Characterization of the interregulation between cyclin-dependent protein kinases and human cytomegalovirus regulatory proteins

vorgelegt von
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Tag der mündlichen Prüfung:               26. Mai 2009
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There are times that ask questions and times that answer.

(Zora Neale Hurston)
# Table of contents

## A Summary

A Zusammenfassung

## B Introduction

B-1 The human cytomegalovirus

B-2 Current state of anti-cytomegaloviral therapy

B-3 Interregulation between HCMV infection and cyclin-dependent protein kinases (CDKs)

B-4 CDKs and the HCMV-encoded CDK ortholog pUL97

B-5 CDK inhibitors

B-6 CDK inhibitor sensitivity of the regulatory HCMV protein pUL69

## C Objectives

## D Results

D-1 Anti-cytomegalovirus potency of a panel of selected CDK inhibitors

D-2 Effect of HCMV replication on the expression levels of individual CDKs and cyclins

D-3 Impact of knockdown of CDK-1, -2, -7, or -9 on replication efficiency of HCMV

D-3.1 Selection of siRNA sequences for specific knockdown of individual CDKs

D-3.2 Generation and characterization of CDK-deficient human fibroblasts

D-3.3 Effect of CDK knockdown on HCMV replication

D-4 Yeast two-hybrid analysis of interactions between CDK/cyclin complexes and HCMV-encoded proteins

D-5 Investigation of the phosphorylation of HCMV-encoded IE2p86 by CDK/cyclin complexes

D-6 Investigation of the interregulation between cellular CDK/cyclin complexes as well as HCMV-encoded CDK ortholog pUL97 and HCMV protein pUL69

D-6.1 CDK inhibitors and inhibitors of the viral CDK ortholog pUL97 induce intranuclear speckled aggregates of pUL69

D-6.2 HCMV variants show quantitative differences in the formation of CDK inhibitor-induced pUL69 aggregates

D-6.3 Induction of pUL69 aggregates is not necessarily a prerequisite of antiviral activity of CDK inhibitors

D-6.4 Activity of pUL97 is also required for the typical intranuclear localization of pUL69

D-6.5 CDK inhibitor-induced speckled aggregates of pUL69 accumulate within replication centers

D-6.6 CDK9 and cyclin T1 colocalize with pUL69 in replication centers and in speckled aggregates

