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Abstract

Epstein-Barr virus (EBV) BamHI-A rightward frame 1 (BARF1) protein is considered a viral oncogene in epithelial cells and has immune-modulating properties. During viral lytic replication BARF1 is expressed as an early gene, regulated by the immediate early EBV protein R. However, in viral latency BARF1 is exclusively expressed in epithelial tumors such as nasopharyngeal (NPC) and gastric carcinoma (GC) but not in lymphomas, indicating that activation of the BARF1 promoter is cell type specific.

Undifferentiated NPC is characterized by high expression of ΔNp63 isoforms of the epithelial differentiation marker p63, a member of the p53 family of transcription factors. Transcription factor binding site analysis indicated potential p53 family binding sites within the BARF1 promoter region. This study investigated ability of various p53 family members to transactivate the BARF1 promoter. Using BARF1-promoter luciferase reporter constructs we demonstrate that only p63 isoform ΔNp63α is capable of transactivating the BARF1 promoter, but not the TAp63 isoforms, p53 or p73. Direct promoter binding of ΔNp63α was confirmed by ChIP analysis. Deletion mutants of the BARF1 promoter revealed multiple ΔNp63 response elements to be responsible for BARF1 promoter transactivation. However, ΔNp63α alone was not sufficient to induce BARF1 in tumor cells harboring full EBV genomes, indicating that additional cofactors might be required for full BARF1 regulation. In conclusion, in EBV positive NPC and GC, BARF1 expression might be induced by the epithelial differentiation marker ΔNp63α, explaining BARF1 expression in the absence of lytic reactivation.

Introduction

Epstein-Barr virus (EBV) is associated with several human malignancies, of B-cell origin such as Burkitt's lymphoma, Hodgkin's disease, and lymphoproliferative disorders in immune-compromised individuals, or in epithelial cells such as nasopharyngeal carcinoma (NPC) and gastric carcinoma (GC) (1,2). In EBV-related malignancies, several distinct gene expression

patterns have been analyzed in detail (1). In addition to well studied genes as EBNA1 (3), and the latent membrane proteins LMP1 and LMP2 (2) EBV-associated carcinomas are characterized by the selective expression of BamHI-A rightward frame 1 (BARF1) (4-6).

The BARF1 gene encodes a 220 amino-acids (aa) protein of which the membrane-associated leaderpeptide, comprising the first 20 aa, is cleaved off, leading to efficient secretion of hexameric BARF1 (7-9). BARF1 may drive carcinogenesis by immortalizing and transforming epithelial cells, and by blocking apoptosis enabling cell survival (10). In addition, secreted BARF1 has immune-modulating properties, arresting growth and differentiation of mononuclear cells by functional inhibition of the macrophage colony stimulating factor (M-CSF) (11-13). In B-cells and lymphomas BARF1 is expressed in the early phase of the viral lytic replication cycle (10,14). In EBV-positive carcinomas, however, BARF1 expression is not restricted to the lytic phase and can be detected in latently infected tumor cells. BARF1 protein is considered a viral oncogene in epithelial cells (5,6,15,16). A detailed review discussing BARF1 was recently published (17).

EBV-positive carcinomas are predominantly undifferentiated and characterized by high expression levels of p63 (18-21). The transcription factor p63 is one of the key regulators of epithelial cell development and differentiation. In multilayered epithelial structures p63 protein diminishes from high levels in basal undifferentiated cells to low levels towards the differentiated cells at the surface (22). p63 is the founding member of the family of transcription factors that also includes p53 and p73, sharing a common protein structure. They consist of a central DNA-binding domain, a transactivation domain (TA), and a tetramerization domain. The strong homology among the different family members results in similar consensus sequences to transactivate target genes (23,24). p63 and p73 contain an extended C-terminal coding region that undergoes complex alternative splicing (22). The p63 gene uses two transcriptional start sites resulting in the expression of two major variants, called TA and ΔN which combined with alternative C-terminal splicing results in at least six transcripts: TAp63α, TAp63β, TAp63ɣ, ΔNp63α, ΔNp63β and ΔNp63ɣ. The existence of multiple p63 isoforms with various domains that can either activate or repress promoters adds to the functional complexity of the p63 gene (25-27). Whether or not a gene is activated or repressed by the specific p63 isoforms is dependent on the cell type and the presence of cell specific cofactors (28-30).

Several cancers, including NPC, show overexpression of ΔNp63. The p63 gene is rarely mutated in cancer, and several studies indicate that the (in)balance between isoforms rather than the reduction of one particular isoform might be important (31). EBV might promote this imbalance by increasing the stability of p63 (19). A number of EBV proteins induce expression of ΔNp63α such as latent membrane protein 2A (LMP2A) (32) and Epstein-Barr virus nuclear antigen leader protein (EBNA-LP, EBNA-5) (19), and EBV downregulates levels of cellular microRNA-203 whose target is p63 (33). ΔNp63α upregulation reduces normal cell differentiation and might act to keep EBV in a latent state. Besides inhibition of differentiation, ΔNp63 is thought to play a role in cancer pathogenesis by inhibiting

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apoptosis, and upregulating specific signaling pathways (18,31,34). Expression of ΔNp63α was found to promote cell proliferation in both GC and NPC cells (20,35).

