



Roseoloviruses manipulate host cell cycle

Niza Frenkel, Eyal Sharon¹ and Haim Zeigerman¹

During lytic infections HHV-6A and HHV-6B disrupt E2F1–Rb complexes by Rb degradation, releasing E2F1 and driving the infected cells toward the S-phase. Whereas upon infection E2F1 and its cofactor DP1 were up-regulated, additional E2F responsive genes were expressed differentially in various cells. E2F binding sites were identified in promoters of several HHV-6 genes, including the U27 and U79 associated with viral DNA replication, revealing high dependence on the binding site and the effect of the E2F1 transcription factor. Viral genes regulation by E2F1 can synchronize viral replication with the optimal cell cycle phase, enabling utilization of host resources for successful viral replication. Furthermore, it was found that infection by roseoloviruses leads to cell cycle arrest, mostly in the G2/M-phase.

Addresses

Department of Cell Research and Immunology and the S. Daniel Abraham Institute for Molecular Virology, Tel Aviv University, Tel Aviv 69978, Israel

Corresponding author: Frenkel, Niza (nfrenkel@post.tau.ac.il)

¹ These authors contributed equally to this work.

Current Opinion in Virology 2014, 9:162–166

This review comes from a themed issue on **Special section: roseoloviruses**

Edited by Laurie Krug

For a complete overview see the [Issue](#) and the [Editorial](#)

Available online 10th November 2014

<http://dx.doi.org/10.1016/j.coviro.2014.10.003>

1879-6257/Published by Elsevier B.V.

Introduction

The mammalian cell cycle is a highly regulated process. At the G1 phase cells undergo a critical check point, ensuring their readiness for DNA synthesis. This is followed by the S-phase during which the cellular genome is duplicated. Following DNA duplication, the cell progresses into the G2 phase, preparing for mitosis. Members of the E2F family serve as transcriptional activators of genes that play significant roles in cell cycle control, including: DNA replication, mitosis, the mitotic checkpoint, DNA-damage checkpoints, DNA repair, differentiation, development and apoptosis [1]. In responsive genes containing the E2F binding sequence transcription begins by binding the E2F and DP heterodimers to the E2F binding site. The E2F-DP transcription complexes are negatively regulated by members of

the retinoblastoma (Rb) protein family, which block the E2F activation domain, preventing the transcription of E2F responsive genes.

The Rb protein is regulated by phosphorylation and degradation. Hypophosphorylated Rb binds E2F1 with a high affinity, leading to inhibition of E2F1 transcription activity. In the G1 phase the Rb protein is inactivated following its phosphorylation by cyclin D/CDK-4/6 and cyclin E/CDK-2 complexes, resulting in its dissociation from E2F1 and cellular entry into the S-phase [2,3].

DNA viruses synthesize significant quantities of nucleic acid during the productive lytic replication. Therefore they have evolved ways to modulate the Rb–E2F pathway. Viral inactivation of the Rb family members enables them to create an environment more accommodating for viral replication. The disassembly of Rb/E2F1 complexes by viral proteins leads to accumulation of free E2F1 transcription factor and induction of S phase entrance. Such viral proteins include the extensively studied human papillomavirus oncoprotein E7, the adenovirus E1A protein, and the SV40 large T antigen. These three viral proteins represent two distinct mechanisms of Rb inactivation: steric disruption of Rb–E2F complexes and Rb degradation [4**].

Herpesviruses encode proteins that use these pathways and additional direct and indirect mechanisms to inhibit Rb family member function.

Cells infected by the *alphaherpesviruses* HSV-1, HSV-2, and VZV, accumulate in the G1 phase of the cell cycle [5,6]. Moreover, while the Rb proteins remain unphosphorylated in HSV-infected cells, the activity of kinases responsible for their phosphorylation, the Cdks, is critical for HSV-1 replication [7]. Thus, the *alphaherpesviruses* may be less dependent on cellular E2F-responsive genes for viral DNA replication than other herpesviruses and may not need to target Rb family members for inactivation.