D-6.7 Cyclin T1 but not CDK9 directly interacts with pUL69 in mammalian cells
# Table of contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-6.8</td>
<td>Direct phosphorylation of pUL69 by CDK/cyclin complexes</td>
<td>40</td>
</tr>
<tr>
<td>D-6.9</td>
<td>Kinetic study of the nucleo-cytoplasmic translocation of CDK1 in HCMV-infected cells</td>
<td>42</td>
</tr>
<tr>
<td>D-6.10</td>
<td>The mRNA export activity of pUL69 requires CDK activity</td>
<td>43</td>
</tr>
<tr>
<td><strong>E</strong></td>
<td>Discussion</td>
<td>45</td>
</tr>
<tr>
<td><strong>F</strong></td>
<td>Material and Methods</td>
<td>52</td>
</tr>
<tr>
<td>F-1</td>
<td>Biological materials</td>
<td>52</td>
</tr>
<tr>
<td>F-1.1</td>
<td>Bacteria</td>
<td>52</td>
</tr>
<tr>
<td>F-1.2</td>
<td>Yeast</td>
<td>52</td>
</tr>
<tr>
<td>F-1.3</td>
<td>Eukaryotic cells</td>
<td>52</td>
</tr>
<tr>
<td>F-1.4</td>
<td>Virus strains</td>
<td>52</td>
</tr>
<tr>
<td>F-1.5</td>
<td>Antibodies</td>
<td>53</td>
</tr>
<tr>
<td>F-2</td>
<td>Nucleic acids</td>
<td>54</td>
</tr>
<tr>
<td>F-2.1</td>
<td>Oligonucleotides</td>
<td>54</td>
</tr>
<tr>
<td>F-2.2</td>
<td>Vectors and expression plasmids</td>
<td>57</td>
</tr>
<tr>
<td>F-3</td>
<td>Enzymes, media and buffers</td>
<td>63</td>
</tr>
<tr>
<td>F-3.1</td>
<td>Enzymes</td>
<td>63</td>
</tr>
<tr>
<td>F-3.2</td>
<td>Media</td>
<td>63</td>
</tr>
<tr>
<td>F-3.3</td>
<td>Standard buffers and solutions</td>
<td>64</td>
</tr>
<tr>
<td>F-4</td>
<td>CDK inhibitors and reference compounds</td>
<td>65</td>
</tr>
<tr>
<td>F-5</td>
<td>Standard molecular biology techniques</td>
<td>66</td>
</tr>
<tr>
<td>F-6</td>
<td>Cell culture techniques</td>
<td>66</td>
</tr>
<tr>
<td>F-6.1</td>
<td>Maintenance of cell cultures</td>
<td>66</td>
</tr>
<tr>
<td>F-6.2</td>
<td>Transfection of cultured cells</td>
<td>67</td>
</tr>
<tr>
<td>F-6.3</td>
<td>Cytotoxicity assay</td>
<td>67</td>
</tr>
<tr>
<td>F-7</td>
<td>Virus infection</td>
<td>67</td>
</tr>
<tr>
<td>F-7.1</td>
<td>Virus stocks</td>
<td>67</td>
</tr>
<tr>
<td>F-7.2</td>
<td>Plaque reduction assay</td>
<td>68</td>
</tr>
<tr>
<td>F-7.3</td>
<td>HCMV GFP-based replication assay</td>
<td>68</td>
</tr>
<tr>
<td>F-8</td>
<td>Generation and characterization of retrovirally transduced cell subpopulations</td>
<td>68</td>
</tr>
<tr>
<td>F-8.1</td>
<td>Generation of retroviral transfer particles</td>
<td>68</td>
</tr>
<tr>
<td>F-8.2</td>
<td>Retroviral transduction and selection of stably transduced cell subpopulations</td>
<td>69</td>
</tr>
<tr>
<td>F-8.3</td>
<td>Flow cytometry analysis</td>
<td>69</td>
</tr>
<tr>
<td>F-9</td>
<td>Western blot analysis</td>
<td>70</td>
</tr>
<tr>
<td>F-10</td>
<td>Indirect immunofluorescence analysis</td>
<td>70</td>
</tr>
<tr>
<td>F-11</td>
<td>Analysis of protein-protein interaction</td>
<td>71</td>
</tr>
<tr>
<td>F-11.1</td>
<td>Yeast two-hybrid analysis</td>
<td>71</td>
</tr>
<tr>
<td>F-11.2</td>
<td>Coimmunoprecipitation</td>
<td>72</td>
</tr>
<tr>
<td>F-12</td>
<td>Analysis of protein phosphorylation</td>
<td>73</td>
</tr>
<tr>
<td>F-12.1</td>
<td><em>In vitro</em> kinase assay (IVKA)</td>
<td>73</td>
</tr>
<tr>
<td>Section</td>
<td>Title</td>
<td>Page</td>
</tr>
<tr>
<td>------------------</td>
<td>--------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>F-12.2</td>
<td>In vivo labeling</td>
<td>74</td>
</tr>
<tr>
<td>F-13</td>
<td>CAT mRNA export assay</td>
<td>74</td>
</tr>
<tr>
<td>G</td>
<td>Abbreviations</td>
<td>75</td>
</tr>
<tr>
<td>H</td>
<td>References</td>
<td>77</td>
</tr>
<tr>
<td>I</td>
<td>Appendix</td>
<td>96</td>
</tr>
</tbody>
</table>
A Summary

Replication of human cytomegalovirus (HCMV) includes various key issues of virus-host cell interaction. Notably, cyclin-dependent protein kinases (CDKs) are functionally integrated into efficient viral gene expression and protein modification. Although CDK1, -2, -7, and -9 have been considered as potential HCMV-regulating kinases, the underlying regulatory mechanisms are still unclear. In this thesis, primary human fibroblasts with siRNA-mediated knockdown of individual CDKs were generated. Subsequent infection experiments demonstrated a substantial reduction of HCMV replication efficiency in CDK knockdown cell populations. In order to gain a deeper insight into the HCMV-CDK interregulation, yeast two-hybrid experiments were performed which defined viral proteins as direct interactors of CDK9/cyclin T1. Moreover, for one of the identified proteins, the mRNA export factor pUL69, coimmunoprecipitation analyses determined the cyclin subunit as the direct binding partner. Interestingly, it was reported that pharmacological inhibition of CDKs induces intranuclear aggregation of pUL69. This phenomenon of subnuclear pUL69 aggregates was thereafter characterized in detail. Infection experiments with therapy-resistant cytomegalovirus variants revealed strain-specific quantitative differences in CDK inhibitor-induced pUL69 aggregates. Further immunofluorescence analyses identified pUL69 aggregates as a constituent of viral replication centers, but not spliceosomes, nuclear pore complexes or aggresome structures. A report describing viral protein kinase pUL97 as a viral CDK ortholog was coincide with the finding that inhibition of pUL97 activity produced similar pUL69 aggregates, indicating a combined fine-regulation of the intranuclear pUL69 localization by the viral CDK ortholog pUL97 along with CDKs. Remarkably, pUL69 entirely colocalized with CDK9 and cyclin T1 in aggregates induced by CDK inhibitors. Subsequent in vitro phosphorylation analyses identified cytomegaloviral proteins, in particular pUL69, as specific substrates of CDK/cyclin complexes. This CDK-mediated phosphorylation of pUL69 appeared to contribute to its mRNA export activity since CDK inhibitors effected a significant decline in pUL69-dependent mRNA export when using a reporter assay. Taken together, this study provides evidence for a distinct regulatory role of CDKs on cytomegaloviral proteins and particularly implies for CDK9/cyclin T1 to have a direct impact on the functionality of pUL69.
A Zusammenfassung

