The hide-and-seek game of the oncogenic Epstein-Barr virus-encoded EBNA1 protein with the immune system: An RNA G-quadruplex tale

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Van-Trang Dinh[1], Nadège Loaëc[1], Alicia Quillévéré[1], Ronan Le Sénéchal[1], Marc Keruzoré[1], Rodrigo Prado Martins[2], Anton Granzhan[3] and Marc Blondel[1*]

[1] Univ Brest; Inserm UMR1078; Etablissement Français du Sang (EFS) Bretagne; CHRU Brest, Hôpital Morvan, Laboratoire de Génétique Moléculaire, 22 avenue Camille Desmoulins, F-29200 Brest, France

[2] ISP, INRAE, Univ Tours, UMR1282, Tours, Nouzilly, France

[3] Chemistry and Modelling for the Biology of Cancer (CMBC), CNRS UMR9187, Inserm U1196, Institut Curie, Université Paris Saclay, F-91405 Orsay, France

* To whom correspondence should be addressed. Tel: +33 (0)2 98 01 83 88; Email: marc.blondel@univ-brest.fr

Abstract

The Epstein-Barr virus (EBV) has been known for almost 60 years as the first oncogenic virus described in human and, nowadays, is still one of the most efficient means for transforming and immortalizing human B-cells. EBV infects more than 95% of the human population worldwide, but most EBV infections are asymptomatic. Nevertheless, when EBV primary infection occurs during adolescence or young adulthood, it can lead to an acute infection known as infectious mononucleosis. After the primary infection, the virus persists lifelong in the memory B cells of the infected individuals. Under certain conditions the virus can cause several human cancers, that include lymphoproliferative disorders such as Burkitt and Hodgkin lymphomas and non-lymphoid malignancies such as 100% of nasopharyngeal carcinoma and 10% of gastric cancers. Each year, about 200,000 EBV-related cancers emerge, hence accounting for at least 1% of worldwide cancers. Like all the gammaherpesviruses, EBV has evolved a strategy to escape the host immune system. This strategy is mainly based on the tight control of the expression of its Epstein-Barr nuclear antigen-1 (EBNA1) protein, the EBV-encoded genome maintenance protein. Indeed, EBNA1 is essential for viral genome replication and maintenance but, at the same time, is also highly antigenic and T cells raised against EBNA1 exist in infected individuals. For this reason, EBNA1 is considered as the Achilles heel of EBV and the virus has seemingly evolved a strategy that employs host cell factors binding on RNA G-quadruplex (rG4) within EBNA1 mRNA to limit its expression to the minimal level required for function while minimizing immune recognition. This review recapitulates in an historical way the knowledge accumulated on EBNA1 immune evasion and discusses how this rG4-dependent mechanism can be exploited as a therapeutic target to unveil EBV-related cancers to the immune system.

Introduction

Although first observed at the beginning of the 20th century [1], the Burkitt lymphoma (BL) was first described in 1958 as a prevalent lymphosarcoma affecting young children in sub-Saharan Africa [2]. It was then rapidly realized that the zone of incidence of BL coincided with the one of malaria, leading to the hypothesis that malaria could be responsible, or at least a cofactor for BL. In this context, Anthony Epstein and his student Yvonne Barr made the first observation, thanks to electron microscopy, of the so-called Epstein-Barr virus (EBV) in cultured lymphoblasts from Burkitt lymphoma ([3, 4]). A couple of years later, translocation and resulting activation of the *c-MYC* oncogene was detected in BL [5, 6]. Altogether these observations have paved the way for the current model that EBV, *c-MYC* and malaria collaborate to cause BL (reviewed in [7]). Indeed, shortly after its discovery in 1964, the hypothesis that EBV may cause cancers has emerged, notably based on the observation that almost 100% of BL in tropical Africa were latently infected by the virus [8] and carry the viral genome as a clonal extra-chromosomal episome [9]. This way, EBV was the first oncogenic virus described in human and is still nowadays one of the most efficient means for transforming and immortalizing human B-cells. EBV infects more than 95% of the human population worldwide, but most EBV infections are asymptomatic even though, when EBV primary infection occurs during adolescence or young adulthood, it can lead to an acute infection known as infectious mononucleosis. After the primary infection, the virus persists lifelong in the memory B cells of the infected individuals. Under certain conditions the virus can cause several human cancers, that includes lymphoproliferative disorders such as Burkitt and Hodgkin lymphomas, non-lymphoid malignancies such as 100% of nasopharyngeal carcinoma, 10% of gastric cancers, rare T- and NK-cells lymphomas as well as leiomyosarcoma [10-12] and, potentially, gliomas [13]. EBV is therefore found in a variety of human cancers whose localisation mostly overlaps with the known tissue tropism of the virus for B and epithelial cells. According to some estimations, about 200,000 EBV-related cancers emerge per year, hence accounting for at least 1% of cancer worldwide [14]. In addition, a link between EBV primo-infection and multiple sclerosis has been suspected for a long time and firmly confirmed recently [15]. For all these reasons, EBV became one of the most studied human viruses so far. As a latent virus, like all the gammaherpesviruses, EBV has evolved a strategy to escape the host immune system. This strategy is mainly based on the tight control of the expression of its Epstein-Barr nuclear antigen-1 (EBNA1) protein, the EBV-encoded genome maintenance protein of the virus [16]. Indeed, EBNA1 is considered as the Achilles heel of EBV due to the fact it is essential for viral genome replication and maintenance but, at the same time, it is also highly antigenic and T cells raised against EBNA1 exist in infected individuals [17-22]. This review aims at recapitulating the knowledge about the EBNA1-based immune evasion of EBV and how the discovered mechanisms can define original therapeutic targets to unveil EBV-related cancers to the immune system.

EBNA1 is the Genome Maintenance Protein (GMP) of EBV

EBNA1 is a multifunctional EBV-encoded protein whose primary function is to ensure EBV genome replication and maintenance, hence being considered the genome maintenance protein (GMP) of the virus [23]. In addition, EBNA1 plays a role in transcriptional activation of the EBV latency genes in latent infection [24, 25]. EBNA1 is a multi-domain protein (**Figure 1**) that contains, from residue 90 to 328 in

its N-term part, a glycine alanine repeat (GAr) that consists of a stretch of single alanines separated by one, two or three glycines. This GAr domain is conserved across all EBV strains and plays a critical role in the ability of EBNA1, and thereby of EBV, to evade the immune system during the latent phase of viral infection (described in details later in this review). Importantly, a natural polymorphism in the length of GAr exists and the ability of GAr to allow immune evasion of EBNA1 is GAr-length-dependent: the longer GAr, the most efficient the immune evasion is. The GAr domain is flanked on each side by a glycine-arginine-rich (RGG) motif. RGG motifs (aka RGG boxes) are low complexity sequences that contain repetitions of the arginine-glycine-glycine tripeptide. RGG motifs can lead to intrinsically disordered regions [26] and have been involved in interactions with nucleic acids and various proteins [27]. The first RGG motif of EBNA1 is located from residue 33 to 64 whereas the second lies between residues 329 and 377 and both are responsible for EBNA1 association to host cell metaphase chromosomes, hence supporting EBV genome replication and maintenance [28-30]. The second RGG motif of EBNA1 is followed by a monopartite nuclear localization signal NLS (KRPRSPSS, residues 379 to 386) and then by a C-terminal DNA-binding and dimerization domain (from residue 459 to 609), which is also responsible for the attachment of the viral genome to the cellular chromatin to ensure the viral replication [16, 31].