The *gammaherpesviruses* are associated with proliferative disorders including a number of cancers. Both EBV and KSHV appear to encode proteins that modulate the Rb–E2F pathway, either directly or indirectly. EBV has multiple proteins (Z, R, LMP-1, EBNA-2,-3C,-5), that could lead to the phosphorylation of Rb by cellular CDKs, and/or may directly phosphorylate Rb through the function of the viral kinase, BGLF-4 (ortholog of HCMV UL97). Moreover, EBV at the lytic infection, but not during latency, was shown to induce cell cycle arrest at

the G1/S with elevated levels of cyclin E and cyclin A [8]. In the KSHV infection E2F1 pathway is activated by LANA-mediated disruption of E2F1/Rb complexes, or by direct phosphorylation of Rb through the action of the v-cyclin and/or the ORF36 proteins [9,10].

Beta-herpesviruses manipulate E2F–Rb pathway during lytic infection

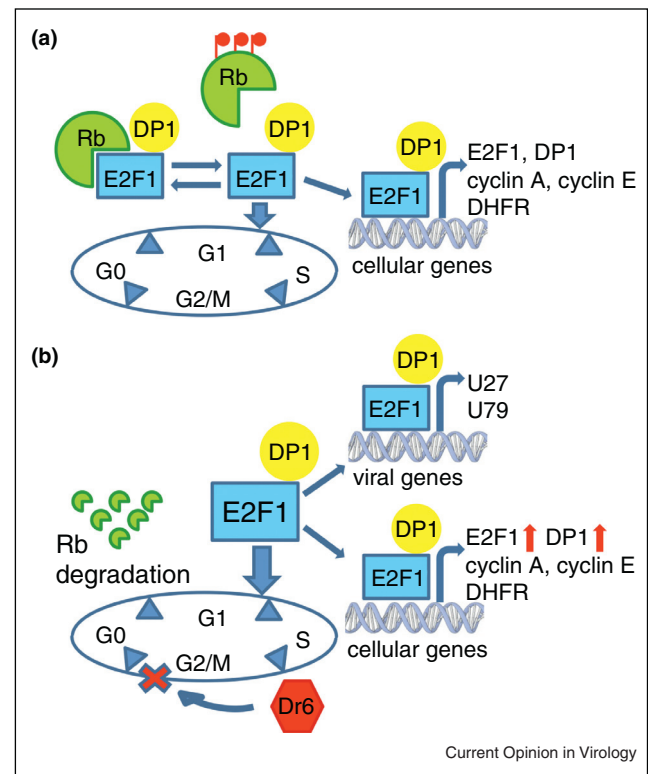
For the HCMV: It was demonstrated that at very early stages of infection hypophosphorylated Rb protein was first degraded, and protein synthesized de novo was then hyperphosphorylated [11]. The HCMV pp71 protein is a prominent component of the viral tegument [12,13]. By binding to Rb protein, pp71 induces Rb degradation in a proteasome-dependent, ubiquitin-independent manner [14]. HCMV UL97 has been shown to phosphorylate multiple residues of Rb, disrupting the E2F1/Rb and Rb/HDAC complexes, rendering Rb inactive. Due to both pp71-mediated degradation and UL97-mediated phosphorylation, Rb is inactivated and E2F responsive genes are highly expressed [15].

