E2F1, ARID3A/Bright and Oct-2 factors bind to the Epstein–Barr virus C promoter, EBNA1 and oriP, participating in long-distance promoter–enhancer interactions

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The Epstein–Barr virus (EBV) C promoter (Cp) regulates several genes required for B-cell proliferation in latent EBV infection. The family of repeats (FR) region of the latent origin of plasmid replication (oriP) functions as an Epstein–Barr nuclear antigen 1 (EBNA1)-dependent distant enhancer of Cp activity, and the enhancer–promoter interaction is mediated by a higher-order multi-protein complex containing several copies of EBNA1. Using DNA-affinity purification with a 170 bp region of the Cp in combination with mass spectrometry, we identified the cell cycle-regulatory protein E2F1, the E2F-binding protein ARID3A, and the B-cell-specific transcription factor Oct-2 as components of this multi-protein complex. Binding of the three factors to the FR region of oriP was determined by DNA-affinity and immunoblot analysis. Co-immunoprecipitation and proximity ligation analysis revealed that the three factors, E2F1, ARID3A and Oct-2, interact with each other as well as with EBNA1 in the nuclei of EBV-positive cells. Using the chromatin immunoprecipitation assay, we showed that E2F1 and Oct-2 interacted with the FR part of oriP and the Cp, but the ARID3A interaction was, however, only detected at the Cp. Our findings support the hypothesis that EBNA1 initiates transcription at the Cp via interactions between multiple EBNA1 homodimers and cellular transcription factors in a large molecular machinery that forms a dynamic interaction between Cp and FR.

INTRODUCTION

Epstein–Barr virus (EBV) is a ubiquitous human gamma-herpesvirus. EBV infection is usually asymptomatic or results in infectious mononucleosis followed by establishment of a lifelong latent infection in approximately 90% of the population worldwide. The viral genome contains six latency promoters [W promoter (Wp), C promoter (Cp), Q promoter (Qp), latent membrane protein (LMP)1 and LMP2A/B promoters] that regulate expression of all viral genes essential for viral genome persistence and cell survival in the infected host. The promoters are active during defined periods of infection and are tightly regulated by viral and cellular transcription factors as well as by epigenetic events. These different patterns of gene expression are referred to as latency types 0, I, II or III, and correlate with cellular context and tumour classification (reviewed by Kieff & Rickinson, 2001; Rickinson & Kieff, 2001). The EBV Cp plays a central role in EBV biology controlling the expression of six EBV-encoded nuclear proteins [Epstein–Barr nuclear antigen (EBNA)1–6] essential for virus establishment and latency. Accordingly, Cp has been extensively studied and several cellular factors involved in regulating its activity have been identified, including CBF1, CBF2, CTCF, C/EBP, Sp1, Egr-1, NF-Y, E2F1/Rb/LSD1 and EBNA2 (Boreström et al., 2003; Chau et al., 2006, 2008; Fuentes-Panana et al., 2000; Grossman et al., 1994; Jin & Speck, 1992; Ling et al., 1993; Nilsson et al., 2001; Sung et al., 1991). The family of repeats region (FR or oriP) of the EBV origin of plasmid replication (oriP) serves as an upstream enhancer of Cp-controlled transcription. The transactivation of Cp by FR is essential in lymphoblastoid cell lines (LCLs) expressing the viral growth programme (latency III). It is mediated by EBNA1, one of the proteins encoded by the multicistronic Cp transcript. EBNA1 lacks a detectable activation domain and transactivation of Cp is indirect through interactions with other proteins, presumably of cellular origin (Frappier & O’Donnell, 1991). We have previously reported large differences in EBNA1-mediated transactivation with regard to the origin and cellular context of the transfected cell line. In general, cells of the B-cell lineage are more efficient in mediating this effect, suggesting that some B-cell-specific factor or factors are involved (Almqvist et al., 2003; Boreström et al., 2003).