The function of EBNA1 in genome replication and maintenance also relies on its capacity to bind to multiple sites on the origin of DNA replication (OriP) of EBV genome. The EBV OriP contains two cis-acting components, a dyad symmetry (DS) element, and a family of repeats (FR) [32, 33]. The DS element is a repetition of four EBNA1 binding sites divided in two by a 9 bp sequence, it is responsible for DNA replication and it has also been shown to be sufficient for EBNA1-dependent EBV replication [31, 32, 34]. The FR element consists of 20 tandem copies of a 30 bp sequence containing a 18 bp palindromic element on which EBNA1 binds, followed by 12 bps corresponding to an AT-rich element [35]. This FR element is not directly involved in DNA replication but, the mitotic segregation of the EBV episome requires binding of EBNA1 to its multiple recognition sites in the FR element for ensuring efficient attachment to host cell chromosomes [31, 36].

In addition, thanks to a yeast-based two-hybrid screen, EBNA1 was shown to specifically interact also with a host cell nuclear protein, the so-called Epstein-Barr binding protein 2 (EBP2). This protein-protein interaction appears to be important for the role of EBNA1 in EBV episome stable segregation through association to host cell chromosomes in metaphase [31, 37, 38]. EBNA1 region that binds to EBP2 has been mapped to the second RGG motif of EBNA1 (residues 329 to 377) whose deletion prevents interaction of EBNA1 with EBP2 and consequently affects EBV genome maintenance [38]. This EBNA1-EBP2 proteins interaction has been furthermore reconstituted in the budding yeast *Saccharomyces cerevisiae*. This way, EBP2 was demonstrated to be crucial for the maintenance of an EBV FR element-containing plasmid maintenance [39-41]. EBNA1 is therefore a crucial protein for EBV DNA replication and episome maintenance, particularly in B cells which are actively dividing cells.

As stated above, EBNA1 is also implicated in transcriptional activation of the EBV latency genes in latent infection [24, 25]. The 65–83 N-terminal EBNA1 residues [42, 43] as well as the second RGG motif (residues 329 to 377) [44-46] are required for transcriptional activation. EBNA1 requires both of these

regions to activate transcription, as deletion of either one abrogates the transcriptional activation function of EBNA1 [43, 44]. Of note, a Δ61–83 EBNA1 mutant was found to be fully active for replication and segregation functions, indicating that transcriptional activation is a distinct EBNA1 function, separable from its GMP role [43].

EBNA1 is the Achilles heel of EBV

Like all the gamma herpesviruses, EBV is a latent virus that evades the host immune system. However, as said above, EBV possesses an Achilles heel: its virally-encoded genome maintenance protein EBNA1 (Epstein-Barr nuclear antigen 1). Indeed, being essential for replication and maintenance of the viral genome, EBNA1 is required for viral persistence but, at the same time, highly antigenic and T cells directed EBNA1 epitopes exist in nearly all EBV-infected individuals [17-22]. Hence, EBV evolved a mechanism by which EBNA1 limits the presentation of EBNA1-derived antigenic peptides. This ability of EBNA1 to evade the immune system has been rapidly attributed to its GAr domain which prevents EBNA1-derived antigenic peptides to be presented to the MHC class I pathway [47]. Importantly, this *in cis* effect of GAr is autonomous and transferable since, when fused to other proteins such as EBNA4, ovalbumin (OVA), or the herpes simplex virus thymidine-kinase, GAr also interfere with their antigenic presentation [47-50]. Of note, the study by Maria Masucci and collaborators represents a milestone since, not only it demonstrated the crucial role of GAr in the immune evasion of EBNA1, but it also represented a proof of principle that interfering with GAr-based immune evasion of EBNA1 may represent an intervention point to unveil EBV-infected cells, in particular tumoral cells from EBV-associated cancers, to the immune system [20, 47].

A historical perspective on the role of GAr in EBNA1 immune evasion: the proteasome era followed by the ribosome era

At the time the role of GAr in immune evasion of EBNA1, and thus of EBV, was discovered, the main dogma was that most antigenic peptides for the MHC class I pathway comes from 26S proteasome-mediated degradation of full-length proteins (reviewed in [51]). In this context, several teams have observed that the GAr domain is able to efficiently inhibit *in cis* protein degradation by the 26S proteasome. This has been shown by observing that the fusion of GAr to several short half-life proteins, that include p53 and Iκβα, interferes with their 26S proteasome-dependent degradation [52-54] and this effect has been shown to be GAr-length-dependent [53, 55, 56]. It was thus quite logical, on this ground, to infer that most of the capacity of GAr to inhibit antigen presentation was due to its ability to inhibit 26S-dependent proteolysis (**Figure 2**, **left part**). However, at the same time, some important problems associated to this model of antigenic presentation, in particular regarding the kinetics of antigenic presentation by the MHC class I pathway, have started to emerge [57-59]. In line, as early as 1996, Jonathan Yewdell has proposed an alternative hypothesis in which the most important source of antigenic peptides would mainly originate from the so-called defective ribosomal products (DRiPs) [58, 59]. DRiPs correspond to abortive byproducts of the translation process that include defective, misfolded

or prematurely terminated polypeptides. The first observation supporting this hypothesis is that most viral proteins are very stable, suggesting that the degradation of full-length proteins (the so-called "retirees") by the proteasome and the consecutive presentation of derived antigenic peptides by the MHC class I would take long time, from hours to days, which is not consistent with the experimental observations that the kinetic of presentation of antigenic peptides by the MHC class I pathway following viral infection is rather rapid (from a few minutes to a few hours) and may happen even before the synthesis of mature full-length proteins. In addition, there is a tight correlation between the onset of protein expression and epitope display for most antigens, which represents a strong indication that antigen presentation is largely linked to translation and not to degradation of mature full-length proteins (retirees) [57, 60]. Indeed, from a number of T cell based-studies, it is clear that numerous antigenic peptides from multiple viruses are rapidly produced following infection [61]. The tight kinetic link between viral gene translation and antigenic peptides generation has been extended to the proteomic level using mass spectrometry, for the vaccinia virus [60] and recently for the influenza A virus (IAV) [62]. Hence, the idea that most viral antigenic peptides come from the degradation of full-length viral proteins did not seem anymore a viable hypothesis. Finally, the ribosome hypothesis was further supported by data showing that the inhibition of *de novo* protein synthesis rapidly reduces the antigenic presentation by MHC class I molecules [63, 64]. All these findings suggest that antigenic peptides have a main source from translation itself (**Figure 2**, **right part**), in particular from DRiPs [57, 65]. Hence, the model in which the ability of the GAr domain of EBNA1 to enable its immune evasion, and therefore immune evasion of EBV, comes from its capacity to inhibit own degradation by the 26S proteasome started to be fragilized. In addition, several studies have found that EBNA1 protein, with or without GAr, is a very stable protein [50, 52, 66] like most viral proteins, although its stability may be tissue-dependent [66]. Also, the steady-state level of EBNA1 protein was strongly increased in cells expressing a version of EBNA1 deleted for its GAr domain (EBNA1ΔGAr) as compared to cells expressing the wild-type EBNA1 (EBNA1wt), which was not expected when removing a domain that would inhibit proteasomal degradation [50, 53]. Finally, when expressing in the budding yeast *Saccharomyces cerevisiae* GAr domains of various lengths fused to destabilized versions of the green fluorescent protein (GFP), harboring N-end rule or ubiquitin fusion degradation signals, the length-dependent effect of the GAr domain on protein stability was confirmed. However, the steady-state level of the various GAr-GFP fusions was inversely correlated to their degree of stabilization: the longer the GAr domain, the more the reporter protein was stable, but the smaller its steady-state level, which appears quite counterintuitive [56]. Altogether, all these indirect data suggested that the ability of GAr to promote immune evasion was not linked to its ability to inhibit the 26S proteasome-mediated proteolysis and, even if GAr is able to inhibit proteasomal degradation of unstable proteins to which it is fused, this effect is not operant in EBNA1 which is a stable protein.