So far, there have been no suggestions in regard to why BARF1 is exclusively expressed in EBV-positive epithelial malignancies and not in lymphomas but the cellular background may point towards a role of dissimilar transcription factors. In this study, the potential role of p53 family members in the epithelial restricted expression of EBV protein BARF1 was evaluated. Multiple potential p53 binding sites were found in the BARF1 promoter region. Specifically the ΔNp63α isoform was capable of inducing the BARF1 promoter. These findings suggests that BARF1 expression in undifferentiated carcinomas is mediated by the epithelial differentiation marker ΔNp63α.

Materials and methods

Cell culture. SNU-719, a naturally derived EBV-infected gastric carcinoma cell line (36) was maintained in RPMI1640 medium. AGS cells were maintained in Ham's F-12 nutrient mixture medium. EBV infected 293 cells and EBV-positive AGS B95.8 (gifts from H.J. Delecluse) and R-stop EBV have been described previously (37,38), and were maintained under conditions of hygromycin B (Roche, Basel, Switzerland) (100 μg/mL) selection. CNE-2 Akata cells (a gift from K. W. Lo), a NPC cell line superinfected with the Akata strain of EBV, were maintained in RPMI1640 under conditions of G418 selection (Invitrogen, Carlsbad, CA) (400μg/mL). C666.1, a NPC cell line consistently harboring EBV was cultured in Dulbecco's modified Eagle's medium (DMEM) in fibronectin-coated flasks (Sigma-Aldrich, Buchs, Switzerland). All media contained 10 % fetal calf serum (FCS), 100 U sodium penicillin, 100 μg/mL streptomycin sulfate and 2 mM L-glutamine.

Plasmids. pSG5 and pcDNA3.1 were obtained from Stratagene (La Jolla, CA) and Invitrogen, respectively. The SG5-R expression vectors (kindly provided by S.D. Hayward) were previously described (14,39). Expression vectors for the various p53 family member isotypes were kindly provided by E.R. Flores (Anderson Cancer Center) and were described previously (40). The BARF1 promoter region from -679 to the ATG start site (164367 to 165045) (GenBank accession no. NC007605) was cloned into the pCpG.LUC, a CpG-free luciferase reporter vector kindly provided by M. Rehli (41), using forward primer with a SpeI site (CTGA CTAGTCTCATCACGCAACACCCACTGTTT), and reverse primer with a BglII site (AATAGATCTGCT CTGGACTCTCCTCACCCAG) (14). Deletion mutants were constructed using forward primers closer to the ATG start site (Table 1). The plasmid was propagated in pir-expressing bacteria (Invitrogen). Plasmid DNA was purified on maxiprep columns according to the manufacturer's protocol (Qiagen, Venlo, Netherlands).

In vitro DNA methylation. The use of a CpG-free reporter construct enables study of effect of promoter methylation without non-specific silencing due to methylation of the vector backbone. In vitro DNA methylation of the luciferase constructs was accomplished by CpG methylase (SssI methyltransferase; New England BioLabs, Ipswich, MA), as recommended by

the manufacturer. Completion of DNA methylation was confirmed by digestion with HpaII (New England BioLabs).

Luciferase assays. Cells were seeded the day prior to transfection. Transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions, except that for the reporter assays the reagent/DNA ratio was 1.5 µl/0.5 µg in 100 µL Opti-MEM for $2x10^5$ cells plated in 1 mL medium in a 12-well plate. Luciferase assays were performed 48 h after transfection by using extracts prepared by freeze-thawing the cell pellet in reporter lysis buffer according to the instructions of the manufacturer (Promega, Madison, WI). Luciferase activity was assayed using the luciferase reporter assay system (Promega).

ATG- n	Forward primers
679	CTGACTAGTCTCATCACGCAACACCCACTGTTT
633	CTGACTAGTAAGTCAGTCAGGCTGGCCAGG
582	CTGACTAGTGATCTTGGCATGCCGCCCAGC
468	CTGACTAGTACCGCAAACACCACTGTGTAGC
410	CTGACTAGTGGTCGTTGTACACTGCGCGCAG
350	CTGACTAGTCGATGTCGGCTGTCCTGCAGG
327	CTGACTAGTAGCTCCGCGTACAGCTTCCTATCC
261	CTGACTAGTGGCAAAGGCAGGTCTTTCTCATCC
220	CTGACTAGTCATGGCCCTGAACATGAGGTAGC
156	CTGACTAGTCACGCCTCGACCGGGGTC
120	CTGACTAGTGTAGACTTGGCTGGCCTCATGG
103	CTGACTAGTCATGGTCTCGTCAGGCCAGC
63	CTGACTAGTTGATAAAATGGGCGTGGCAG

Table 1. Primers BARF1 promotor reporter vector. Forward primers used to construct deletion mutants of the BARF1 promoter luciferase reporter vector starting 679 nucleotides upstream the ATG start site. Underlined, SpeI restriction site.