For the Roseoloviruses: We have shown [16*,17**] that in SupT1 T cells infected with HHV-6A the E2F1 protein and its co-factor DP1 increased whereas the Rb protein underwent massive degradation without hyperphosphorylation at 3 sites, Ser-780, Ser-807 and Thr-821, known to control E2F/Rb association (Figure 1). The degradation of Rb started simultaneously with E2F1 and DP1 up-regulation. Furthermore, it correlated with the accumulation of the viral p41 protein that functions in viral DNA replication. Increased E2F1 expression was also described employing a microarray assay of HHV-6B infected adult T-cell leukemia cell line [18]. Because HHV-6A infection induced elevation of free E2F1, it was of interest to monitor the expression of the E2F1 target genes and alterations of cell cycle during the infection. Although E2F1 and DP1 levels were elevated we found that cyclin A, cyclin E, DHFR and MCM5 were not up-regulated [16*,17**]. These results differ from the results of De Bolle and coworkers, who found that late post HHV-6A infection of human cord blood mononuclear cells there was increased accumulation of cyclin A without up-regulation of cyclin E [19]. Furthermore, analysis of the regulatory proteins which are involved in the cell cycle in HSB-2 cells [20], indicated that cyclins A2, B1, E1 and MCM5 were increased in HHV-6-infected cells, but there was no difference in cyclin D1. Hence, the expression of E2F1 target genes during HHV-6A infection varies in different cells/tissues examined.

Enhanced transcription of viral genes by E2F1

As described above, HHV-6A infection induces Rb degradation, up-regulation of E2F1 and DP1. However, there was no increased expression of additional E2F1-responsive genes. It was thus of interest to test whether the virus exploited E2F1 for viral gene transcription. Scanning of

Figure 1



Mechanisms used by HHV-6 to modulate the Rb–E2F pathway and cell cycle progression. (a) E2F1 regulation in uninfected cells involves Rb phosphorylation and E2F1 release, leading to transcription of cellular genes and entry to S-phase. (b) HHV-6 infection leads to massive Rb degradation and to E2F1 release. Active E2F1 induces up-regulation of itself and its co-factor DP1 which are utilized for transcription of cellular genes as well as viral genes (U27, U79). DR6 gene product causes cell cycle arrest in G2-phase.

the HHV-6A genome revealed several genes that contained the consensus E2F binding sequence TTTSSCGC, where S is either a G or a C upstream of the ATG start codon. This included: (i) U18, a transcriptional regulator in the IE-B/E gene class. (ii) U33, a viral tegument protein that is a critical mediator of metabolic stress. (iii) U52 gene which promotes the accumulation of late transcripts. (iv) U74 gene encoding a portion of the helicase/primase complex. (v) The U27 and the U79 genes, both functioning in viral DNA synthesis [17**]. The U27 gene encodes the P41 viral DNA polymerase processivity factor [21]. The U79-80 early gene encodes a family of nuclear proteins that were found to be essential for viral DNA replication [22].

We concentrated on the U27 and U79 genes and tested whether the E2F1 transcription factor and E2F binding site were utilized for their expression [17**]. We constructed vectors containing a GFP reporter gene driven by wild-type viral promoter or by mutant promoter that

abolished the binding of E2Fs. It was found that the expression of the U27 promoter was dependent on the presence of the intact E2F binding site. Abolishing the E2F site led to significant decrease in promoter activity. Moreover, treatment of the cells with siRNA to E2F1 resulted in decreased U27 promoter activity whereas over-expression of E2F1 led to a substantial increase of the promoter activity. This indicated that additional E2F transcription factors may play a role in the induction of the promoter. High activity of the U79 promoter was detected in SupT1 cells depending on the presence of E2F binding site because the mutation of the site led to 90% reduced activity. The involvement of E2F1 in U79 promoter activity was demonstrated by siRNA treatment and over-expression of the E2F1 protein.

The involvement of E2F1 in transcription of the viral U27 and the U79, β and α genes, respectively, is a novel outlook of the regulation of HHV-6 gene expression. E2F1 studies are of additional interest because approximately 30% of cellular genes contain promoters with E2F1 binding site and analyses of thousand promoters revealed that more than 20% of promoters were bound by E2F1. These results place the E2F1 as a factor that contributes to the regulation of a large fraction of human genes [23]. A possible mechanism for the utilization of E2F1 transcription factor in viral replication might involve viral protein(s) recruiting the E2F1 and directing it to viral promoter(s) or to selected cellular promoters. Examples for such processes include the E2 adenovirus promoter that is activated by an E2F1/E4 complex [24]. In addition, bovine herpes virus 1 (BHV-1) infection leads to increased E2F1 protein levels and the activity of the bovine ICP0 early promoter is increased dramatically by E2F1 [25].