The ability of GAr to interfere with EBNA1 immune evasion is linked to its capacity to interfere *in cis* with the translation of its own mRNA

In a seminal study published in 2003, the use of *in vitro* expression systems and cell-based metabolic labeling led to the demonstration that the increase in EBNA1ΔGAr, as compared to EBNA1wt, was due to an increase in the rate of EBNA1 synthesis [50]. Not only this striking result explained all the previously

described results that did not fit with an effect of GAr on proteasomal degradation, but, more importantly, it was the starting point for other studies that unambiguously demonstrated that GAr does in fact inhibit *in cis* the translation of its own mRNA (EBNA1 mRNA) or of any messenger RNA (mRNA) to which it is fused, without affecting *in trans* the translation of other mRNA [48, 67]. This important demonstration was fully in line with the growing role of translation and of DRIPs in the genesis of antigenic peptides for the MHC class I pathway and readily explained the effect of GAr on immune evasion of EBNA1, and therefore of EBV [68]. These important results have then been confirmed [66] and converged to the model in which the endogenous presentation of EBNA1-derived antigenic peptides is dependent on the newly synthesized EBNA1 protein rather than on the long-lived stable EBNA1 protein, hence highlighting the inhibitory role of GAr on translation as the main mechanism explaining its inhibitory effect on antigen presentation (**Figure 2b**, **right part**). Importantly, the GAr-encoding sequence does not represent *per se* a hurdle to mRNA translation by the ribosome since the insertion of the *c-Myc* internal ribosome entry site (IRES) in the 5' untranslated transcribed region (UTR) of EBNA1 or GAr-OVA fusion-coding mRNA abolishes GAr-based inhibition of both translation and antigen presentation [48, 67]. This result also indicated that the GAr-inhibitory effect on translation requires cap-dependent translation initiation factors.

Tools to monitor the GAr inhibitory effect on translation: setup of a yeast-based assay amenable to high throughput drug and genetic screenings

Once the GAr inhibitory effect on translation firmly established, it rapidly appeared as a relevant intervention point to unveil EBV-infected cells, in particular tumor cells of cancers linked to EBV, to the immune system, hence the need for robust cell-based systems allowing both drug and genetic screenings. For various reasons, the budding yeast *Saccharomyces cerevisiae* has emerged as a relevant model system: notably, the study by the Masucci group already discussed above and based on the expression in yeast of destabilized GFP fused to GAr domains of various lengths indirectly suggested that the length-dependent inhibitory effect of GAr on translation is operant in yeast [56]. In addition, the budding yeast *S. cerevisiae* is an eukaryote as easy to handle as a prokaryote and is most certainly the model system for which the most molecular tools are available. Finally, for decades, yeast has been harnessed by biomedical research, notably on the Epstein-Barr virus itself (reviewed in [41]) and on virus immune evasion [69], and widely used for modeling various pathophysiological mechanisms responsible for a large variety of human disorders [70, 71]. A number of these yeast-based models have been successfully used for performing large-scale pharmacological screening that aimed at identifying candidate drugs or chemical probes (for examples see [72] or [73]) as well as genetic screenings that focused on deciphering novel key factors involved in the considered diseases (for examples see [74] or [75]). Therefore, a yeast model for monitoring the GAr-based inhibition of the translation of its own mRNA *in cis* has been set up [76]. In this model (described in **Figure 3a**), the yeast protein Ade2 belonging to adenine biosynthesis pathway was used as a reporter protein since: (i) like EBNA1 it is a stable protein and (ii) a convenient colorimetric system allows to easily (without any treatment) determine its steady-state level in living yeast cells. Indeed, whereas yeast cells that express

Ade2 at a functional level form white colonies, yeast cells that do not express Ade2 (for example cells deleted for the *ADE2* gene -termed *ade2Δ*-) readily form red colonies on rich YPD medium and any intermediate level of Ade2 leads to the formation of pink colonies whose color intensity is inversely proportional to the level of Ade2 expressed. Hence, this simple reporter system allows to easily monitor subtle changes in the level of Ade2. For this reason, and based on the fact that GAr-based inhibition of translation is probably operant in yeast, fusions of GAr domains of various lengths (21, 43 or 235 amino acids) to Ade2 were tested in yeast and led to the formal demonstration that GAr inhibits *in cis* and in a length-dependent manner the translation of its own mRNA in yeast, without any significant effect on mRNA level nor on protein stability (which was expected given that Ade2 is a very stable protein). Hence, the 43GAr-Ade2 fusion leads to pink yeasts, thereby allowing to easily monitor the effect of GAr and serving as the screening basis for identifying compounds or genes able to interfere with its *in cis* inhibitory effect on translation: modifiers that counteract the GAr effect on translation would lead to whiter yeast colonies, whereas those exacerbating the GAr effect would lead to redder yeast colonies. This yeast-based assay has first been used for drug screening (**Figure 3b**, **left part**) and led to the identification of doxorubicin as a compound specifically counteracting the inhibitory effect of GAr on the expression of GAr-Ade2. This effect was GAr-dependent since doxorubicin had no effect on the expression of Ade2. The yeast assay was then definitively validated when doxorubicin was not only shown to stimulate EBNA1 expression in a GAr-dependent manner in human cells, but also to overcome the GAr-dependent restriction of MHC class I antigen presentation [76]. These results also suggested that, should host cell factors involved in GAr-based translation inhibition exist, they would be functionally conserved from yeast to human. This conclusion was the ground for the use of the yeast-based assay to perform a genetic screening (**Figure 3b**, **right part**) that aimed at identifying those potential host factors. The same readout (changes in the pink color of the 43GAr-Ade2 yeast strain) was used and the effect of overexpressing every single yeast gene was thus tested and led to the identification of the yeast *NSR1* gene [75, 77] which encodes the Nsr1 protein, the yeast ortholog of the human nucleolin (NCL). When overexpressed in yeast, Nsr1 or NCL led to a darker color of the 43GAr-Ade2 yeast strain due to the exacerbation of the GAr-based inhibition of translation, whereas the 43GAr-Ade2 yeast strain deleted for the *NSR1* gene form white colonies that express a higher level of the 43GAr-Ade2 fusion protein. Similar results were obtained when overexpressing or downregulating human NCL in human cells expressing EBNA1 of GAr-OVA fusions [75]. Altogether these results have highlighted nucleolin (NCL) as the first identified host cell factor required for GAr-based inhibition of translation, the mechanism at the basis of EBNA1 immune evasion. The question that came next was the precise role of NCL in GAr-based inhibition of translation.