B Introduction

B-1 The human cytomegalovirus

The human cytomegalovirus (HCMV), also referred to as human herpesvirus 5 (HHV-5), belongs to the family of herpesviridae which is divided into three subfamilies: alpha (α), beta (β) and gamma (γ) (Pellett and Roizman, 2007; Roizmann et al., 1992; Roizman et al., 1981). The classification is based on sequence comparison, genome structure and biological properties. HCMV, the prototype of the β-subfamily, is characterized by a strict species specificity with humans being the only natural host, along with a prolonged life cycle in both infected organisms and cell culture (Mocarski et al., 2007). In response to productive lytic infection, cells characteristically develop cell enlargement (cytomegaly), intranuclear inclusions and further distinct features of the cytopathic effect (CPE).

HCMV is one of the most complex pathogenic viruses. The virion possesses the morphology typical for herpesviruses and comprises four structural elements: (i) the double-stranded (ds) DNA genome of about 230 kbp which is surrounded by (ii) the icosahedral capsid (Mocarski et al., 2007; Wright et al., 1964). This nucleocapsid is then embedded into (iii) the tegument, a pleomorphic layer composed of RNA, viral and cellular proteins, which in turn is enclosed by (iv) the host cell-derived envelope modified by inclusions of viral glycoproteins (Mocarski et al., 2007; Gibson, 2001; Gibson, 1996).

Infection of permissive cells with HCMV affects many aspects of host cell metabolism including DNA, RNA and protein synthesis. Interwined with this virus-host interaction is a defined temporal order of viral gene expression. The lytic replication cycle is subdivided into three distinct phases: immediate early (IE), early (E) and late (L) (Tenney and Colberg-Poley, 1991; McDonough and Spector, 1983; Wathen and Stinski, 1982; Wathen et al., 1981). That is, the expression of the viral open reading frames (ORFs) is effected by a coordinated progression and is temporally regulated at the transcriptional, translational and posttranslational level. In the first instance, viral proteins transported into the infected cells with the virion, directly interfere with the cellular regulatory machinery and initiate the expression of IE proteins such as IE1p72 and IE2p86. Proteins of the IE phase are required for the progression into the E phase due to their regulatory properties as transactivators of promoters of the E genes as well as others. While the products of E genes mostly participate in viral DNA replication they are also a prerequisite for the switch to the L phase which is characterized by the expression of structural proteins and enzymes involved in virion assembly, maturation and release of infectious progeny virus from the cell (Mocarski et al., 2007).

As for all herpesviruses, infection with HCMV, being a worldwide distributed human pathogen (seroprevalence 50%-100%; Mocarski et al., 2007; Krech, 1973), is followed by a
Introduction

lifelong persistence, accompanied by intermittently occurring periods of reactivation (Drew and Lalezari, 1999). After the primary infection, persistent infection is established and latent viral genomes can be maintained in lymphoid cells as well as tissues. Primary or reactivated infection with HCMV proceeds mostly asymptomatic with rare incidences of mild mononucleosis-like symptoms in immunocompetent individuals (Sissons and Carmichael, 2002; Cohen and Corey, 1985; Jordan et al., 1973). However, infection can cause a range of severe or even life-threatening pathological manifestations such as haematological, hepatic and gastrointestinal abnormalities and interstitial pneumonia in patients with suppressed immune functions like transplant recipients or AIDS patients (Vancikova and Dvorak, 2001; Drew, 1992a; Drew, 1992b). Furthermore, HCMV is in discussion to be associated with age-related immunosenescence (Koch et al., 2007; Koch et al., 2006) and proliferative diseases like arteriosclerosis and coronary restenosis (Mueller et al., 2003; Bason et al., 2003; Melnick et al., 1996; Speir et al., 1995). Besides the consequences of infection described for adults or infants, HCMV is still the major viral cause for birth defects comprising mental retardation, seizures, progressive hearing and vision loss (Dolland et al., 2007; Revello and Gerna, 2002; Revello et al., 2002). 50% of women who get infected in the course of pregnancy vertically transmit infectious virus intrauterine during pregnancy or childbirth (Stagno and Whitley, 1985). The majority of transmission, however, occurs horizontally via aerosol drops, sexual contact, nursing, blood transfusion or organ transplantation (Hamprecht et al., 2005; Stagno et al., 1986; Pass, 1985).