The region between oriP and Cp has been hypothesized to loop out in order for the two regions to get into contact...
to initiate or enhance transcription (Mackey & Sugden, 1999; Zetterberg et al., 2004). Tempera et al. (2011) elegantly proved this hypothesis by showing the physical interaction of oriP and Cp in EBV-positive cells of a latency III expression pattern using a chromosome conformation capture (3C) assay. This interaction was abolished in latency I cells, in favour of the interaction between oriP and Qp. The CTCF factor was essential for the loop formation between oriP and the promoters at both Cp and Qp. However, the role played by EBNA1 in the interaction between oriP and the Cp, and the additional factors involved in the transcriptional activation of Cp remain unknown. In the present work we extend the work of Tempera et al. (2011) by identifying proteins binding both the FR and the core Cp, possibly aiding in the interactions that initiate transcription. Using DNA-affinity purification in combination with mass spectrometry (MS) we have identified three factors that bind Cp as well as FR: E2F1, ARID3A and Oct-2. Two of these proteins have previously been linked to EBV, Oct-2 (Almqvist et al., 2005) and E2F1 (Chau et al., 2008; Maser et al., 2001), while ARID3A (Bright/Drill/E2FBP1) is a new player in the EBV field. Taken together, our results show that E2F1, ARID3A, Oct-2 and EBNA1 are vital components of a multi-protein complex in the nuclei of EBV-positive cells that initiates transcription from the Cp.

RESULTS

E2F1, ARID3A and Oct-2 factors bind to the EBV Cp and the FR

Biotinylated PCR-amplified baits corresponding to either the Cp region spanning EBV coordinates 11156–11374 (Baer et al., 1984) or an equally sized fragment of the ampicillin-coding region (Amp) were coupled to magnetic beads and used for affinity purification of DNA-binding proteins in nuclear extracts from WW1-LCL cells (EBV-positive, latency III phenotype). The Cp region studied (−170Cp, Fig. 1a) was previously identified as the minimal Cp fragment with retained ability to activate transcription enhanced by EBNA1-bound oriP in transient transfections of B-cells (Nilsson et al., 2001). The affinity-purified proteins were separated by gel electrophoresis, digested with trypsin and analysed by liquid chromatography (LC) tandem MS. The results from the MS analysis were used to identify proteins binding to the Cp region but not the Amp region. Among the identified proteins Oct-2, E2F1 (two proteins previously linked to the oriP) and ARID3A (a transcription factor expressed mainly in B-cells) were selected for further studies.

Binding of the factors was validated by immunoblotting, showing that ARID3A, E2F1 and Oct-2 were present in the eluates when Cp was used as bait but not when the Amp...
fragment was used (Fig. 1b). The experiment was further extended to include affinity purifications using the original −170Cp bait split into halves: baits A and B (shown schematically in Fig. 1a), and ΔFR fragments containing eight or three EBNA1-binding sites. We have previously shown that eight or more EBNA1-binding sites in reporter plasmids are necessary for efficient transactivation of Cp in transient transfections, and that fewer than four repeats abolish enhancer activity of the construct (Zetterberg et al., 2004). ARID3A, E2F1 and Oct-2 bound to bait B, the distal half of the −170Cp, as well as to the ΔFR-fragments (Fig. 1c). The protein binding to bait B was weaker than that to the full-length −170Cp bait. This might be due to the decreased length of the bait: the shorter distance between the protein-binding site and the magnetic bead might cause steric hindrance and decreased protein binding. The signal obtained using bait B is clearly above background, as seen when compared with the signal obtained with bait A. NF-Y has previously been reported to bind to Cp at position −70 (Boreström et al., 2003), located in probe A, and was therefore used as a control for bait A protein binding (Fig. 1c), and as a negative control in the FR purifications. The amount of ARID3A obtained with the activation-competent ΔFR construct with eight EBNA1-binding sites was remarkably higher than that obtained with the activation-incompetent ΔFR fragment with three EBNA1-binding sites. These results were reproduced in cbc-Rael cells, another lymphoblastoid cell line with a latency III expression pattern (data not shown).