Crucial role of RNA G-quadruplex (rG4) and of their interaction with NCL in GAr-based inhibition of translation and of antigen presentation

G-quadruplexes (G4) are non-canonical secondary structures that may assemble in guanine-rich DNA or RNA. G4 are formed by the stacking of at least two G-quartets which consist of a planar arrangement of four guanines connected by Hoogsteen hydrogen bonds and stabilized by a central cation, most

often K^+. G4 have been involved in gene regulation where they can affect transcription, splicing or translation [78-82]. Importantly, the central regions of most gamma herpesviruses genome maintenance proteins are highly homologous in terms of nucleotide sequence and are purine enriched [83]. At about the time NCL was identified as the first host factor involved in GAr effect on translation, the ability of the GAr-encoding sequence of EBNA1 mRNA to form a cluster of up to thirteen RNA G-quadruplex (rG4) was demonstrated and the possibility that these rG4 could impede ribosome progression has been proposed [84]. In line, in the same study, it was shown that destabilization of these rG4 by antisense oligonucleotides enhances both EBNA1 translation and presentation of EBNA1-derived antigenic peptides. However, as stated above, the fact that driving the translation initiation of a GAr-OVA mRNA by the *c-Myc* internal ribosome entry site (IRES) abolishes GAr-based inhibition of both translation and antigen presentation suggests that the GAr-encoding sequence does not represent *per se* a hurdle to the ribosome progression [48, 67]. In addition, at a low concentration (less than 1 µM), EBNA1 mRNA was translated *in vitro* as efficiently as the EBNA1ΔGAr mRNA [10]. In line, GAr-based inhibition of translation is not operant in a yeast *nsr1Δ* strain deleted for nucleolin [75, 77] also suggesting that the series of rG4 that may assemble in GAr-encoding RNA are not *per se* able to significantly impede ribosome progression. Importantly, human nucleolin (NCL) has long been known to interact with G-quadruplex [85, 86], hence opening the possibility that NCL interacts with rG4 of the EBNA1 mRNA and that this interaction is crucial for GAr-based inhibition of EBNA1 translation and antigenic peptides presentation. This potential NCL/rG4 of EBNA1 mRNA interaction has been evidenced using both RNA pulldown [75, 87] and an adaptation of the proximity ligation assay (PLA) for monitoring RNA/protein interactions [75, 88]. Following these various observations, the benchmark G4-ligand PhenDC3 was tested and shown to interfere with NCL binding to rG4 of EBNA1 mRNA, thereby relieving GAr-based inhibition of both translation and antigenic peptide presentation by the MHC class I [75]. This result has motivated the screening of a series of 20 cationic bis(acylhydrazone) derivatives and led to the identification of two original G4-ligands, PyDH2 and PhenDH2, more active and less cytotoxic than PhenDC3 and interfering with EBV immune evasion also by disrupting the interaction between NCL and rG4 of EBNA1 mRNA [89]. Of note, doxorubicin, the compound isolated in the yeast assay that models GAr-based inhibition of translation described above [76] was recently shown to be a G4-ligand [90, 91], thus giving a plausible explanation for its ability to interfere with EBNA1 immune evasion. Also, it has been recently observed that insertion of the *c-Myc* IRES to the 5' UTR of GAr-OVA RNA prevents the interaction between NCL and rG4 of the GAr-encoding RNA, thus readily explaining its ability to abolish GAr-based inhibition of both translation and antigen presentation [92]. In addition, this result further confirms the importance of the NCL/rG4 of EBNA1 mRNA interaction for EBNA1 immune evasion.

Finally, the mRNA encoding LANA1, the genome maintenance protein of the Kaposi virus, another oncogenic herpesvirus that also evades the immune system, has been proposed to also form rG4 in a central region presenting sequence similarity with the one encoding GAr, readily suggesting rG4 could represent a common feature that may allow immune evasion of various stealthy oncoviruses [84, 93]. The existence and the crucial role of the rG4 of LANA1 mRNA in immune evasion of the Kaposi virus have recently been shown [94, 95].

The C-terminal RGG motif of NCL is crucial for its role in GAr-based inhibition of both translation and antigen presentation

The importance of the various domains of NCL protein for GAr-based inhibition of translation has been tested. NCL is composed of three domains: (i) an N-terminal domain that contains acidic stretches; (ii) a central region that contains four tandem RNA recognition motifs (RRM1 to RRM4); and (iii) a C-terminal RGG motif. Most nucleolin homologues known to date share the same organization, with some variation in the number of RRMs. Hence, the yeast nucleolin Nsr1 contains only two RRMs but possesses the acidic N-terminal domain as well as the C-terminal RGG motif. Importantly, and in contrast to NCL in human, Nsr1 is not essential in yeast as *nsr1Δ* strains, although growing rather poorly, are viable [96]. In addition, human nucleolin NCL can complement the effect of the loss of yeast Nsr1 on GAr-based inhibition of translation in yeast [75, 77]. For these reasons, an approach combining the use of the yeast model for GAr-based inhibition of translation in which the *NSR1* gene has been deleted (*43GAr-ADE2 nsr1Δ* strain) and the expression of either Nsr1 or NCL, or of parts of them, has been exploited to determine which domain of nucleolin is involved in this process and has identified the C-terminal RGG motif of both yeast and human nucleolin. Then, this RGG motif has been shown to interact with the rG4 of EBNA1 mRNA, thus being essential for EBNA1 ability to self-limit its expression [97].

One or two methyl groups can be attached to the nitrogen atoms of arginine, a process termed arginine methylation. This post-translational modification is conducted by protein arginine methyltransferases (PRMTs) [98]. These enzymes catalyze the transfer of a methyl group from *S*-adenosylmethionine (SAM) to the guanidino nitrogen atoms of arginine. There are nine PRMTs in human that are divided into three groups: type I, type II and type III which differ in the pattern of arginine methylation catalyzed [99]. Arginine methylation occurs in a variety of protein motifs, but the RGG motifs are the most commonly reported [100, 101] and, in budding yeast, it is estimated that the majority of arginine methylation occurs in RGG motifs [102]. For these reasons, the potential role of PRMTs in GAr-based inhibition of translation and antigen presentation has been tested. Hence, using drug-based or siRNA-mediated inhibition of PRMTs, it has been shown that type I PRMTs are required for the crucial role of nucleolin in GAr-based inhibition of translation and antigen presentation [97]. Next, the role of methylation of the arginines of the RGG motif of both yeast and human nucleolins has been directly evaluated by changing the eight arginines of the RGG motif of Nsr1, or the ten arginines of the RGG motif of NCL, by either alanines (to prevent methylation), lysines (to prevent methylation while maintaining the positive charge), or phenylalanines as mimics of methylated arginines. Indeed, phenylalanine has been shown to mimic methylated arginine as it carries a bulky hydrophobic moiety [103, 104]. Changing arginines into alanine or lysine suppress the interaction of NCL and Nsr1 with rG4 of EBNA1 mRNA, and thereby GAr-based inhibition of translation, whereas replacement of arginines by phenylalanines maintains both these phenomena [97]. Altogether, these results indicate that methylation of the arginines of the C-terminal RGG motif of nucleolin is necessary for its interaction with rG4 of the GAr-encoding sequence of EBNA1 mRNA, an interaction which is required for GAr-based inhibition of translation and, as a consequence, for EBNA1 immune evasion. Following these observations, two possible models for the role of methylation of the arginines of the C-terminal RGG motif of nucleolin could be envisioned: either this methylation directly promotes the interaction of NCL with rG4 of EBNA1

mRNA, or this methylation may interfere with the ability of the RGG motif of nucleolin to interact, and thus to be sequestered, by some of its partners (that can be proteins, DNA or RNA). In favor of this second possibility is the recent observation that Scd6, a yeast protein involved in translation inhibition, is able to self-associate via its RGG motif and that this self-association prevents its ability to inhibit translation and is negatively regulated by arginine methylation catalyzed by Hmt1, the main yeast type I PRMT [26]. *In vitro* experiments based on arginine methylation of bacterially produced recombinant NCL, or on either methylated or non-methylated RGG peptides have shown that methylation of the arginines of the C-terminal RGG motif of NCL does not significantly change its ability to interact with rG4 of EBNA1 mRNA [97]. Hence, it seems that the RGG motif of NCL may be trapped by an unknown partner, thereby preventing its interaction with rG4 of EBNA1 mRNA and, upon type I PRMTs-mediated arginine methylation, the RGG motif of NCL is released and thus free to interact with rG4 of EBNA1 mRNA, hence leading to immune evasion of EBNA1 (**Figure 4**, **left part**). Of note, the unknown partner of the RGG motif of NCL could be L3, a ribosomal protein that has been shown to interact with the RGG motif of NCL [105].