After the first direct contact with infectious virus, it is suggested, that epithelial cells of the upper pharyngeal, respiratory and genito-urinary tracts are sites for primary infection. Afterwards, leukocyte- and endothelial cell-associated viremia seems to be important for the transfer of virions within the host organism. Although spread throughout the body, HCMV replicates productively only in a subset of cell types including fibroblasts, macrophages, smooth muscle cells, epithelial and endothelial cells (Cheeran et al., 2005; Tsutsui et al., 2005; Plachter et al., 1996; Sinzger and Jahn, 1996; Sinzger et al., 1995). In contrast, the reservoir for HCMV during latency is represented by haematopoietic progenitor cells of the myeloid lineage (Khaiboullina et al., 2004; Sindre et al., 1996; Mendelson et al., 1996). In the latent state, the viral genome exists as an extrachromosomal episome in the absence of gene expression (Bolovan-Fritts et al., 1999). Differentiation of these progenitor cells activates the virus to change from latent into lytic replication (Sinclair and Sissons, 2006; Soderberg-Naucler et al., 1997; Taylor-Wiedeman et al., 1994).

B-2 Current state of anti-cytomegaloviral therapy

Productive replication of HCMV at high levels is associated with symptomatic progress of infection. Especially in individuals with suppressed immune system, the appearance of cytomegalovirus-associated disease is of serious concern. However, the implementation of a
general HCMV prophylaxis is barely realizable as HCMV is a ubiquitous human virus. Although various candidates for HCMV vaccine are under investigation at preclinical level, no vaccine is licensed so far. Currently, there are only five compounds approved for the treatment of acute HCMV infections: (i) ganciclovir (GCV), (ii) valganciclovir (ValGCV), (iii) cidofovir (CDV), (iv) foscarnet (FOS) and (v) fomivirsen (Tab. 1).

<table>
<thead>
<tr>
<th>compound</th>
<th>drug class</th>
<th>drug target</th>
<th>approval status</th>
</tr>
</thead>
<tbody>
<tr>
<td>ganciclovir</td>
<td>purin nucleoside</td>
<td>viral DNA polymerase (competitive)</td>
<td>1989</td>
</tr>
<tr>
<td>valganclovir</td>
<td>purin nucleoside</td>
<td>viral DNA polymerase (competitive)</td>
<td>2001</td>
</tr>
<tr>
<td>cidofovir</td>
<td>pyrimidine nucleosides</td>
<td>viral DNA polymerase (competitive)</td>
<td>1996</td>
</tr>
<tr>
<td>foscarnet</td>
<td>phosphonooformates</td>
<td>viral DNA polymerase (non-competitive)</td>
<td>1991</td>
</tr>
<tr>
<td>fomivirsen</td>
<td>antisense RNA</td>
<td>major IE transcript</td>
<td>1998</td>
</tr>
<tr>
<td>artesunate</td>
<td>artemisinins</td>
<td>n.d.</td>
<td>clinical trials</td>
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<tr>
<td>maribavir</td>
<td>benzimidazoles</td>
<td>pUL97/pUL27(?)</td>
<td>clinical trials</td>
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</table>

(modified from Steininger, 2007)

GCV, the most conversant anti-HCMV drug, along with ValGCV, a derivative of GCV with additional esterifications increasing oral bioavailability, target viral DNA polymerase pUL54. So do CDV and FOS albeit through slightly different mechanisms. GCV, ValGCV and CDV are prodrugs and require modulation for conversion into the actual active drug, the triphosphate derivatives. GCV and ValGCV are nucleoside analogs needing three kinase activities. Of crucial importance is the initial monophosphorylation ensured by HCMV-encoded protein kinase pUL97 (Sullivan et al., 1992). Cellular kinases afford further phosphorylation into di- and triphosphates. Incorporation of the active triphosphate derivatives of GCV and ValGCV lacking the 3’-OH group of the polysaccharide moiety leads then to the termination of viral DNA synthesis (Biron, 2006; Marschall et al., 2001; Crumpacker, 1996; Littler et al., 1992; Matthews and Boehme, 1988). While GCV and ValGCV exert antiviral activity in infected cells due to requirement of the activity of pUL97, CDV is already a monophosphorylated nucleotide.
analog and therefore independent of pUL97 activity. Apart from that, CDV acts through a very similar mechanism described for GCV and ValGCV (Lea and Bryson, 1996).