### Binding of E2F1, ARID3A and Oct-2 is abolished by a 15 bp mutation in Cp

In a previous study we identified two mutations that abolished oriP-independent Cp activity in transient transfections in EBV-positive latency I cell lines (Nilsson et al., 2001). Both mutations [mutation 4 (m4) and mutation 5 (m5), Fig. 2a] are located in the distal region of −170Cp. Biotinylated baits carrying m4 or m5 were used for DNA-affinity purification and the eluted proteins were analysed by immunoblotting using NF-Y, E2F1, ARID3A or Oct-2 antibodies (Fig. 2b). Binding of ARID3A, E2F1 and Oct-2 was abolished by m4, whereas binding was unaffected by m5. NF-Y was used as a control and its binding was unaffected by both mutations. Performing a transcription factor binding site search using TF Search and Transfac we identified overlapping binding sites for E2F1 and Oct-2 factors in the region encompassed by m4 (Fig. 2a).

To evaluate the effect of m4 on Cp activity in a latency III environment, we performed transient transfections in WW1-LCL and cbc-Rael cell lines using reporter plasmids with the luciferase reporter gene under the control of the mutated or non-mutated −170Cp region, with or without

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**Fig. 2.** Binding of ARID3A, E2F1 and Oct-2 is abolished by a 15 bp mutation. (a) Locations and the sequences of mutations m4 and m5 and the proposed locations of the binding sites E2F1 and Oct-2 as well as for NF-Y (control). PCR-amplified biotinylated probes containing these mutations were used for DNA-affinity purification assays. (b) Purified protein complexes were analysed by immunoblotting. (c) Results from transient transfections in cbc-Rael (EBV-positive, latency III), using luciferase (Luc) reporter plasmids with the luciferase reporter gene under the control of the mutated or non-mutated −170Cp region. RLU, relative luciferase units. The values are means of three independent experiments and error bars indicate SEM.
oriP. Mutation m4 decreased oriP-independent Cp activity by 98% and oriP-dependent activity by 90% (Fig. 2c).

To further define the factor-binding region of Cp, a series of electrophoretic mobility-shift assays (EMSAs) was performed using nuclear extracts of EBV-positive cell lines with latency III expression patterns (WW1-LCL and cbc-Rael). A probe [(-149/−109)Cp], including the m4 region, was used and unlabelled Cp fragments, E2F-, OCTA- and ARID3A-consensus site-carrying fragments, were used as competitors (Fig. 3). In previous studies we have noticed that Sp factors bind strongly to the region of interest (Nilsson et al., 2001), and that this might interfere with the detection of weaker bands. Binding of Sp factors was therefore reduced in these experiments by competition with the Sp-consensus fragment. All competitors significantly reduced or abolished specific bands (Fig. 3), strengthening the observation that ARID3A (band I), Oct-2 (band I), Sp (bands I and II) and E2F1 (band III) are part of a multi-protein complex formed at the (−149/−109) region of the Cp.

**ARID3A upregulates oriP-dependent Cp activity in transient transfections**

To further evaluate the functional significance of the interaction of ARID3A with the Cp and oriP, we performed co-transfections in the EBV-negative B-cell line DG75. These cells express E2F1 and Oct-2 endogenously, but ARID3A is not expressed at a detectable level (Fig. 8). The reporter plasmids p(-170Cp)Luc or p(-170Cp/oriP)Luc were co-transfected with 1 µg pcDNA3-HA-ARID3A or an equimolar amount of the empty vector pcDNA3. Ectopic expression of ARID3A upregulated transcription from the p(-170Cp)Luc by approximately 30%, and the expression from the FR-containing vector was upregulated by 100% (Fig. 4), indicating that this effect is FR-dependent. Worth noting is the significantly lower basal activity of the -170Cp/oriP vector in comparison to the -170Cp variant, due to the transcriptional repression effect of the oriP sequence in the absence of the viral protein EBNA1 (Nilsson et al., 1993).