Finally, all these results bring out an intriguing point regarding the role of NCL in GAr-based self-limitation of EBNA1 expression and antigenic presentation. Indeed, NCL has also been positively involved in EBV episome maintenance and transcription through a direct interaction with the EBNA1 protein [106]. Hence, NCL appears to positively control both EBV episome maintenance and transcription on the one hand, and the self-limitation of the EBV GMP (EBNA1) expression on the other. As for EBV, one can consider it makes sense to have the same host cell protein regulating these two key aspects of EBV's latency. Indeed, if NCL level is low, then the maintenance and transcription of EBV episome should be compromised but, as a result of NCL role in GAr-based limitation of EBNA1 expression, EBNA1 mRNA should be more efficiently translated, which may compensate for its reduced level and favour the maintenance of EBV genome. On the contrary, if NCL level is high, then EBV episome will be efficiently maintained and transcribed, hence leading to a high level of EBNA1 mRNA, but then the increased NCL could further downregulate its translation, thereby limiting the level of EBNA1 protein and therefore its detection by the immune system. Importantly, as stated above, the role of NCL in EBNA1 immune evasion involves its ability to interact with G4 structures present in EBNA1 mRNA, whereas its role in episome maintenance and transcription involves its ability to interact with EBNA1's N-terminal 100 amino acids (hence upstream of the GAr domain of EBNA1 protein) [106]. Therefore, targeting the NCL-EBNA1 mRNA interaction should specifically affect EBNA1 immune evasion.

Therapeutic perspectives: potential intervention points and therapeutic avenues

Based on the current model of GAr-based inhibition of both EBNA1 expression and antigen presentation, two intervention points may be envisioned to unveil EBV-infected cells, in particular tumor cells of EBV-related cancers, to the immune system: (i) the interaction between rG4 of EBNA1 mRNA and the C-terminal RGG motif of NCL, and (ii) type I PRMTs (**Figure 5**). Importantly, in both cases proof-of-principles exist as G4 ligands that interfere with the rG4/NCL interaction on the one hand, and drugs

targeting type I PRMTs on the other, interfere with the GAr-based inhibition of translation and antigen presentation [75, 89, 97]. Of note, for both these therapeutic avenues, selectivity may be an issue (as for most of the drugs). Indeed, type I PRMTs may appear as relatively non-specific therapeutic targets as their inhibition could, in principle, affect the methylation of many proteins, similar to what has been initially considered for drugs inhibiting protein kinases or phosphatases. Of note, this potential lack of specificity has not prevented the development to the clinics of a number of kinases inhibitors [107], in particular in the field of cancer, and several inhibitors of PRMTs are currently being evaluated in clinical trials. Also, since RGG motifs are the main substrates of PRMTs and as, in various cell-based assays, type I inhibitors did not exhibit significant toxicity at concentration ranges in which they significantly affect both GAr-based translation inhibition and the interaction between NCL and rG4 of EBNA1 mRNA, the lack of specificity may not be an issue that could preclude their potential further developments to the clinics [97]. As for G4 ligands, an inherent limitation in their development to the clinics may also be their potential lack of specificity given that, in addition to the numerous rG4, there are hundreds of thousands potential DNA G4 (dG4) that may form in the human genome. Importantly however, G4 in the RNA context are often in a dynamic equilibrium with other structural isoforms, and even low doses of G4 ligands may have a profound impact on this equilibrium, preventing or leading to the recruitment of the corresponding structure-specific binding factors (nucleolin in the case of the rG4 of EBNA1 mRNA). Another important point is that, besides the G-quartets, which are the constant elements constitutive of G4, there are other structural characteristics which vary from one sequence to another: the loops and the flanking regions. These elements ensure that each G4 is, in principle, unique within the whole genome or transcriptome and, as such, could be exploited as key targets to gain specificity through, for instance, the coupling of complementary oligonucleotides to G4-ligands [108]. Hence, it is not excluded that some specificity toward particular G4, notably rG4, may be reached and various methods to assess G4 ligands specificity have been described [109, 110]. Finally, the combined use of G4-ligands and inhibitors of type I PRMTs may also help to solve the specificity issue by potentially allowing the use of much reduced doses of both types of drugs.

The citrullination of arginines of the RGG motif of NCL may also be involved in the GAr-based mechanism of inhibition of both EBNA1 translation and antigen presentation. Indeed, deamination, also called citrullination, is the process of conversion of arginine into citrulline through enzymatic activation of peptidyl arginine deiminases (PADIs), notably PADI4 (aka PAD4), the main peptidyl arginine deiminase in human, thus preventing their methylation by PRMTs [111, 112]. Citrullination of RGG motifs was observed in several RGG-containing proteins, including NCL [113]. Interestingly, it has been reported that EBV infection activates several PRMTs, especially PRMT1, the main type I PRMTS, and at the same time inactivates PADI4 [114]. Hence, a possibility exists that EBV infection, by activating PRMT1 as well as inactivating PADI4, activates arginine methylation of the NCL C-terminal RGG motif, thus promoting the interaction of this protein with the G4s of EBNA1 mRNA, leading to limitation of its translation, and therefore to the evasion of EBNA1/EBV from the immune system (**Figure 4**).

Importantly, in addition to the GAr-encoding mRNA sequence, the GAr peptide could also participate to the inhibition of its own expression. For example, the addition of antibodies directed against the GAr domain can enhance the translation of GAr-carrying mRNAs, which supports the idea that the nascent