On the contrary, FOS is a pyrophosphate analog that can directly bind to pUL54 pyrophosphate binding site. By this, elimination of pyrophosphate deoxynucleoside triphosphate is blocked, thus inhibiting the viral DNA synthesis in a non-competitive manner (Öberg, 1989; Öberg, 1982).

Fomivirsen (ISIS 2922) is an anti-sense RNA molecule acting through an unique mode of action. It consists of 21 phosphorothioate-linked nucleosides which can bind to complementary mRNA sequence transcribed from the major immediate early transcriptional unit (Perry and Balfour, 1999). This binding inhibits translation of IE products of HCMV and results in suppression of the initiation of viral replication cycle as these proteins are functionally essential (Anderson et al., 1996).

Overall, the use of these drugs has decreased the burden of HCMV disease, especially in transplant patients and AIDS patients, where the treatment significantly improved survival and quality of life. Nevertheless, the clinical use is restricted due to several obstacles. Besides low oral bioavailability with exception of ValGCV, high cytotoxicity can lead to severe adverse effects including neutropenia, anemia, bone marrow cytotoxicity, nephrotoxicity as well as thrombocytopenia. Carciogenic and teratogenic effects have been described for GCV, ValGCV and CDV. Thus, all drugs are poorly suitable for long-term use and can only be applied very limited to congenital infected infants. Moreover, drug-resistant variants may appear spontaneously which are then selected after extended exposure during therapy. Resistant HCMV mutants usually show reduced replicative fitness, however, they can cause fatal infections in immunodeficient and immunocompromised individuals. Due to the fact that most drugs target the viral DNA polymerase pUL54, cross resistance represents an accessory challenge in clinical application (Gilbert and Boivin, 2005).

Combined, alternative strategies and development of novel compounds in anticytomegaloviral therapy are urgently needed. A very interesting compound is maribavir, also referred to as 1263W94, belonging to the group of benzimidazoles. It has been demonstrated maribavir inhibits HCMV DNA synthesis as well as nuclear egress (Krosky et al., 2003a). Furthermore, laboratory strains as well as clinical isolates showed high sensitivity towards this drug. So did GCV-resistant variants which was promising as pUL97 was postulated to be the main target (Biron et al., 2002). However, maribavir-resistant isolates were identified with the mutation mapped to pUL97 or, alternatively, to a fairly unknown viral protein, pUL27 (Chou et al., 2004; Komazin et al., 2003), suggesting a more complex mode of antiviral action. Maribavir has been subject to preclinical pharmacokinetic as well as toxicological studies (Koszalka et al., 2002) and phase I as well as phase II clinicals trials with encouraging results (Ma et al., 2006; Selleseth et al., 2003; Wang et al., 2003; Koszalka et al., 2002). It exhibited
auspicious safety profile, excellent oral bioavailability and lower toxicity compared to available anti-HCMV drugs. Antiviral activity also appeared promising (Winston et al., 2008; Lalezari et al., 2002), however, very recently, a first public release of data from the ongoing phase III evaluation could not confirm the initial promising results (www.viropharma.com).

Further approaches are given by research on substances from natural resources (Rechter et al., 2006) or semi-synthetic derivatives like artesunate. This is a derivative of artemisinin and approved for antimalarial therapy. In recent years, it has been demonstrated artesunate also possesses broad antiviral activity inhibiting replication of several herpesviruses such as HCMV, HSV-1, EBV and HHV-6 (Milbradt et al., 2009; Shapira et al., 2008; Jansen and Soomro, 2007; Kaptein et al., 2006; Efferth et al., 2002). Some of these reports also indicated sensitivity of non-related viruses towards artesunate (Paeshuyse et al., 2006; Romero et al., 2005; Fillebeen et al., 2005; Qian et al., 1982). Moreover, the antiviral activity of artesunate seemed additive when applied in combination with conventional drugs such as GCV, CDV and FOS (Kaptein et al., 2006). Although the mode of action is not known in detail, a targeting to cellular proteins was shown (Siedle et al., 2004; Efferth et al., 2002; Bork et al., 1997). Further drugs currently in development, such as protein kinase inhibitors, target protein or pathways of the host cell. This strategy which is an issue of current interest (Johansson and Persson, 2008; Wang and Fischer, 2008; Schang et al., 2006; Schang, 2005a; Schang, 2005b; de la Fuente et al., 2003), promises advantages compared to conventional drugs.