**E2F1, ARID3A and Oct-2 bind to the -170Cp and the FR in vivo**

The binding of E2F1, ARID3A and Oct-2 to the Cp, FR and oriLyt regions was analysed in vivo by chromatin immunoprecipitation (ChIP) assay in cbc-Rael and WW1-LCL cells. The immunoprecipitated DNA was quantified in triplicate.
using quantitative (real-time) PCR (qPCR) with primers covering \(-170\text{Cp}\) and FR. Primers targeting or\(\text{Lyt}\) were used as a negative control. The amount of precipitated DNA corresponding to the Cp and FR regions was higher using antibodies against E2F1 and Oct-2, relative to the amount precipitated with non-immune rabbit IgG (Fig. 5). ARID3A enrichment in the Cp region was only obtained when an additional cross-linker ethylene glycol bis(succinimidyl succinate) (EGS) with an extended spacer arm (16 Å) was used. No ARID3A enrichment was detected in the FR region. There was little or no enrichment in the or\(\text{Lyt}\) region, supporting the notion that the interactions were specific.

**EBNA1 interacts with E2F1, ARID3A and Oct-2**

The putative interaction of the factors with EBNA1 was studied in co-immunoprecipitation (co-IP) experiments. Protein complexes were pulled down from the EBV-positive latency III cell lines WW1-LCL and B95-8-LCL as well as the EBV-negative cell line DG75, using specific antibodies against E2F1, ARID3A and Oct-2 (Fig. 6a). The precipitated proteins were analysed by immunoblotting using an anti-EBNA1 antibody. The anti-E2F1, ARID3A and Oct-2 antibodies pulled down significant amounts of EBNA1. No specific bands were observed in the immunoprecipitates obtained with DG75 cells (data not shown), nor with the IgG fraction from normal rabbit serum (Fig. 6a).

Immunoprecipitations from haemagglutinin (HA)-tagged EBNA1-transfected WW1-LCL and B95-8-LCL cells using HA-specific antibodies were also performed. E2F1, ARID3A and Oct-2 were detected in all fractions whilst no bands could be detected in the control samples from mock-transfected cells (Fig. 6b). Furthermore, ARID3A and Oct-2 were also found in the E2F1 precipitate, E2F1 and ARID3A in the Oct-2 precipitate, and E2F1 and Oct-2 in the ARID3A precipitate (Fig. 6c). The results suggest that all three factors along with EBNA1 are present in the same multi-protein complex.

To verify the interactions at single-molecular resolution we used in situ proximity ligation assay (in situ PLA) (Jarvius et al., 2016).
et al., 2007; Söderberg et al., 2006). PLA is a sensitive method to detect proteins in close proximity to one another (maximum distance 40 nm). An interaction generates an amplifiable DNA reporter molecule that is detected as a red spot. Interactions between endogenous EBNA1 on the one hand and E2F1, ARID3A and Oct-2, respectively, on the other, were investigated in WW1-LCL, B95-8-LCL and DG75 (negative control) cells (Fig. 7). Specific spots representing single protein interactions could be detected in the nuclei of all EBV-positive cells, but not in cells from the DG75 control samples where no EBNA1 is expressed. The results demonstrated that there is a close interaction in the nucleus between EBNA1 and E2F1, ARID3A and Oct-2 in vivo. The subcellular localization of the individual proteins was examined to ensure that the distribution pattern agrees with previous reports (Daikoku et al., 2004; Ivanova et al., 2007; Kim & Tucker, 2006; Maruo et al., 2005) (data not shown).

Expression of ARID3A in B-cells correlates with Cp-induced EBNA2 expression

To investigate whether expression of any of the identified factors correlate with Cp activity, we performed immuno-blots analyses of whole-cell extracts from several different cell lines that were EBV-negative (HepG2, HEK293, HeLa and DG75) as well as EBV-positive (Akata, Daudi, Elijah, Rael, MutuI, P3HR1, Raji, B95-8, MutuIII, B95-8-LCL, WW1-LCL and cbc-Rael), of different latencies and with different tissue origins (Fig. 8). All three non-B-cell lines (HepG2, HeLa and HEK293) lacked detectable expression of Oct-2, while E2F1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were present. ARID3A was seen in HepG2 as well as a faint signal in HEK293. Oct-2 was expressed in all B-cell lines tested. In the B-cell lines, ARID3A was only expressed at detectable levels in EBV-positive cells with a latency III pattern and a transcriptionally active Cp (B95-8, MutuIII, B95-8-LCL, WW1-LCL and cbc-Rael) and one latency I cell line (MutuI). Notably, MutuI is known to be able to drift in its gene expression pattern to form a latency III cell type (Gregory et al., 1990); this transition could possibly be facilitated by the expression of ARID3A. In summary, ARID3A expression correlates with Cp-induced EBNA2 expression.