GAr polypeptides could participate to the translation inhibition of its own mRNA [48, 67]. In addition, the nascent GAr peptides have recently been shown to interact with the nascent polypeptide-associated complex alpha (NACA) and displace it from the ribosome [115]. Endogenous NACA, in addition to bind to the GAr peptides, also interacts with GAr mRNA sequence as well as with NCL in the cell nucleus, thus facilitating NCL-EBNA1 mRNA interaction, thereby favoring mRNA translation inhibition *in cis*. Importantly, siRNA-mediated silencing of NACA expression interferes with the interaction between NCL and the rG4 of GAr mRNA and thus on GAr-based inhibition of both translation and antigenic peptides production [115]. Also, as stated above, the EBNA1 protein itself contains two RGG motifs: one upstream and one downstream of the GAr domain, and it has been shown that EBNA1 could bind to its own mRNA through its two RGG motifs [116] and also that these two RGG motifs could bind to rG4 [117]. Moreover, these RGG domains are encoded by G-rich sequences in the EBNA1 mRNA that also have potential to form G-quadruplexes. These observations open two non-mutually exclusive possibilities that would implicate the RGG domains of EBNA1 in the mechanism of regulation of its expression. Firstly, like NCL, the EBNA1 protein could bind to the rG4 of its own mRNA through its two RGG motifs, and thus participate in the inhibition of its translation. The other possibility would be that, like the GAr-encoding sequence, the EBNA1 mRNA sequences encoding the two RGG motifs of EBNA1 would be capable of forming rG4 that may also recruit NCL and/or EBNA1 protein(s) and would therefore participate to the inhibition of EBNA1 translation as well as the production of EBNA1-derived antigenic peptides, leading thus to the immune evasion of EBNA1/EBV. Of note, in both cases, type I PRMTs and PADIs may play a regulatory role. Interestingly, NCL and EBNA1 proteins have been reported to interact [106], hence opening the possibility of a cooperative binding on rG4 of EBNA1 mRNA, and thereby of a cooperative inhibitory effect of these two proteins on EBNA1 mRNA translation. All these possibilities clearly deserve detailed investigations.

Importantly, in addition to its crucial role in EBV genome replication and maintenance and in the virus immune evasion, EBNA1 has also been linked to cell transformation and, if the mechanism underlying its oncogenic activity is still unclear [16], it may involve the c-Myc oncogene. Indeed, the EμEBNA1 mice that express EBNA1 in their B-cells thanks to the immunoglobulin heavy chain intronic enhancer (Eμ) develop and succumb to B-cell lymphoma [118]. Importantly, the B cell tumours of these mice are monoclonal and remarkably similar to those induced by transgenic c-Myc expression (Eμmyc transgenic mice, [119, 120]). However, contrary to the characteristic translocations observed in the *c-MYC* locus which are linked to the deregulation of its expression, in B cells tumours arising from EμEBNA1 transgenic mice the murine *c-MYC* locus was intact with no 5' rearrangements detected whereas the expression of c-Myc is also deregulated [118]. Hence, EBNA1 is able to induce overexpression of the *c-MYC* oncogene and, as such, may participate to the oncogenicity of EBV. Interestingly, recently a direct link between EBNA1 and C-Myc expression was revealed with the identification of a unique mechanism involving the GAr domain of EBNA1 [121]. In this model, the *in cis* GAr-mediated mRNA translation suppression causes a translational stress that activates the E2F1 transcription factor expression *via* phosphoinositide-3-kinase-δ- (PI3Kδ-) dependent signalling. This increase in the level of E2F1 causes the induction of c-Myc. In support of this model are the observations that drug-based or siRNa-mediated specific inhibition of PI3Kδ abrogate EBNA1-dependent induction of both E2F1 and c-

Myc. Hence, in addition to be the GMP of EBV and to play a central role in the ability of the virus to evade the immune system, EBNA1 participate to the oncogenicity of EBV. Interestingly, both oncogenicity and immune evasion of EBV depend on the GAr domain of EBNA1. For this reason, pharmacological means that would result in exacerbating the GAr inhibitory effect on translation, by further decreasing EBNA1 level, may also be of therapeutic value for EBV-related cancers.

Finally, beyond cancer, EBV, and more particularly EBNA1, have been involved in other diseases, notably multiple sclerosis (MS). Indeed, the long-time suspected link between EBV and MS has recently been firmly confirmed in a large-scale study in which the authors analysed serum EBV antibodies from individuals who developed MS among a cohort of more than 10 million young adults in a more than 20 years follow-up, and the results indicated a 32-fold increase in the risk of developing MS after EBV infection [15]. In parallel, a role for EBNA1 in MS has been proposed, notably via molecular mimicry leading to the development of cross-reactive antibodies and T cells [122, 123]. In line, a vaccine against EBNA1 appears a relevant therapeutic option to treat EBV-related disorders, in particular EBV-related cancers and MS, and several experimental vaccines have been developed against EBV, including against EBNA1 [124-126]. Notably, vaccination against EBNA1 has been shown efficient in eliciting comprehensive $CD4^+$ and $CD8^+$ T cell responses which can translate into protection against EBV antigen expressing lymphomas [125, 127].

Concluding remark

Beyond the clear interest of all the studies on EBNA1 biological roles and immune evasion for defining intervention points and novel therapeutic avenues to treat EBV-related diseases, in particular EBV-related malignancies, they also represent another illustration that “viruses are the best cell biologists” in that their study quite often leads to seminal discovery in cell biology.

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Figure Legends

Figure 1: Schematic representation of the EBV-encoded EBNA1 protein highlighting the essential role of the GAr domain in immune evasion of the virus

The first 32 amino acids of EBNA1 full length are not associated to a particular function. The two RGG motifs (Arginine-Glycine-Glycine) are highlighted in gold (from amino acids 33 to 64 and 329 to 377), the GAr domain (Glycine-Alanine repeat) is shown in orange (from amino acids 90 to 328), the nuclear localization signal (NLS) is located between amino acids 378 and 386 and colored in black. The DNA-binding and dimerization domain (amino acids 459 to 609) is colored in purple. As EBNA1 full length, EBNA1ΔGAr is able to fulfill its essential functions for EBV genome replication and maintenance.

Figure 2: The proteasome and the ribosome era parallel with the two consecutive models for EBNA1 immune evasion

On the left (**Proteasome era**) is represented the former model in which processing of the full-length EBNA1 protein by the 26S proteasome would be the main source of EBNA1-derived antigenic peptide. On this ground, the ability of GAr to inhibit *in cis* 26S proteasome-mediated proteolysis gave a plausible explanation for its role in immune evasion of EBNA1.

On the right (**Ribosome era**) is represented the current model in which most of antigenic peptides would originate from byproducts of the translation (the so-called defective ribosomal products -DRIPs-) of EBNA1 mRNA. Hence, in this model, the ability of GAr to limit *in cis* the translation of its own mRNA fully explains its inhibitory effect on antigen presentation.

Figure 3: Rationale of the yeast-based assay for GAr-based inhibition of translation and use for both drug and genetic screenings

a. Rationale of the use of the Ade2 reporter protein in yeast. When Ade2 is expressed at a functional level yeast cells are white. When not expressed (*ade2Δ* cells), yeast cells are red. Any intermediate level of Ade2 leads to pink colonies whose colour intensity is inversely proportional to the level of Ade2 expression. This allows to easily monitor the GAr-inhibitory effect on translation in yeast.

b. Examples of use of the Ade2 reporter system for drug screening (left panel) aiming at identifying compounds that counteract the *in cis* inhibitory effect of GAr on translation, or genetic screening (right panel) to identify host genes involved in GAr-based inhibition of translation.

Figure 4: Model for the role of NCL and of the arginine methylation and citrullination of its C-terminal RGG motif in EBNA1 immune evasion

In this model on the left, NCL would be trapped by an unknown factor (Protein X) which interacts with the unmethylated C-terminal RGG motif of NCL. Upon type I PRMTs-mediated methylation of the arginines of this RGG motif, the NCL/Protein X complex would be dissociated. Hence, the RGG motif of NCL would now be available to interact with the RNA G-quadruplexes (rG4) of EBNA1 mRNA, which is required for GAr-based limitation of translation, a phenomenon at the basis of EBNA1 immune evasion.