B-3 Interregulation between HCMV infection and cyclin-dependent protein kinases (CDKs)

HCMV cell entry, gene expression, replication and intracellular virion maturation are processes that require viral as well as cellular factors. Thus, an intricate set of interactions between the virus and the host cell are required for productive infection. Thereby, HCMV not only replicates in a subset of cell types, but also favors a specific cell cycle phase. The different cell cycle phases are depicted in Fig. 1. Each phase is characterized by the activity of distinct cyclin-dependent protein kinases (CDKs) in complex with respective regulatory subunits termed cyclins. G1 represents a preparation step in which the cell is primed for cellular DNA synthesis and is regulated by D-typed cyclins in complex with CDK4 or CDK6 (Sherr, 1995; Meyerson and Harlow, 1994; Kato et al., 1993). The transition to S phase and initiation of DNA replication is associated with CDK2/cycE-mediated events (Geng et al., 1996; Dynlacht et al., 1994; Hinds et al., 1992). Once the cell enters S phase, the replication machinery for duplication of cellular DNA is activated and regulated by CDK2 in complex with cyclin A (Takeda and Dutta, 2005). This complex is active until late in G2 which is defined as a further preparation step before mitosis takes place (M phase). Here, the activity of CDK1/cycB is required for the cell to commit to mitosis resulting in chromosome segregation and cell
division (Pines and Hunter, 1991; Nurse, 1990). An additional stage, G0, is a state of quiescence in which the cell has transiently withdrawn from the cell cycle.

HCMV interferes with this ordered process (Bresnahan et al., 1996; Jault et al., 1995). As can be seen in Fig. 1, the virus acts inhibitory on CDK4/cycD, CDK6/cycD and CDK2/cycA, and stimulatory on CDK1/cycB as well as CDK2/cycE. The activation of cycE-associated kinase activity stimulates the expression of enzymes involved in cellular DNA replication and forces infected cells to enter S phase. Then, HCMV induces cell cycle arrest at that point mainly by inhibiting progression through the S phase. Particularly the inhibitory effect on CDK2/cycA has been described as important mechanism for suppressing duplication of cellular DNA. These HCMV-induced events ensures adequate resources for its own replication at the expense of the host DNA replication.

Fig.1: HCMV-induced cell cycle arrest. Cell cycle-regulatory cyclin-dependent protein kinases are suppressed (CDK4/cycD, CDK6/cycD, CDK2/cycA) or activated (CDK1/cycB, CDK2/cycE) in HCMV-infected cells. The initial HCMV-mediated events result in a very early S phase arrest.

At the time of HCMV infection, usually not all cells are present in the cell cycle phase favorable for viral replication. This has consequences for the initial efficiency of HCMV infection (Salvant et al., 1998). Two conditions can be distinguished. First, when the virus infects the cell within G0 or G1, productive replication can commence immediately leading to cell cycle arrest very early in S phase. Thereby, the virus concomitantly inhibits potentially competing cellular DNA synthesis allowing cellular precursors to be used for viral but not cellular DNA replication. Second, when the cells are infected during S phase, a delay in the initiation of viral gene expression can be observed due to cells proceeding through both S and M prior to virus-mediated cell cycle arrest. Therefore, it has been postulated that HCMV gene expression and cellular DNA synthesis are mutually exclusive.

HCMV-encoded proteins involved in hijacking the host cells’ regulatory systems have been identified. A stimulatory activity is attributed to the tegument protein pp71 (Kalejta, 2004; Kalejta et al., 2003; Kalejta and Shenk, 2003). Another virion-associated protein, pUL69
(Winkler and Stamminger, 1996), is required for the cell cycle arrest (Hayashi et al., 2000; Lu and Shenk, 1999; B-7). Additionally, also proteins that need to be expressed very early in the replication process such as immediate early protein IE2p86 have been described to contribute to these events (Petrik et al., 2006; Song and Stinski, 2005; Wiebusch et al., 2003; Wiebusch and Hagemeier, 2001; Wiebusch and Hagemeier, 1999). However, whether these proteins directly interact with CDKs or cyclins has not been defined yet.