DISCUSSION

To further investigate the nature of the multi-protein complex responsible for oriP-EBNA1 enhanced activation of Cp, we employed affinity purification in combination with high-resolution MS to identify proteins binding the Cp core. We show that E2F1, ARID3A and Oct-2 bind the core promoter sequence of the EBV Cp as well as the minimal FR sequence containing eight EBNA1-binding sites. In DNA-affinity purifications, using mutated Cp sequences, and EMSA we were able to narrow down the binding region to a 15 bp sequence, previously shown to be essential for Cp activity in transient transfections. This 15 bp Cp sequence contains putative binding sites for E2F1 (−132/−141) and

![Fig. 7. ARID3A, E2F1 and Oct-2 interact with EBNA1 in the nuclei of EBV-positive cells. The interaction of EBNA1 with ARID3A, E2F1 and Oct-2 by in situ PLA is visualized as red dots where one dot corresponds to one protein complex. The nuclei were stained with Hoechst 33342 (blue). Interactions are detected in WW1-LCL and B95-8-LCL cells, but not in DG75 cells (negative control).](image-url)
Oct-2 (−121/−133), found through a transcription factor-binding site search using the TransFac database.

The ARID3A–FR interaction was clearly demonstrated by DNA-affinity experiments using conditions stabilizing native protein–protein interactions but could not be demonstrated by ChiP analysis. ChiP enrichment in the −170Cp region could only be detected when a long-arm cross-linker was used, indicating that the ARID3A–FR and −Cp interactions are mediated at a distance. Using co-IP and in situ PLA we showed that ARID3A, Oct-2, E2F1 and EBNA1 are part of a multi-protein complex in the nuclei of EBV-positive cells with a latency III expression pattern.

ARID3A was initially shown to directly bind E2F1 to activate transcription as a heterodimer (Suzuki et al., 1998). This transcription factor has a restricted expression pattern and is in adults primarily expressed in B-lymphocytes (Nixon et al., 2004; Webb et al., 1998). The function of human ARID3A is not well-characterized, but studies of the mouse orthologue Bright makes ARID3A an interesting candidate in the search for proteins linking Cp to oriP. Bright is a nuclear matrix-associated transcription factor that increases transcription through binding of the immunoglobulin heavy-chain enhancer (Eμ) (Herrsch et al., 1995; Webb et al., 1991), has the ability to induce DNA bending, and has been implicated in facilitating spatially separated promoter–enhancer interactions (Kaplan et al., 2001). Bright binds the promoter as well as the enhancer as a multimeric complex that includes multiple copies of the protein (Herrsch et al., 1995) in a manner analogous to the mechanism proposed for oriP-EBNA1-induced activation of Cp (Mackey & Sugden, 1999; Tempera et al., 2011; Zetterberg et al., 2004). Ectopic expression of ARID3A in transient transfections in the EBV-negative DG75 cell line showed that this factor could upregulate FR-dependent Cp activity in the absence of EBNA1. The level of Cp upregulation using ARID3A was, however, not comparable to that one achieved previously using EBNA1 (Zetterberg et al., 2004). Transient transfections in combination with reporter plasmid assays is a well-established system to study gene regulation and promoter activity. The drawback of this simplified system is, however, that the spatial requirements for protein–protein interactions are not fulfilled. This might influence the amplitude of the effect seen when studying individual factors.