Cell cycle arrest in roseoloviruses infection

A variety of viruses were found to induce the G₂/M arrest, including DNA viruses, RNA viruses, and retroviruses

[26]. Inhibition of the G₂/M checkpoint by viruses may serve to maintain the cell in a pseudo S-like phase increasing viral replication [20]. Viruses may also utilize the DNA damage responses to maximize viral replication [27]. For the many of viruses it is difficult to define precisely the mechanism(s) of the G₂/M arrest. The cell cycle is controlled by complex interactions that are not yet fully understood. In a number of instances, G₂ arrest has been linked to the inhibition or delay in the activation of the Cdk1/cyclin B1 kinase activity [28]. Another mechanism is the interference with mitotic progression. It was shown that HHV-6A infection induced cell cycle arrests at the G₂/M phase in different cell types, as summarized in Table 1, including: glial cell, epithelial cells, HSB-2 cells, cord blood mononuclear cells and SupT1 T cells.

HHV-6B infection led to cell cycle arrest in G₁/S and/or G₂/M depending on the cells that were tested [19,29–31,32*]. A new report showed that DR6 protein can induce accumulation of cells in G₂/M and also the cytoplasmic accumulation of cyclin B1 [32*]. This function was dependent on the N-terminal part of the protein, which is also required for nuclear localization. Thus, a possible role of DR6 during HHV-6B infection might be that DR6 functions as a chaperone facilitating the assembly of viral replication units or facilitating cell proliferation arrest in order to enhance viral replication.

It was reported by Zauli G. and co-worker [33] that HHV-7 infection of both primary CD4+ T lymphocytes and SupT1 T-cell line induced various alteration of cell cycle regulation. Specifically, elevated level of cyclin B and Cdk1 were observed late post infection and were accompanied by G₂/M arrest. To the best of our knowledge, no additional studies were reported for HHV-7 involvement in cell cycle arrest.

Mechanisms for cell cycle arrest at G₂/M may involve the cellular response to DNA damage [26]. More specifically,

Table 1

Cell cycle arrest induced by HHV-6A and HHV-6B

Virus	Host cell line	Phase of arrest	Assay	Mechanism, viral protein involved	Ref.
HHV-6B	Human epithelial cell (HCT 116)	G1/S 24 hpi	FACS: tDNA, V	p53 independent	[31]
HHV-6B (DR6 exp. v.)	Human epithelial cell (HCT 116)	G2/M 24, 48 hpi	FACS: tDNA	DR6, p53 independent WB of cyclins and CDK	[32*]
HHV-6A	Human glial precursor cell	G1/S 72 hpi	FACS: tDNA, V	Not analyzed	[29]
HHV-6B	Human CBMC	G2/M 48, 72, 96 hpi	FACS: tDNA, V	WB of cyclins and CDKs	[19]
HHV-6B	T cells (MOLT 3)	G1/S; G2/M 24, 48 hpi	FACS: tDNA	p53 pathway	[30]
HHV-6A	T cells (SUPT1)	G2/M 24; 48 hpi	FACS: tDNA	Rb modification	[16*]
HHV-6A	T cells (HSB-2)	G2/M 24, 48, 72 hpi	FACS: tDNA	WB of Cyclins	[33]

Abbreviations: exp. v., cells were transfected with expression vector; hpi, hours post infection; WB, western blot analysis; tDNA, total DNA fluorescent staining; V, fluorescent staining of viral components.

checkpoint kinases 1 and 2 (Chk1 and Chk2) can be responsible for the G2/M phase arrest by phosphorylation of CDKs in response to either ssDNA or dsDNA breakage [34,35]. Massive DNA breakage occurs during the cleavage and packaging of viral concatemeric DNA. In addition, in viral infections there is uncoupling between cell cycle phase and the appearance of cellular proteins characteristic of the particular phase [36].