Citrullination (on the right), by preventing arginine methylation, would interfere with type I PRMTs-mediated methylation of the C-terminal RGG motif of NCL, hence interfering with GAr-based inhibition of translation and presentation of EBNA1-derived antigenic peptides.

Figure 5: Possible intervention points to unveil EBNA1 to the immune system.

Based on the model presented in **Figure 4**, at least to intervention points could be exploited to unveil EBV-infected cells (in particular tumour cells of EBV-related cancers) to the immune system: G4 ligands such as PhenDC3 that interfere with the binding of NCL on rG4 of EBNA1 mRNA (left) and compounds inhibiting type I PRMTs (right). By distinct modes of action, both types of drugs interfere with the binding of NCL on rG4 of EBNA1 mRNA, and thereby with the ability of GAr to self-limit the translation of its own mRNA. This, in turn, relieves the limitation of the production of EBNA1-derived antigenic peptides, ultimately interfering with immune evasion of EBNA1 and thus of EBV.

Figure 1

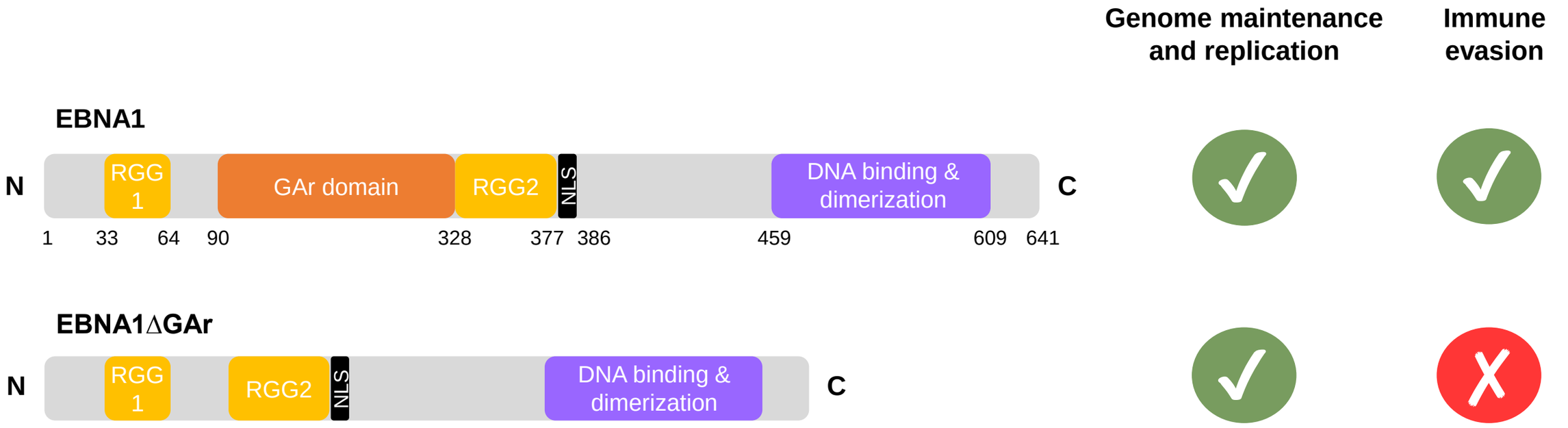
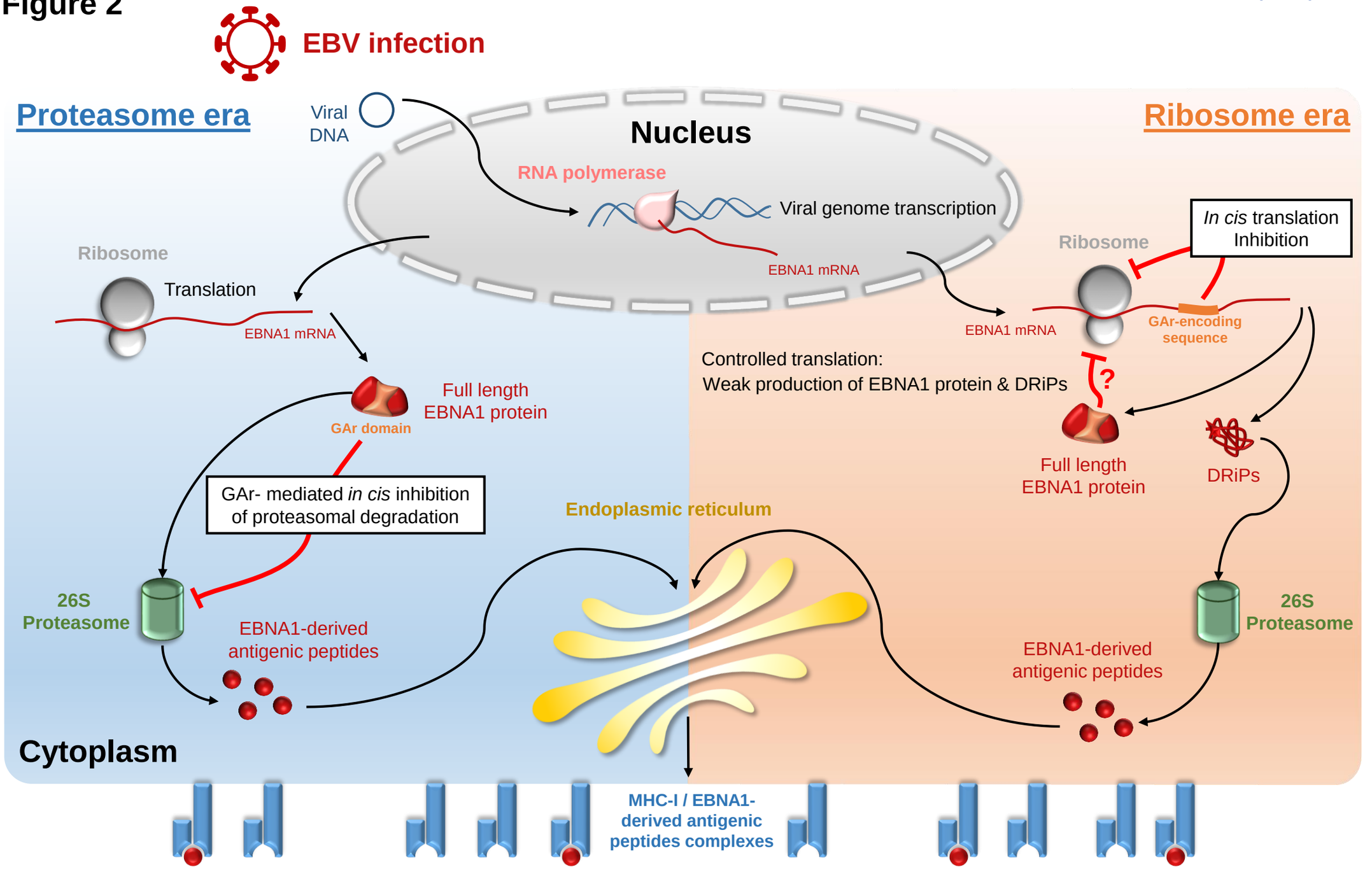