The EBV episome is relatively large (172 kb) and most likely interacts with a multitude of cellular factors to form
the loops necessary for transcriptional activation. EBNA1 bound to the FR has been shown to direct the viral DNA to the perichromatin regions of the nucleus (Deutsch et al., 2010), i.e. the most active site of transcription within the cell (Niedojałdo et al., 2011). Kanda et al. (2007) have previously shown extensive nuclear co-localization of EBNA1 and the EBV genome, suggesting that the EBNA1-interacting proteins also would co-localize with the viral genomes. These results were extended by Deutsch et al. (2010), who combined immunofluorescence and FISH (fluorescent in situ hybridization) in order to visualize these co-localizations in more detail. They found that the majority of EBV and EBNA1 signals co-localized, and that no EBV signal was detected without EBNA1.

There were, however, EBNA1 signals in the absence of EBV signal, which might be explained by the abundance of EBNA1 protein in relation to EBNA1-binding sites within oriP (Deutsch et al., 2010).

The protein–protein interactions found in this paper present many new possible avenues of research. The mechanism underlying the transcriptional activation potential of EBNA1 has eluded researchers for years, and the present interactions that have been found will add important information to this puzzle. In latency I, the EBV Qp is regulated by both EBNA1 (Sample et al., 1992) and E2F1 (Davenport & Pagano, 1999); the interactions between the proteins could possibly have different effects in different forms of viral latency. Furthermore, Paulson et al. (2002) showed that EBV adopts a type I latency programme in 293 cells and Tempera et al. (2011) recently showed that EBV bacmids adopt a chromatin formation that links the oriP to the Qp in these Oct-2-negative cells. Since the Cp is regulated by Oct-2, this suggests that Oct-2 could aid in the selection process of the chromatin formation in the latency III expression pattern. Moreover, the correlation between endogenous ARID3A expression, latency type III and Cp-induced EBNA2 expression in EBV-positive cell lines indicates that ARID3A may play a role in this selection process as well. Furthermore, little is known about ARID3A outside of the most commonly studied mouse model. The interactions with Oct-2, EBNA1 and E2F1 will shed light on alternative functions of this protein.

As a final note, a model can be put forward where expression of EBNA2 and EBNA5 from the Wp pushes the EBV-infected B-cell into the cell cycle through upregulation of c-Myc. The cycling of the cell induces expression of E2F1, ARID3A and NF-Y. As the levels of EBNA2 rise and it binds to the Cp, chromatin-remodelling complexes are recruited, inducing a more relaxed chromatin structure. This change in structure enables binding of EBNA1 and other proteins that may initiate transcription at Cp via interactions between multiple EBNA1 homodimers and cellular transcription factors in a large multi-protein complex and in a dynamic interaction between Cp and FR via a bridging DNA loop. Further functional and detailed molecular studies will reveal the details of this model.

METHODS

Cell culture. All lymphoid cell lines were maintained as suspension cultures in RPMI 1640 medium (Invitrogen). All non-B-cell lines were cultivated in Dulbecco’s modified Eagle’s medium (Invitrogen). All cultures were supplemented with 10% FCS (Invitrogen) and 100 units penicillin/streptomycin ml$^{-1}$ (Invitrogen). For more information regarding cell lines, see Fig. 8.

DNA-affinity purification. DNA-affinity purification was performed as described by Atanasia et al. (2005), with minor modifications. All baits were amplified from published reporter plasmids by PCR using biotinylated primers. We used the minimal Cp (~170Cp), spanning EBV coordinates 11156–11374 (Nilsson et al., 2001) as a specific bait (Fig. 1a) and a fragment from the ampicillin-coding region (Amp) as a non-specific control. To further map the region of the ~170Cp that binds the identified proteins, we split the original ~170Cp-bait in half (baits A and B). The minimal FR containing eight EBNA1-binding repeats (A$\Delta$FR 7784–8190) and the control containing three EBNA1-binding repeats were defined by Zetterberg et al. (2004). The A$\Delta$FR containing eight EBNA1 repeats (A$\Delta$FR(E1)) is able to transactivate Cp in transient transfection experiments, whereas the one containing three EBNA1-binding repeats (A$\Delta$FR(E3)) is not. The specific baits and the control baits of all affinity purifications were of comparable sizes. The locations of all baits are presented in Fig. 1. Primers were designed using MIT Primer3 software (http://frodo.wi.mit.edu/) and acquired from Invitrogen. The sequences are available upon request.