In HHV-6 infections there are uncertainties with regards to the cell cycle arrest at the G2/M phase: first, the cell cycle is defined by measuring total DNA content of the infected cells (Table 1) without considering the viral DNA replication. Second, we expect that the majority of infected cells will be accumulated in the arrested phase. However, in previous reports only moderate increase in the percentage of cells in the G2/M phase were observed. Finally, the infection of HHV-6 leads to fusion of infected cells and formation of syncytia with ballooning shape, causing significant alterations in cell morphology and function.

Summary of the findings and future directions

It was shown that HHV-6 infection of T cells resulted in Rb degradation. The released E2F1 was associated with some cellular transcription as well as transcription of viral genes (Figure 1). The HHV-6 genome encodes a number of genes containing in their promoters the E2F binding site. These include the E2F binding sites in the U27, U74 and U79 promoters which are conserved in the HHV-6A and HHV-6B genomes. A comparison between HHV-6A and HHV-6B regarding the expression of these genes, in primary infection as well as following reactivation from latency, can contribute to the understanding of variation of viral replication in different cell types.

Future studies could involve analyses of additional genes containing the E2F1 binding site in their promoters. Furthermore, the kinetics of synthesis should be followed so as to determine whether the transcription employing E2F binding site follows the regular cascade regulation of immediate early, early and late viral genes expression. An increasing body of evidence demonstrated that HHV-6 induces the accumulation of infected cells at G2/M phase. However, these experiments were based mostly on total DNA analysis. It is necessary to test additional aspects characterizing the phase of the cell cycle as well as the metabolic state of the infected cells. These analyses would potentially increase our understanding of roseolovirus involvement in human diseases and engineering of new antiviral therapeutics.

Acknowledgements

This review was supported by the Israel Science Foundation grant no.187/12; the S. Daniel Abraham Institute for Molecular Virology; and the S. Daniel Abraham Chair for Molecular Virology and Gene Therapy, Tel Aviv University.