ChIP assay. CNE-2 Akata and AGS B95.8 cells were transfected with expression vectors for TAp63α, ΔNp63α or an empty control expression vector using Lipofectamine 2000 (Invitrogen). Cells were first cross-linked in 1.5 mM EGS (ethylene glycolbis[succinimidyl succinate]) for 30 min at room temperature followed by cross-linking in fresh 1 % paraformaldehyde for 10 min at room temperature. The cross-linking reaction was quenched using 125 mM glycine. Following IGEPAL (CA-630) mediated cell lysis and DNA fragmentation by sonication, DNA-protein complexes were immunoprecipitated with anti-p63 (NeoMarkers, Fremont, CA), and control anti-IgG (Santa Cruz, Santa Cruz, CA) antibodies. Protein-DNA cross-linking was reversed at 65°C overnight, and DNA was purified using the Qiagen gel extraction kit. The presence of BARF1 promoter DNA fragments in each precipitate was detected with PCR using forward primer GGCCCTGAACATGAGGTAGC and reverse primer GCCAACAGGAGGAGCTGAGC, those for E-cadherin were forward primer CATGGCTCACACCTGAAATCC and reverse primer AGTACAGGTGCACACCACCA, and JAG-1 forward primer: ACCTTTCACCATTCCCCTAC and reverse primer GCCCAAGGACAAAATAGCCA, as previously used by Testoni et al. (42). Primers for the negative control β2M were forward primer AGGGCTGGGCATAAAAGTCA and reverse primer GCCTCACCACCAACTTCATC.
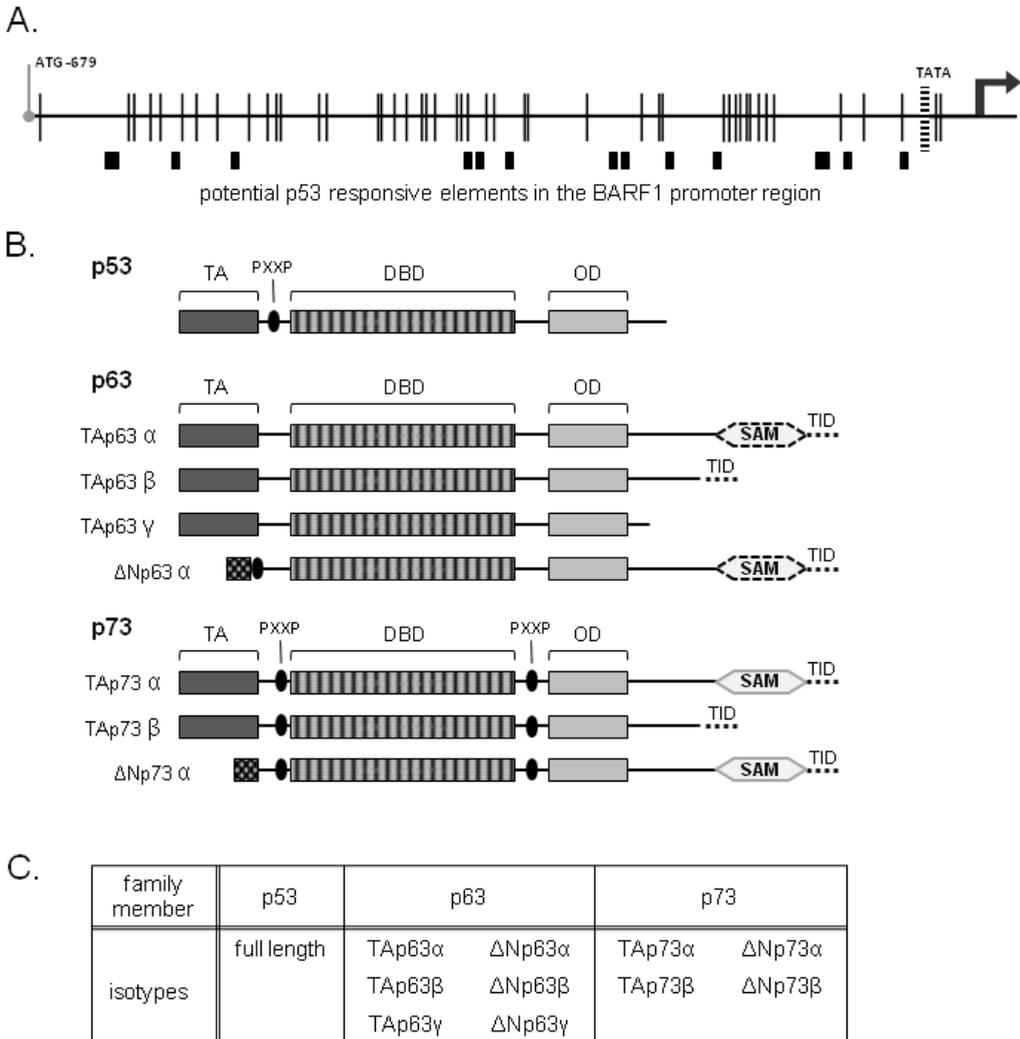
<u>Quantitative RT-PCR.</u> Cells were plated in 6-well plates and expression vectors for TAp63α, ΔNp63α or an empty control expression vector using Lipofectamine 2000 (Invitrogen). After 48 hours cells were harvested in 1 ml TRIzol (Invitrogen). Guanidinium isothiocyanate-phenol-chloroform extraction was performed to isolate total cellular RNA, followed by DNase (Promega) treatment and ethanol-precipitation. cDNA was synthesized using avian myeloblastosis virus (AMV) reverse transcriptase (RT) (Promega) and sequence specific primers: BARF1 forward (GCCTCTAACGCTGTCTGTCC) and reverse (GAGAGGCTCCCATCCTTTTC), U1A forward (CAGTATGCCAAGACCGACTCAGA) and reverse (GGCCCGGCATGTGGTGCATAA). RT-PCR was performed with SybrGreen (Roche) and afore mentioned primers using a LightCycler 480 system (Roche) After quantification to known concentrations of the corresponding gene constructs, values were normalized to U1A.