Sample preparation and mass spectrometry (MS). Eluates from the DNA-affinity purification with the Cp and Amp baits were concentrated by ultrafiltration using Microcon Centrifugal Filter Devices YM-3 (Millipore). Proteins were separated on a 10% NuPAGE Novex Bis–Tris gel (Invitrogen) and subjected to in-gel digestion with trypsin (Forsman et al., 2008). MS analysis of the extracted tryptic peptides was performed by liquid chromatography (LC)-MS/MS analysis on a Hybrid Linear Ion Trap (LTQ)-Fourier transform (FT-ICR) mass spectrometer (Thermo Electron) coupled online to a nano-LC instrument (MDLC; GE Health Care). Protein identity was established by database searches using the Mascot software (version 2.2.01, Matrix Science, http://www.matrixscience.com). Data files corresponding to each DNA-affinity experiment, Cp or Amp, were merged and searches were performed against the SwissProt database (version 51.6). Results from Mascot database searches were imported into the ProteinCenter software for further data reduction, resulting in a list of proteins filtered to only contain annotated transcription factors.

Immunoblot analysis. Immunoblot analysis was carried out according to standard protocols and the antibodies used were: rabbit-anti-ARID3A (Abcam, ab4445), rabbit-anti-Oct-2 (Santa Cruz, sc-233x), mouse-anti-E2F1 (Santa Cruz, sc-251), mouse-anti-EBNA2 (Abcam, ab90543) and rabbit-anti-GAPDH (Santa Cruz, sc-25778). Anti-mouse-HRP and anti-rabbit-HRP (Cell Signaling Technology) were used as secondary antibodies. The membranes were probed with GAPDH and stained with Ponceau S (Sigma) to confirm equal loading and transfer of proteins. Membranes were developed using SuperSignal West Dura Extended Duration Substrate (Pierce) and visualized using a CCD camera (Bio-Rad).

Plasmids, transient transfections and reporter assays. The luciferase reporter vector for Cp activity p(−170Cp)Luc and p(−170Cp/oriP)Luc have been described previously (Almqvist et al., 2005). The Cp transcription initiation site numbered as +1 (see Fig. 1a) is located at position 11336 of strain B95-8 EBV DNA (Baer et al., 1984). The mutated vectors p(−170Cp/m4)Luc and p(−170Cp/m5/oriP)Luc were constructed by PCR amplification of the mutated Cp
from the previously described pgCp(−170/m4)CAT (Nilsson et al., 2001). The correct sequences of all constructs were verified using the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction kits (Applied Biosystems). DG75, W1-LCL and cbc-Rael cells were transfectected by electroporation with a Gene Pulser system (Bio-Rad), using 5 × 10^6 cells and 10 μg reporter plasmid and 1 μg pcDNA3-HA-ARID3A (Fukuyo et al., 2004) (DG75). The cells were harvested 48 h post-transfection and lysed in 200 μl RLB (Promega). Luciferase activity was analysed using the Luciferase Assay System (Promega), as described by the manufacturer.

**Electrophoretic mobility-shift assay (EMSA).** The EMSAs were performed using standard protocols according to Nilsson et al. (2001). A double-stranded, blunt-ended, synthetic oligonucleotide covering EBV coordinates 11187–11227 was used as a probe.

In previous studies we have noticed that Sp factors bind strongly to the region of interest (Nilsson et al., 2001), and that this might interfere with the detection of weaker bands. Binding of Sp factors was therefore reduced by competition using 3 pmol of an unlabelled oligonucleotide containing the Sp consensus, in addition to the other competitors. For the sequences of the probe and the competitors, see Fig. 3.

**Transcription factor-binding site search.** Transcription factor binding site search was executed using TF Search (http://www.cbrc.jp/research/db/TFSERcH.html) and PROMO (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoi niti.cgi?dirDB=TF_8.3) (Farre et al., 2003; Messeguer et al., 2002).