References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- of special interest
 - of outstanding interest
1. Polager S, Ginsberg D: **E2F — at the crossroads of life and death.** *Trends Cell Biol* 2008, **18**:528-535.
 2. Mundle SD, Saberwal G: **Evolving intricacies and implications of E2F1 regulation.** *FASEB J* 2003, **17**:569-574.
 3. Singh S, Johnson J, Chellappan S: **Small molecule regulators of Rb–E2F pathway as modulators of transcription.** *Biochim Biophys Acta* 2010, **1799**:788-794.
 4. Hume AJ, Kalejta RF: **Regulation of the retinoblastoma proteins** •• **by the human herpesviruses.** *Cell Div* 2009, **4**:1.
In-depth review of the alterations in cell cycle during the infections of different human herpesviruses. This review summarizes the data that was found on the matter up to 2009.
 5. Song B, Liu JJ, Yeh KC, Knipe DM: **Herpes simplex virus infection blocks events in the G1 phase of the cell cycle.** *Virology* 2000, **267**:326-334.
 6. Leisenfelder SA, Moffat JF: **Varicella-zoster virus infection of human foreskin fibroblast cells results in atypical cyclin expression and cyclin-dependent kinase activity.** *J Virol* 2006, **80**:5577-5587.
 7. Schang LM, Phillips J, Schaffer PA: **Requirement for cellular cyclin-dependent kinases in herpes simplex virus replication and transcription.** *J Virol* 1998, **72**:5626-5637.
 8. Rodriguez A, Jung EJ, Flemington EK: **Cell cycle analysis of Epstein–Barr virus-infected cells following treatment with lytic cycle-inducing agents.** *J Virol* 2001, **75**:4482-4489.
 9. An FQ, Compitello N, Horwitz E, Sramkoski M, Knudsen ES, Renne R: **The latency-associated nuclear antigen of Kaposi's sarcoma-associated herpesvirus modulates cellular gene expression and protects lymphoid cells from p16 INK4A-induced cell cycle arrest.** *J Biol Chem* 2005, **280**:3862-3874.
 10. Kuny CV, Chinchilla K, Culbertson MR, Kalejta RF: **Cyclin-dependent kinase-like function is shared by the beta- and gamma-subset of the conserved herpesvirus protein kinases.** *PLoS Pathog* 2010, **6**:e1001092.
 11. Hume AJ, Finkel JS, Kamil JP, Coen DM, Culbertson MR, Kalejta RF: **Phosphorylation of retinoblastoma protein by viral protein with cyclin-dependent kinase function.** *Science* 2008, **320**:797-799.
 12. Kalejta RF: **Functions of human cytomegalovirus tegument proteins prior to immediate early gene expression.** *Curr Top Microbiol Immunol* 2008, **325**:101-115.
 13. Kalejta RF: **Tegument proteins of human cytomegalovirus.** *Microbiol Mol Biol Rev* 2008, **72**:249-265.
 14. Winkler LL, Kalejta RF: **The 19S proteasome activator promotes human cytomegalovirus immediate early gene expression through proteolytic and non-proteolytic mechanisms.** *J Virol* 2014, **88**:11782-11790.
 15. Kalejta RF: **Human cytomegalovirus pp71: a new viral tool to probe the mechanisms of cell cycle progression and oncogenesis controlled by the retinoblastoma family of tumor suppressors.** *J Cell Biochem* 2004, **93**:37-45.
 16. Mlechkovich G, Frenkel N: **Human herpesvirus 6A (HHV-6A) and HHV-6B alter E2F1/Rb pathways and E2F1 localization and cause cell cycle arrest in infected T cells.** *J Virol* 2007, **81**:13499-13508.
 - First report that showed alterations of the E2F1-Rb pathways during HHV-6A infection, including: a high level of free E2F1, a disassembly of Rb–E2F1 complexes and accumulation of the infected cells in G2/M phase.
 17. Sharon E, Volchek L, Frenkel N: **Human herpesvirus 6 (HHV-6) alters E2F1/Rb pathways and utilizes the E2F1 transcription factor to express viral genes.** *Proc Natl Acad Sci U S A* 2014, **111**:451-456.

This manuscript reports additional findings regarding the manipulation on E2F1-Rb pathway during HHV-6A infection, showing a Rb degradation, a selective induction of the E2F1 responsive cellular genes, a presence of E2Fs binding sites in HHV-6A genome and utilization of E2F1 and E2Fs binding sites for viral genes expression.