<u>SDS-PAGE and Western blot.</u> Lysates from luciferase assay and ChIP were diluted in 2x loading buffer (Biorad) with β-mercaptoethanol, denatured for 5 min at 95ºC and separated on a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis SDS-PAGE gel. After transfer to Hybond ECL nitrocellulose membrane (GE Healthcare, Little Chalfont, United Kingdom), the membrane was blocked in phosphate buffered saline (PBS) with 0.05 % Tween-20 (PBST) containing 3 % non-fat dry milk for 1 h at room temperature, after which anti-p63 (SantaCruz) was incubated for 2 h at room temperature in PBST containing 5 % bovine serum albumin (BSA). After incubation with peroxidase-labeled secondary antibody rabbit anti-mouse (Dako, Glostrup, Denmark) bands were visualized with ECL Plus (GE Healthcare). Positive control lysates for endogenous p63 were obtained by pBABE-LIC-ΔNp63α transfected 293T cells. Briefly, ΔNp63α was PCR amplified from pcDNA3.1-ΔNp63α and cloned into pBABE-LIC vector (obtained from Priyamvada Rai, University of Miami), as described in (43).

Results

<u>Potential p53 family response elements found in the BARF1 promoter.</u>
EBV protein BARF1 is selectively transcribed in undifferentiated NPC and GC associating with viral latency, but not in EBV associated B cell malignancies unless the lytic cycle is activated. To analyze which cell type specific transcription factor could potentially transactivate the BARF1 promoter, a transcription factor search was performed on the BARF1 promoter region from -680 bp through the promoter area of the BARF1 gene to the ATG start site, as defined recently (14). Using Match Matrix Search for Transcription Factor Binding Sites (44) a list of potential transcription factors was generated which was evaluated for cell type specific factors. The search revealed that the BARF1 promoter harbors several binding sites for p53 transcription factor family members (Figure 1A).

A.

potential p53 responsive elements in the BARF1 promoter region

B.

C.

family member	p53	p63		p73	
isotypes	full length	TAp63α TAp63β TAp63γ	ΔNp63α ΔNp63β ΔNp63γ	TAp73α TAp73β	ΔNp73α ΔNp73β

Figure 1. The p53 family of transcription factors and potential binding sites in the BARF1 promoter region. [A] Schematic overview of p53 family proteins. Transactivating domain, TA; DNA binding domain, DBD; oligomerisation domain, OD; sterile alpha motif, SAM; transactivation inhibitory domain, TID; PXXP motif. [B] Potential p53 family member binding sites in the BARF1 promoter region. Black vertical lines represent methylation sites. A BARF1 promoter reporter construct was created by inserting the promoter sequence, up to -679 nucleotides from the ATG start site upstream of the luciferase gene in a CpG-free reporter construct. [C] Overview of expression vectors containing the various isoforms of the different p53 family members used in the reporter assays.

ΔNp63α, but no other p53 family members, transactivated the BARF1 promoter in luciferase reporter assays.

The epithelial differentiation marker ΔNp63α, a member of the p53 family, is upregulated by EBV and shows elevated levels in NPC and GC (18-21). Individual p53 family members (Figure 1B) were cloned and expressed in epithelial cells co-transfected with various BARF1 promoter-reporter constructs. To study the transactivation potential of the p53 family

members on BARF1 activation, the BARF1 promoter sequence, up to -679 nucleotides from the ATG start site, was inserted upstream of the luciferase gene in a pCpG-free reporter construct. The reporter construct was cotransfected with individual expression vectors containing the various isoforms of the different p53 family members (Figure 1C) in AGS cells, an EBV-negative GC cell line, and SNU-719 cells, a naturally derived EBV-infected GC cell line. Induction of luciferase activity was evaluated 48 h after transfection. Expression of comparable levels of p53 and p73 isoforms showed no induction of luciferase activity (Supplementary Figure 1).
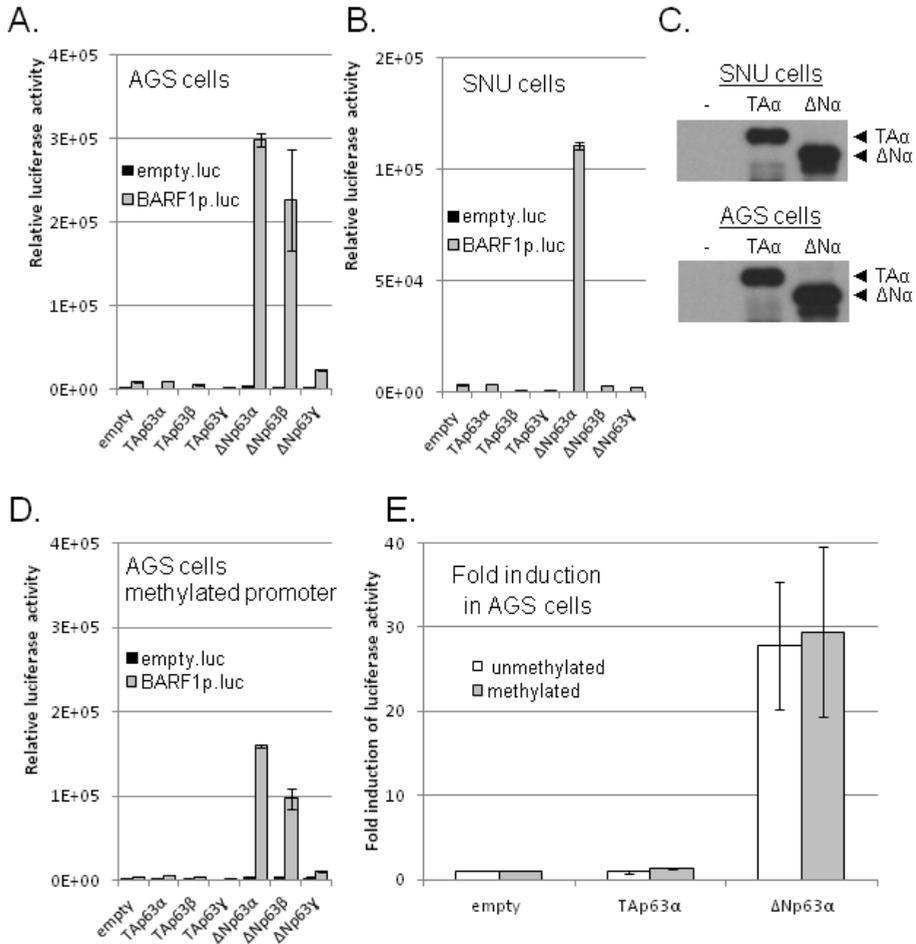