Figure 2



Limited presentation of EBNA1-derived antigenic peptides

Figure 3

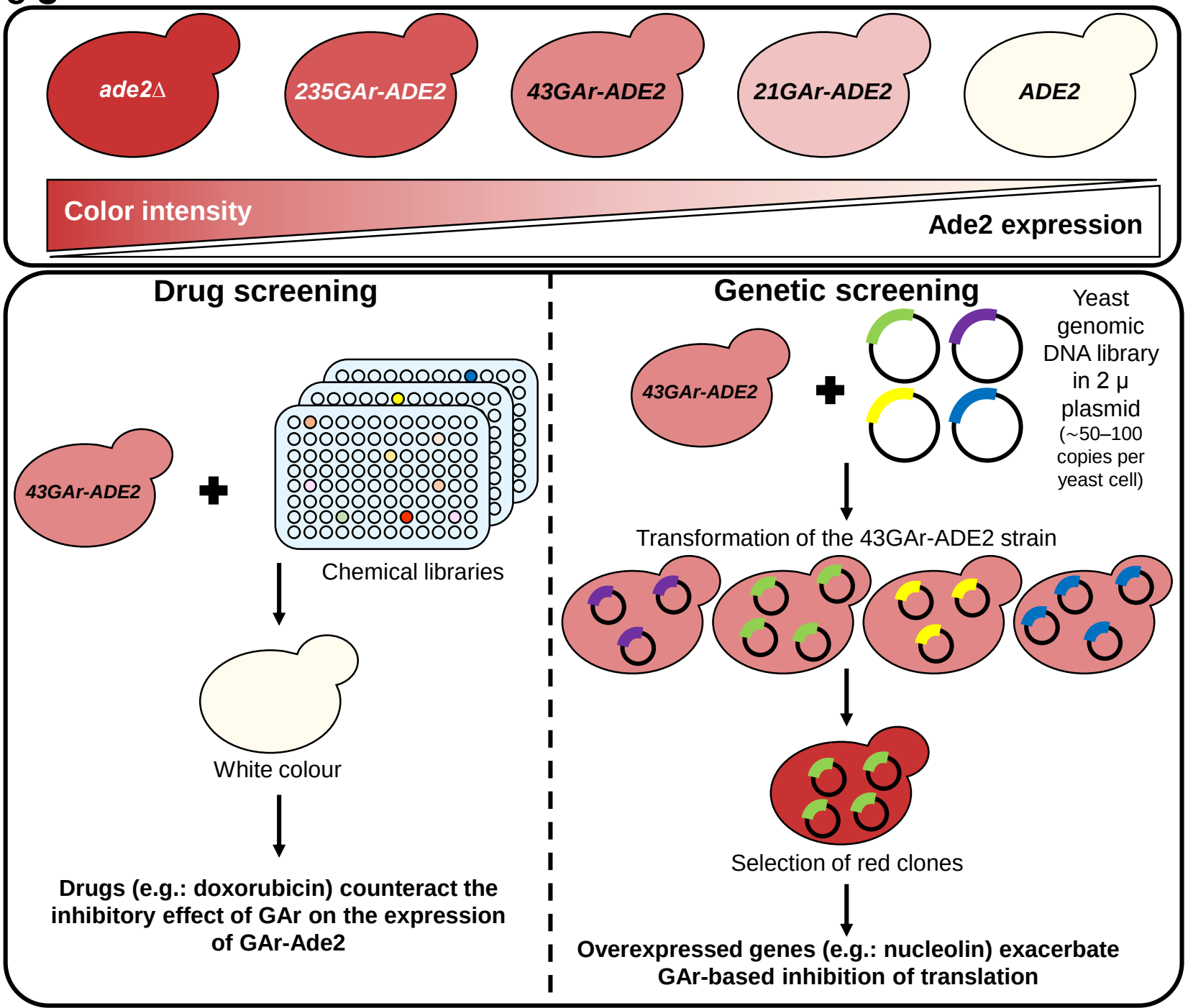


Figure 4

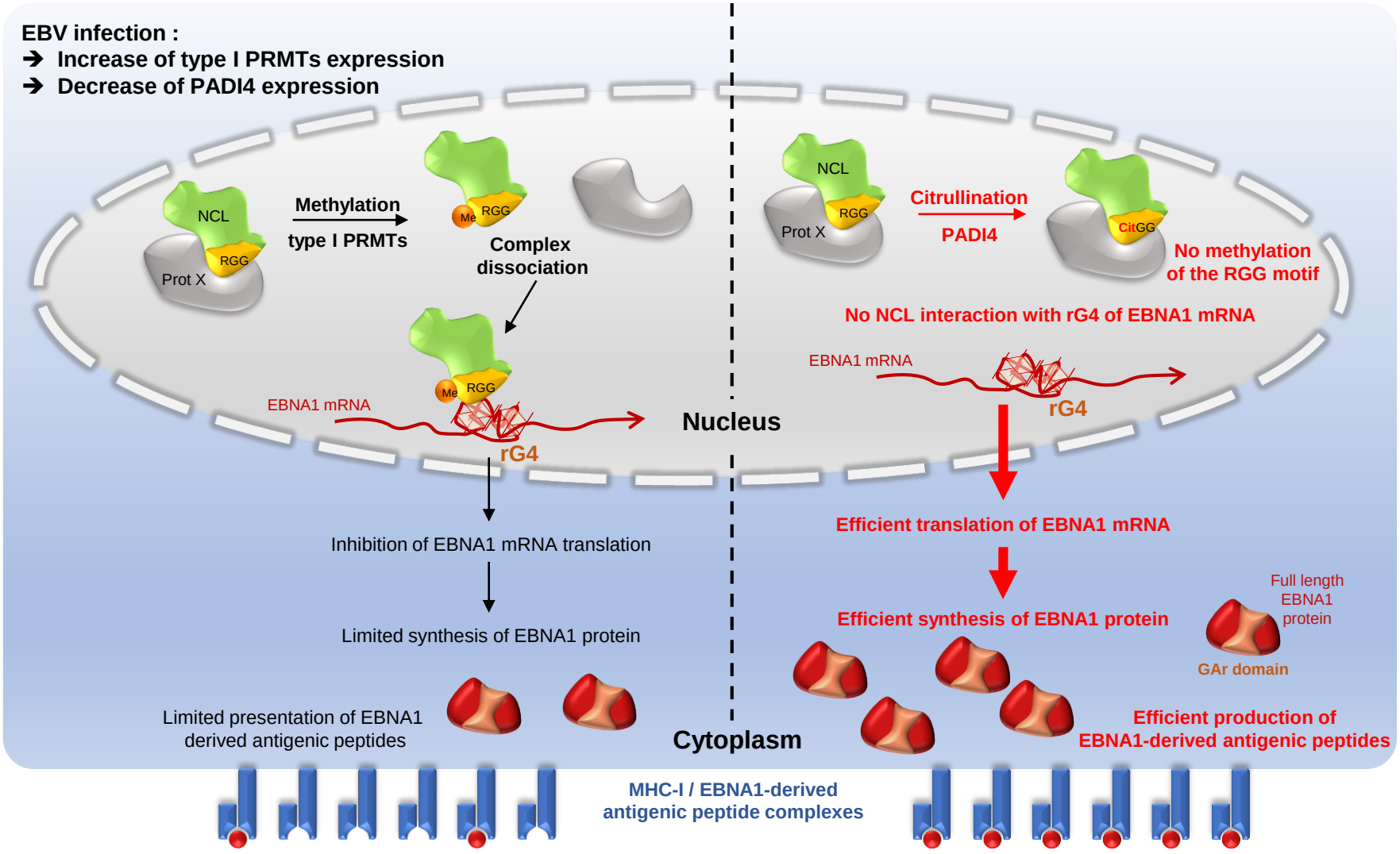


Figure 5