18. Takaku T, Ohyashiki JH, Zhang Y, Ohyashiki K: **Estimating immunoregulatory gene networks in human herpesvirus type 6-infected T cells.** *Biochem Biophys Res Commun* 2005, **336**:469-477.
 19. De Bolle L, Hatse S, Verbeken E, De Clercq E, Naesens L: **Human herpesvirus 6 infection arrests cord blood mononuclear cells in G(2) phase of the cell cycle.** *FEBS Lett* 2004, **560**:25-29.
 20. Li L, Gu B, Zhou F, Chi J, Feng D, Xie F, Wang F, Ma C, Li M, Wang J *et al.*: **Cell cycle perturbations induced by human herpesvirus 6 infection and their effect on virus replication.** *Arch Virol* 2014, **159**:365-370.
 21. Lin K, Ricciardi RP: **The 41-kDa protein of human herpesvirus 6 specifically binds to viral DNA polymerase and greatly increases DNA synthesis.** *Virology* 1998, **250**:210-219.
 22. Taniguchi T, Shimamoto T, Isegawa Y, Kondo K, Yamanishi K: **Structure of transcripts and proteins encoded by U79-80 of human herpesvirus 6 and its subcellular localization in infected cells.** *Virology* 2000, **271**:307-320.
 23. Bieda M, Xu X, Singer MA, Green R, Farnham PJ: **Unbiased location analysis of E2F1-binding sites suggests a widespread role for E2F1 in the human genome.** *Genome Res* 2006, **16**:595-605.
 24. Neill SD, Hemstrom C, Virtanen A, Nevins JR: **An adenovirus E4 gene product trans-activates E2 transcription and stimulates stable E2F binding through a direct association with E2F.** *Proc Natl Acad Sci U S A* 1990, **87**:2008-2012.
 25. Workman A, Jones C: **Productive infection and bICP0 early promoter activity of bovine herpesvirus 1 are stimulated by E2F1.** *J Virol* 2010, **84**:6308-6317.
 26. Davy C, Doorbar J: **G2/M cell cycle arrest in the life cycle of viruses.** *Virology* 2007, **368**:219-226.
 27. Chaurushiya MS, Weitzman MD: **Viral manipulation of DNA repair and cell cycle checkpoints.** *DNA Repair (Amst)* 2009, **8**:1166-1176.
 28. Planz O, Pleschka S, Oesterle K, Berberich-Siebelt F, Ehrhardt C, Stitz L, Ludwig S: **Borna disease virus nucleoprotein interacts with the CDC2-cyclin B1 complex.** *J Virol* 2003, **77**:11186-11192.
 29. Dietrich J, Blumberg BM, Roshal M, Baker JV, Hurley SD, Mayer-Proschel M, Mock DJ: **Infection with an endemic human herpesvirus disrupts critical glial precursor cell properties.** *J Neurosci* 2004, **24**:4875-4883.
 30. Oster B, Bundgaard B, Hollberg P: **Human herpesvirus 6B induces cell cycle arrest concomitant with p53 phosphorylation and accumulation in T cells.** *J Virol* 2005, **79**:1961-1965.
 31. Oster B, Kaspersen MD, Kofod-Olsen E, Bundgaard B, Hollberg P: **Human herpesvirus 6B inhibits cell proliferation by a p53-independent pathway.** *J Clin Virol* 2006, **37**(Suppl. 1):S63-S68.
 32. Schleimann MH, Hoberg S, Solhøj Hansen A, Bundgaard B, Witt CT, Kofod-Olsen E, Hollberg P: **The DR6 protein from human herpesvirus-6B induces p53-independent cell cycle arrest in G2/M.** *Virology* 2014, **452-453**:254-263.
- Latest article showing that the viral DR6 gene induces a cell cycle arrest and accumulation of treated cells at G2/M phase.
33. Secchiero P, Bertolaso L, Casareto L, Gibellini D, Vitale M, Bemis K, Aleotti A, Capitani S, Franchini G, Gallo RC *et al.*: **Human herpesvirus 7 infection induces profound cell cycle perturbations coupled to dysregulation of cdc2 and cyclin B and polyploidization of CD4(+) T cells.** *Blood* 1998, **92**:1685-1696.
 34. Li L, Gu B, Zhou F, Chi J, Wang F, Peng G, Xie F, Qing J, Feng D, Lu S *et al.*: **Human herpesvirus 6 suppresses T cell proliferation through induction of cell cycle arrest in infected cells in the G2/M phase.** *J Virol* 2011, **85**:6774-6783.
 35. Patil M, Pabla N, Dong Z: **Checkpoint kinase 1 in DNA damage response and cell cycle regulation.** *Cell Mol Life Sci* 2013, **70**:4009-4021.
 36. Machida K, Liu JC, McNamara G, Levine A, Duan L, Lai MM: **Hepatitis C virus causes uncoupling of mitotic checkpoint and chromosomal polyploidy through the Rb pathway.** *J Virol* 2009, **83**:12590-12600.