa University Hospital (Ishikawa, Japan) were used for the detection of LMP1 and SATB1. Specimens obtained at biopsy were from eight men and two women, ages 45–78 years (mean 58.7 years), and classified histologically as follows: five non-keratinizing carcinoma (type II) and five undifferentiated carcinoma (type III) [5th TNM classification system of the International Union Against Cancer (1997)]. There were two stage IIB, seven stage III and one stage IVC. SATB1 levels were examined by immunohistochemistry in the NPC tissues of the ten patients (Fig. 3). Pearson's correlation coefficient was used to detect correlations among the expression scores of each protein analysed in NPC. The expression score of each protein in relation to clinical data was analysed with the Mann–Whitney U-test as described previously (Horikawa *et al.*,

2011). Expression scores for SATB1 and LMP1 are closely correlated ( $r=0.731$ ;  $P<0.001$ ). These data demonstrate a significant *in-vivo* correlation between LMP1 and SATB1 levels in NPC, which supports the hypothesis that expression of SATB1 is associated with a highly aggressive and metastatic phenotype of several human carcinomas (Han *et al.*, 2008; Kuo & Chao, 2010; Peng *et al.*, 2011; Tu *et al.*, 2012).

Recently, we showed that LMP1 contributes to the cancer progenitor-like phenotype of NPC (Kondo *et al.*, 2011), and that SATB1 is required for proper stem-cell differentiation and negatively regulates pluripotency genes (Savarese *et al.*, 2009). Considering that SATB1 expression is lost as the progenitor cells further differentiate (Agrelo & Wutz, 2009; Wen *et al.*, 2005), it is possible that expression of SATB1 is part of an embryonic pathway for establishing epigenetic patterns that are reactivated in somatic progenitors (Agrelo & Wutz, 2009). Upregulation of SATB1 by LMP1 strengthens these recent findings, and SATB1 could have some role in the context of the cancer stem/progenitor cell axis in the oncogenesis of NPC. The data presented here underscore also a series of findings that support the contribution of LMP1 to upregulation of metastasis via inducing epithelial mesenchymal transition (EMT) (Horikawa *et al.*, 2007, 2011).

SATB1 expression and the ability of Xist induction to initiate X chromosome inactivation are two aspects that provide a link between the epigenetic context of embryonic cells with that of somatic progenitors and tumour cells

(Agrelo *et al.*, 2009). Haematopoietic progenitors are probably at a critical transition when cell identity is unstable. This epigenetic context thus defines the biology of a class of cancer progenitors that distinguishes them from normal cells and could be a novel target for cancer therapies. The data presented here underscore also a series of findings that support the contribution of LMP1 to upregulation of metastasis via inducing EMT (Horikawa *et al.*, 2007, 2011). Induction of SATB1 by LMP1 in epithelial cells sheds light on a more precise role of LMP1 in the cancer stem cell theory (Kondo *et al.*, 2011). It will be of interest to investigate the clinical implications of LMP1-mediated SATB1 induction in future studies.

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