In general, it is accepted that HCMV infection causes changes in the regulation of the cell cycle and CDKs. In particular, it has been reported that several regulatory steps of HCMV replication require the activity of CDK1, -2, -7, and -9 along with the respective regulatory cyclins (Kapasi and Spector, 2008; Sanchez and Spector, 2006; Tamrakar et al., 2005; Sanchez et al., 2004; Sanchez et al., 2003; Salvant et al., 1998; Bresnahan et al., 1997; Jault et al., 1995). In infected cells, CDK1/cycB1 kinase activity is induced as early as 8 hours post infection (hpi) and maintained throughout the viral replicative cycle. Here, cyclin B1 accumulates due to increased synthesis along with reduced degradation while CDK1 accumulates in its active form (Sanchez et al., 2003). Furthermore, it was shown that this mitotic kinase translocates from the nucleus to the cytoplasm. The cytoplasmic pattern of CDK1 was detected 48 hpi (Sanchez et al., 2003). For CDK2/cycE, a dramatic increase in activity was shown, whereby the viral immediate early protein IE2p86 contributes to the transactivation of the cyclin E promoter (Wiebusch and Hagemeier, 2001). Also for CDK7 and -9 along with their respective subunits cyclin H, MAT-1 (CDK7) and cyclin T1 (CDK9), augmented protein levels with intensified kinase activity have been detected resulting in enhanced phosphorylation of the cellular RNA polymerase II (RNAPII; Tamrakar et al., 2005). In addition, at early times, CDK9 is localized with input viral DNA and hereby aggregates of CDK7, CDK9, cyclin T1 and a subset of phosphorylated RNAPII are detectable along with IE1p72 and IE2p86 adjacent to nuclear structure referred to as nuclear domain 10, the site where viral RNA and DNA synthesis starts (Tamrakar et al., 2005; Ishov et al., 1997). These IE transcription sites are further composed of viral pUL69 and cellular proteins involved in chromatin modification (bromodomain protein Brd4, histone deacetylases HDAC1 and HDAC2; Kapasi and Spector, 2008). The localization pattern of these components alters in the course of infection. Tamrakar and colleagues (2005) reported that at late times, CDK9 is defined in a nuclear punctate pattern whereas CDK7 resides in replication centers. RNAPII was detected in nuclear accumulations, within or at the periphery of replication centers dependent on its phosphorylation status. Taken together, the studies on effects of HCMV on CDKs and cyclins clearly indicate that the activity of individual CDK/cyclin complexes is required at various steps during lytic replication.

**B-4 CDKs and the HCMV-encoded CDK ortholog pUL97**

CDKs are serine/threonine protein kinases that are characterized by the typical
arrangement of 11 subdomains contained within the functional unit of the kinase domain (Pavletich, 1999; Morgan, 1997). Heterodimeric complexes are constituted by a catalytic subunit (CDK) and a regulatory subunit (cyclin). The assembly of a CDK with its corresponding cyclin induces important structural changes in the catalytic subunit but yields only a partially active complex. Full activity is achieved by phosphorylation and/or dephosphorylation of the CDK on specific threonine residues (Orzaez et al., 2009; O’Farrell, 2001; Espinoza et al., 1996; Russo et al., 1996; Morgan, 1995; Fisher and Morgan, 1994). Activating phosphorylation typically occurs at a threonine residue of the T-loop located close to the ATP binding site; the CDK-activating kinase (CAK) is the complex composed of CDK7, cyclin H and MAT-1 (Espinoza et al., 1996; Russo et al., 1996; Morgan, 1995; Fisher and Morgan, 1994; Kato et al., 1994; Poon et al., 1993; Solomon et al., 1993; Fesquet et al., 1993). The removal of inhibitory phosphorylation (tyrosine residues near the N termini of all CDKs) is mostly performed by the action of the Cdc25 group of phosphatases (Obaya and Sedivy, 2002). Additional mechanisms regulating CDK activity are represented by expression levels as well as localization of cyclins and furthermore, the binding of inhibitory factors such as members of the INK4 or Cip family (Mailand et al., 2000; McConnell et al., 1999; Hengst and Reed, 1998; Carnero and Hannon, 1998). So far, 13 CDKs and 25 cyclins encoded in the human genome have been described, however, the complete range of CDK and cyclin pairing is not fully defined yet (Murray and Marks, 2001).

Fig. 2: Scheme of functional network of CDKs participating in multiple cellular processes. The main functional attribution of each CDK is indicated by a green line.

Besides the cell cycle, multiple cellular processes are subject to CDK regulation including transcription, apoptosis and cell differentiation (Fig. 2). The subdivision into cell cycle-associated CDKs (i.e., CDK1 and -2) or transcription-associated CDKs (i.e., CDK7 and -9) may represent a simplified classification since, on the one hand, individual CDKs participate in diverse cellular processes and, on the other hand, each of these processes is regulated by various protein kinases, indicating a complex functional network (Fig. 2). Overall, however, CDK1 and CDK2 are mainly linked to cell cycle regulation, whereas CDK7 and CDK9 are mainly associated with transcription regulation. Notably, although CDKs exhibit conserved kinase domains (44% homology; Table 2B), the overall homology of CDK1, -2, -7, and -9 is only 22% for the total protein sequence (Table 2A). Considering pair-wise homology between
two particular CDKs, the percentages range from 32%-65% for total protein sequences (Table 2A) and 56%-84% for kinase domains (Table 2B).