**Chromatin immunoprecipitation (ChIP) assay.** ChIP was carried out according to the protocol provided by Upstate Biotechnology, with minor modifications according to Nelson et al. (2006). For the ChIP assay of ARID3A an additional cross-linking reagent ethylene glycol bis(succinimidyl succinate) (EGS) was used. EGS has a spacer arm of 16.1 Å, in comparison to formaldehyde which produces cross-links of about 2.0 Å, and was used as described by Zeng et al. (2006). Isolated DNA fragments were quantified by quantitative (real-time) PCR (qPCR) using Quantitect SYBR Green RT-qPCR kit (Qiagen) on a Rotor-Gene real-time rotary analyser (Corbett Life Sciences). Melting curve analysis was performed for each primer pair to ensure the specificity of the PCR primers. Primers were designed using MIT Primer3 software (http://frodo.wi.mit.edu/) and acquired from Invitrogen. The sequences are available upon request. A primer pair in the oriLyt-region of EBV was used as a negative control. The antibodies used for ChIP were: rabbit-anti Oct-2 (sc-233x, Santa Cruz), rabbit-anti-E2F1 (sc-193x, Santa Cruz), rabbit-anti Drill (ab4445, Abcam) and normal rabbit IgG (Upstate).

**Immunoprecipitation (IP).** Exponentially growing DG75, WW1-LCL and B95-8-LCL cells or cells transfected with 2 μg HA-EBNA1 were harvested (50 × 10^6 cells/IP). The cells were lysed in 2 ml RIPA buffer (150 mM NaCl, 1 % NP-40, 0.5 % sodium deoxycholate, 0.1 % SDS, 50 mM Tris-HCl, pH 8.0, 50 μL SUPERase-In mL^-1^-). The proteins of interest were immunoprecipitated with protein A-Sepharose beads pre-conjugated with specific antibody at 4 °C overnight. The immunoprecipitated complex was washed with RIPA buffer, eluted using 4 × LDS Sample buffer (Novex; Invitrogen) and immunoblotted according to standard protocols. Specific antibodies used were: rabbit-anti Oct-2 (sc-233x; Santa Cruz), rabbit-anti E2F1 (sc-193x; Santa Cruz), rabbit-anti Drill (ab4445; Abcam), rabbit-anti HA (ab9110; Abcam) and normal rabbit IgG (Upstate). To detect co-IP proteins, immunoblotting was performed using mouse-anti EBNA1 (clone 1B5; Acris GmbH), mouse-anti Oct-2 (sc-36822; Santa Cruz), mouse-anti-E2F1 (sc-251; Santa Cruz), mouse-anti Drill (sc-101030) and mouse-anti HA (12CA5; Roche).

**In situ proximity ligation assay (PLA).** The in situ PLA uses two separate antibodies that recognize two different proteins or two different epitopes of the same protein. The PLA probes bind to the primary antibodies that are bound to the protein, generating a DNA surrogate of the protein that can be amplified if the probes are in close proximity (<40 nm) using a ligation and amplification solution. Briefly, the cells were centrifuged on glass slides in a Cytospin centrifuge at 750 g for 3 min. Cells were fixed with Histofix (HistoLab Products AB), 0.2 % Triton X-100 at ~20 °C for 20 min and then rehydrated in 1 × PBS for 2 × 5 min. Blocking, antibody hybridizations, proximity ligations and detections were performed according to the manufacturer’s recommendations (Duolink IQ; OLINK Bioscience). Antibodies used for protein and protein–protein detection were mouse-anti EBNA1 (clone E1-2.5; Acris GmbH), rabbit-anti Oct-2 (sc-233x; Santa Cruz), rabbit-anti E2F1 (sc-193x; Santa Cruz) and rabbit-anti Drill (ab4445; Abcam). Preparations were mounted in Duolink mounting medium (OLINK Bioscience). Images were collected using a Zeiss LSM510 META confocal microscope system and the figures were assembled in Adobe Illustrator CS2.

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