Figure 2. The BARF1 promoter reporter construct is transactivated by the ΔNp63α isoform.
[A] Representative graphs of a cotransfection of the reporter construct with individual p63 isoforms in AGS and [B] SNU-719 cells. Only the ΔNp63α isoform shows transcriptional activity in both cell lines. [C] Representative SDS-PAGE Western blot assay of both the TAp63α and ΔNp63α isoform demonstrates comparable protein levels. [D] Methylation of the BARF1 promoter reduces relative luciferase activity induced by ΔNp63α, representative graph. [E] Luciferase activity is 28-fold upregulated by the ΔNp63α isoform and not by the TAp63α isoform (n=7). Fold induction induced by the ΔNp63α isoform compared with constitutive activity of the methylated promoter is unchanged (29-fold) (n=3).

However, among the p63 family isoforms, particularly expression of the ΔNp63α isoform showed specific transactivation of the BARF1 promoter in both AGS and SNU-719 cells (Figure 2A and B). Next to ΔNp63α, in AGS cells expression of the ΔNp63β isoform also induced luciferase activity. Expression levels of individual p63 isoform proteins were similar as analyzed with a SDS-PAGE Western blot assay (Figure 2C). The TAp63 isoforms, considered to be the transcriptionally active p63-form with a proper transactivation domain, did not induce luciferase activity. The difference between the TAp63α and ΔNp63α isoforms lies in absence of the TA1 transactivating domain and the gain of a putative TA2 transactivating domain with different characteristics in ΔNp63α.

The EBV episome is highly methylated in latent carcinoma (45) and previous research by our group demonstrated a high level of CpG methylation of the BARF1 promoter region (14). To investigate to which level BARF1 promoter transactivation by ΔNp63α is affected by methylation, the pCpG luciferase construct containing the BARF1 promoter was methylated *in vitro* using methyltransferase. In transfected AGS cells, both the basal induced activity and the ΔNp63α induced activity of the BARF1 promoter-reporter construct was only slightly weaker in the methylated construct (Figure 2D), leaving the fold induction by ΔNp63α mostly unaffected by methylation (Figure 2E). Repeated experiments showed that expression of the TAp63α isoform has no effect on the BARF1 promoter while expression of the ΔNp63α isoform gave rise to an average 28-fold induction irrespective of the promoter methylation status (Figure 2E).
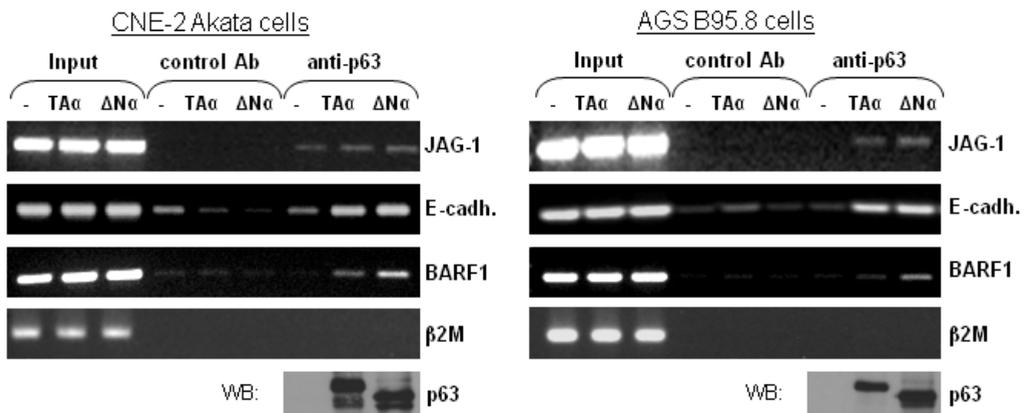
Figure 3. ΔNp63α directly complexes with the BARF1 promoter. ChIP assays were performed using extracts from CNE-2 Akata and AGS B95.8 cells transfected with the TAp63α isoform, the ΔNp63α isoform, or a control expression vector (-). p63 was immunoprecipitated by a control antibody or a non-isotype specific anti-p63 antibody, and coimmunoprecipitated DNA was PCR amplified. JAG-1 and E-cadherin served as positive controls for p63 binding and beta-2 microglobulin (β2M) served as negative control. The band in lane 9, indicates that the promoter region DNA is precipitated with ΔNp63α.

ΔNp63α was complexed with the BARF1 promoter *in vivo*

The ΔNp63α-mediated activation of the BARF1 promoter could either involve direct binding via the DNA-binding domain, or via the previously defined interaction of ΔNp63α with a CCAAT binding factor (30,46). Evaluation of the BARF1 promoter region showed us that no CCAAT box is present in the sequence. To determine if ΔNp63α was complexed with the BARF1 promoter region in EBV harboring cells, chromatin immune precipitation (ChIP) assays were performed. Gastric carcinoma AGS B95.8 cells and nasopharyngeal carcinoma CNE-2 Akata cells, both containing EBV episomes, were transfected with TAp63α or ΔNp63α expression vectors or a control vector, treated with a crosslinking agent and lysed. Both p63 isoforms were precipitated by anti-p63 antibody, which binds all isotypes. Binding of p63 to the known JAG-1 p63 intronic binding site and the E-cadherin promoter served as positive controls (42). Cross-linked DNA was PCR amplified using primers for the BARF1 promoter, the E-cadherin promoter and the JAG-1 intronic binding site. The ChIP assay showed that p63 bound to the BARF1 promoter region or in its immediate proximity, as well as to the E-cadherin and JAG-1 promoters (Figure 3). Since the p63 antibody does not differentiate between the isoforms, the bands visible in TAp63α transfected cells were probably due to non-activating binding of the TAp63α isoform.

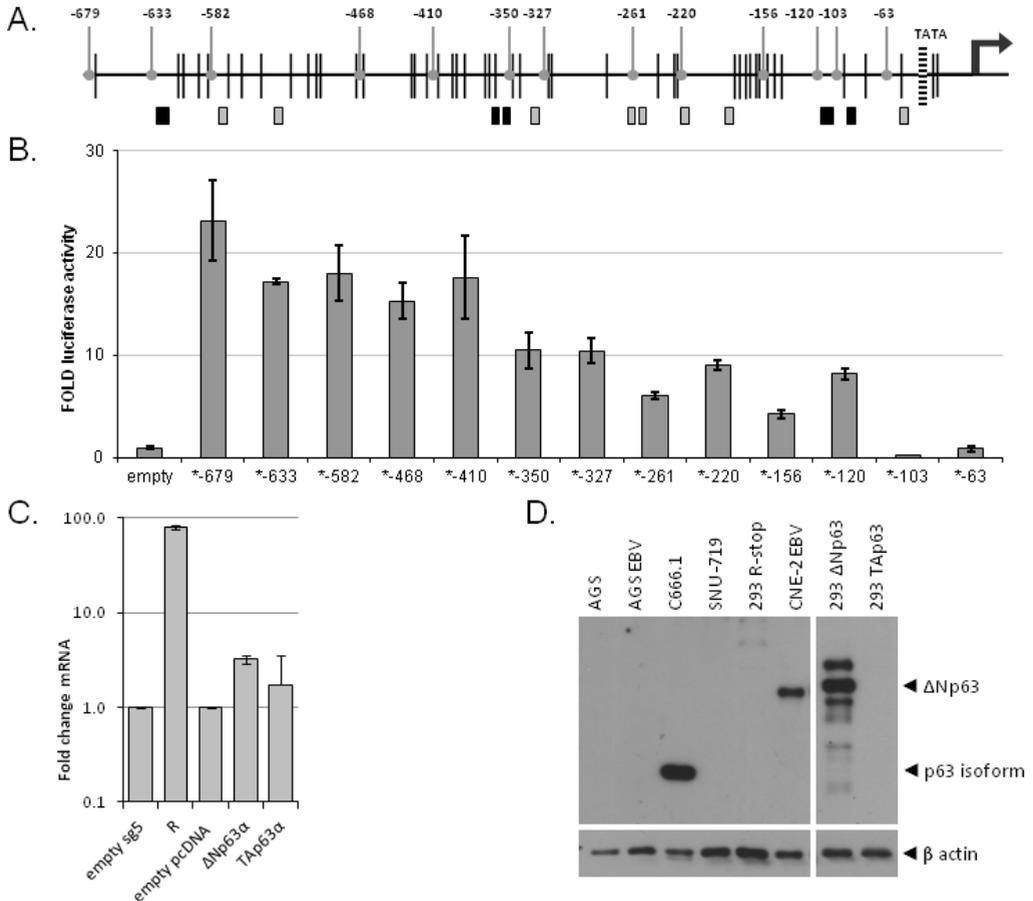### Multiple p63 response elements are responsible for BARF1 promoter transactivation

To identify the transcription factor binding site responsible for BARF1 promoter activation by ΔNp63α, deletion mutants of the reporter construct were made, shortening the BARF1 promoter sequence starting from the original 679 nucleic acids upstream of the ATG start site (ATG-679) to ATG-63 in small steps (Figure 4A). After removal of the region between ATG-679 and -410 an almost two fold drop in luciferase activity was noticed (Figure 4B). Luciferase activity was completely lost when only 103 nucleotides of the BARF1 promoter remained, indicating that multiple ΔNp63α response elements exist on the BARF1 promoter in close proximity to the TATA-box. This is in agreement with the distribution of the potential p53 family member binding sites indicated as boxes in Figure 4A. The black boxes indicate binding sites likely to be most important for ΔNp63α BARF1 transactivation.

ΔNp63α alone was not sufficient to induce BARF1 in context of the viral genome

To obtain evidence that ΔNp63α is capable of transactivating BARF1 expression in the context of EBV infection, two different EBV-positive epithelial cells were transfected with ΔNp63α, TAp63α or empty control vectors. As a positive control, an R expression vector was used to induce BARF1 mRNA as described before (14). Quantitative RT-PCR of BARF1 mRNA was used to analyze whether BARF1 mRNA could be induced in context of the intact wild type viral genome. At 48 h after transfection with the ΔNp63α expression vector neither AGS B95.8 nor C666.1 cells showed induction of BARF1 mRNA above the natural basal levels in each cell line (Supplementary Figure 2). Similar studies were done in 293-cells carrying recombinant-EBV. In order to prevent background levels of BARF1 transactivation by the lytic transactivator R, 293HEK cells carrying recombinant R-stop EBV (293RKO) were used (14,38).

Only in these cells a minor induction (3-fold) of BARF1 mRNA by ΔNp63α could be detected (Figure 4C). Previous studies showed low p63 levels in 293HEK cells as compared to C666.1 (19,32). Possibly the steady-state p63 levels in the AGS B95.8, CNE-2 Akata, and C666.1 cell lines are at maximal levels above which additional BARF1 transactivation cannot be detected. However, we were not able to detect endogenous p63 using the available antibody on Western blot (Figure 4D).

Figure 4. Multiple p63 response elements are responsible for BARF1 promoter transactivation. [A] Potential p53 family responsive elements are depicted on the BARF1 promoter region (boxes). Black vertical lines represent methylation sites. Rounded gray indicators point to the deletion mutants made from the BARF1 reporter construct, shortening the BARF1 promoter sequence from the original -679 to -63 relative to the ATG start site. [B] AGS cells were transfected with the deletion mutant luciferase constructs and with or without ΔNp63α expression vector. The ΔNp63α induced luciferase activity (fold) was measured 48 h after transfection. A representative experiment is shown. [C] 293RKO cells demonstrate that, unlike R, ΔNp63α has only minor transactivating activity (3-fold) of BARF1 in the context of the intact viral genome. [D] Endogenous p63 levels as detected by SDS-PAGE Western blot assay. HEK293 cells stably expressing either TAp63 or ΔNp63 were used as positive control.

Discussion

In vivo, the EBV-encoded BARF1 gene is exclusively transcribed in EBV-positive undifferentiated epithelial malignancies, like NPC and GC. In these tumors EBV remains in a latent state and the regulation of BARF1 expression remains undefined (5). We recently demonstrated that BARF1 expression is induced by the immediate-early transactivator R during early stages of lytic EBV reactivation in both epithelial and B-cells (14). Here we identified the differentiation specific transcription factor, ΔNp63α as a the putative regulator of constitutative latent BARF1 expression in the epithelial background.

EBV associated nasopharyngeal and gastric cancers are characterized by high levels of the p53 family member ΔNp63α (18-21). Analysis for potential transcription factor binding sites revealed that the BARF1 promoter region has multiple potential binding sites for the p53 family of transcription factors. Using promoter reporter constructs we showed that ΔNp63α was capable of transactivating the BARF1 promoter up to 30-fold where other p53 family members have no effect. Chromatin immunoprecipitation with cells harboring the full EBV genome demonstrated that ΔNp63α bound to the BARF1 promoter region or in its immediate proximity.

The protein p63 is essential for epithelial cell development and differentiation. Previous studies have shown that ΔNp63 is responsible for maintaining the proliferative potential of basal epithelial cells, while the interplay of TAp63 with ΔNp63α would facilitate epithelial differentiation (23). EBV-positive carcinomas are predominantly undifferentiated and have high p63 expression levels (18-21,35). In latent B-cells, which are the natural reservoir for EBV, the dominant p63 isoform is the TAp63α isoform and not ΔNp63α. BARF1 expression is linked to viral latency in epithelial cells, but not in B lymphocytes and therefore ΔNp63α may be one of the responsible cell type specific transcription factors regulating latent BARF1 gene expression.

A number of viruses utilize the epithelial differentiation process for productive viral replication and a tight control of p63 expression is essential for the survival of these viruses. Kaposi's sarcoma-associated herpesvirus (KSHV) (47), human papilloma virus (HPV) (48) and EBV (49-51) all produce their viral progeny in differentiated cells albeit major differences exist in execution of this viral reproductive route. The HPV productive process is studied the most intensively using organotypic raft cultures, which duplicate epithelial differentiation *in vitro* (52). Mighty et al found that regulation of p63 is essential for HPV replication (53). ΔNp63α is affected by multiple HPV proteins such as E6, which is thought to influence ΔNp63α function by enhancing its activity (54), and E5 and E7 that increase ΔNp63α levels by downregulation of skin-specific cellular microRNA203 (miR-203) expression (55,56). High expression of ΔNp63α is also related with HPV carcinogenesis, accentuated by the finding that it correlates with a higher rate of progression of cervical low grade lesions and carcinoma (57).

Likewise, in the EBV life cycle the differentiation status of the host cell is essential in determining whether new viral progeny will be made, or whether the virus remains in a

latent state and thereby hiding from the immune system. A differentiation responsive element can be found in the promoter of the lytic switch protein BZLF1 (Z, Zta, ZEBRA) (58), turning on lytic replication when the host cell initiates differentiation. Prevention of normal differentiation by elevated ΔNp63α levels might act to keep EBV in a latent state (49-51), and multiple EBV proteins have been found to influence p63. Early after infection Epstein-Barr virus nuclear antigen 5 (EBNA5, EBNA-LP) interacts and stabilizes p63 (19,59), reducing normal differentiation and bringing EBV to a immunogenic-safe latent state. The stabilizing function of EBNA5 on p63 is later taken over by other latent EBV proteins. MicroRNA profiling of EBV-infected epithelial cells and NPC tissues demonstrated that, similar to HPV infection, EBV causes downregulation of cellular miR-203 levels (33). LMP1 is responsible for this miR-203 downregulation, and it is plausible that this will contribute to higher levels of its target ΔNp63α. LMP2A physically associates with ΔNp63α, increasing protein level and stability (32). Both LMP1 and LMP2A have been shown to increase β-catenin transcriptional activity in epithelial cells (60,61) and shortly hereafter β-catenin proved to be a key regulator of ΔNp63α expression (62). These findings suggest that LMP1 and LMP2A act via the β-catenin pathway involved in activation of ΔNp63α transcription.

The differentiation status of EBV-positive cells is also influenced by cyclin D1 (63), whose expression is activated by the earlier mentioned β-catenin pathway (64), as well as by BARF1 (65). In addition, microRNA-34a/c targeting cyclin D1, is downregulated by p63. Although ΔNp63α and cyclin D1 colocalize in the basal layer and family member p73 interacts with certain cyclins (66), no direct interaction has been established between p63 and cyclin D. The exact mechanism leading to inhibition of differentiation might differ among cell types (14).

Overexpression of ΔNp63α was not sufficient to activate BARF1 expression in the context of the intact viral genome, although ΔNp63α was capable of transactivating the BARF1 promoter reporter construct. The inability of solely the ΔNp63α isoform to activate BARF1 expression indicates that specific chromatin modeling as well as other cofactors might be required for full BARF1 regulation. Previous research by our group indicated that the BARF1 promoter is almost completely methylated both in NPC and Burkitt's lymphoma cells (2,67). Methylation of the BARF1 promoter region does not seem to have a direct effect on the transactivating capability of ΔNp63α. In addition to DNA methylation, histone modification of the promoter will also regulate the accessibility to transcription factors, and this may be cell type dependent (19,32).

The inability of ΔNp63α to upregulate BARF1 expression can also be explained if the maximum BARF1 promoter transactivating capacity of ΔNp63α was already expressed. The cell lines tested are all undifferentiated (293, AGS, C666.1) or poorly differentiated (CNE-2) expressing at base line already p63 (29), leaving the possibility that the BARF1 mRNA expression induced by ΔNp63α in these cells was already at its peak. However, these endogenous p63 levels were not detected with Western blot assay. Future studies might elucidate the identity and importance of the unknown host or viral factors in BARF1 gene regulation.

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In conclusion, this study provides the first indication that expression of the BARF1 protein during viral latency in epithelial cells is potentially regulated by the epithelial differentiation factor ΔNp63α.
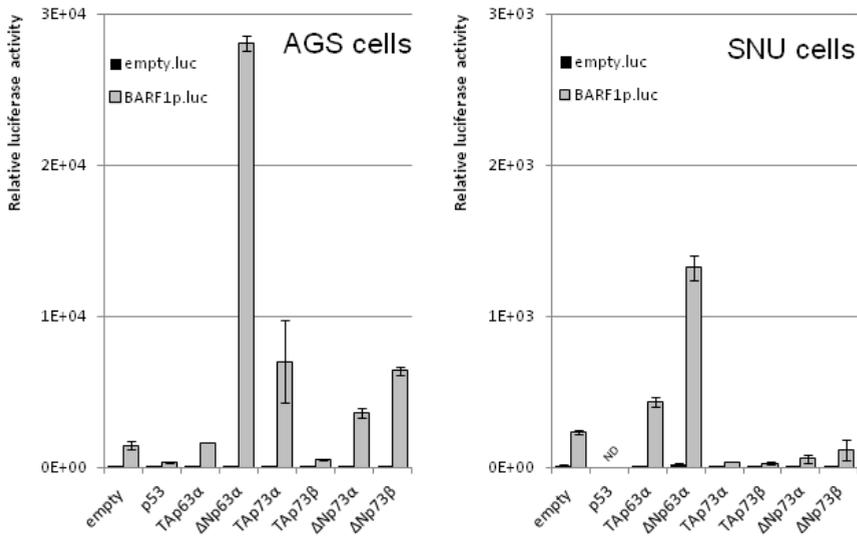
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Supplemental data

Supplemental Figure 1

Supplemental Figure 2