**Table 2: Homology of total protein sequence (A) and kinase domains (B) of CDK1, -2, -7, and -9 and between individual CDKs.** Primary sequences were taken from protein library. Accession numbers: NP_001777 (CDK1), NP_001789 (CDK2), NP_001790 (CDK7) and NP_001252 (CDK9). Overall means the percentage of identity between all four analyzed CDKs.

<table>
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<th>CDK7</th>
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<td><strong>A</strong> total protein sequence</td>
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<td>overall: 21.8%</td>
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<td>CDK2</td>
<td>64.9%</td>
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<td>36.4%</td>
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<td>CDK9</td>
<td>32.7%</td>
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<td><strong>B</strong> kinase subdomains</td>
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<td>overall: 44.0%</td>
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<td>CDK2</td>
<td>84.2%</td>
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<td>CDK7</td>
<td>58.2%</td>
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<td>CDK9</td>
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Cell cycle-associated CDK1 (Cdc2) forms complexes with cyclin B1, B2 or A and is required for entry into mitosis and correct segregation of cellular material between daughter cells during mitosis (Merrick et al., 2008; Miyazaki and Arai, 2007; Pines and Hunter, 1991; Maller, 1991; Nurse, 1990). It phosphorylates various nuclear substrates during S and G2 phase such as regulators necessary for the control and timing of mitotic events (Porter and Donoghue, 2003; Endl and Gerdes, 2000; Macaulay et al., 1995; Keryer et al., 1995; Blangy et al., 1995; Eggert et al., 1993; Hoffmann et al., 1993), further kinases (Roskoski, Jr., 2005; Shenoy et al., 1989), components of the cytoskeleton (Okata et al., 1995) and proteins of the secretory pathways (Lowe et al., 2000), at which substrate specificity is given by association with the respective cyclin (Draviam et al., 2001). A further cell cycle-associated CDK, CDK2, binds to cyclin E and cyclin A. In complex with cyclin E, CDK2 regulates S phase entry and initiation of DNA replication by phosphorylating S phase-specific substrates like NPAT, an enzyme participating in histone gene transcription (Zhao et al., 2000; Ma et al., 2000; Zhao et al., 1998) as well as proteins of the retinoblastoma protein (Rb) family (Bartek and Lukas, 2001; Lundberg and Weinberg, 1998; Zarkowska and Mittnacht, 1997; Kitagawa et al., 1996; Hatakeyama et al., 1994). CDK2/cycE targets CDK inhibitor p27 and cyclin E resulting in proteolytic degradation of these phosphorylated substrates ( Muller et al., 1997; Vlach et al., 1997; Sheaff et al., 1997; Clurman et al., 1996; Won and Reed, 1996). Transition through S phase and DNA replication are then controlled by CDK2 associated with cyclin A (Ishimi et al., 2000; van den Heuvel and Harlow, 1993; Cardoso et al., 1993). Known substrates of this complex are HIRA which losses repressor activity on histone genes Hir1p and Hir2p upon phosphorylation (Hall et al., 2001; Magnaghi et al., 1998) and components of proteolytic pathways involved in cell cycle progression (Ohtoshi et al., 2000; Yam et al., 1999).

Concerning the regulation exerted by transcription-associated CDKs, a prominent example is the phosphorylation of the C-terminal domain (CTD) of RNA polymerase II (RNAPII) by CDK7/cycH/MAT1 and CDK9/cycT1 (Majello and Napolitano, 2001; Reinberg et al., 1998).
In human cells, CTD is composed of 52 repeats of the consensus heptapeptide sequence Tyr-Ser-Pro-Thr-Ser-Pro-Ser, and is subject to high levels of phosphorylation. Functionally, this domain is described as a binding domain and transporter of factors involved in RNA synthesis initiation, elongation, 5' capping, splicing, cleavage and polyadenylation (Meinhart et al., 2005). Differential recruitment and binding of individual factors seem to be dependent on the pattern of phosphorylation of serines at positions 2 and 5 which varies considerably during the transcription process. Typically, hypophosphorylated RNAPII binds to transcription initiation complex. Then, CDK7/cycH/MAT1, as a component of the basal transcription factor complex TFIIH, phosphorylates serine 5. It is followed by serine 2 phosphorylation by CDK9/cycT1, also referred to as positive transcription elongation factor B (P-TEFb) which is associated with commitment of RNAPII to productive transcription elongation.

For HCMV, it was shown that the HCMV-encoded protein kinase pUL97 possesses structural resemblance (Romaker et al., 2006) and activities similar to CDKs, thus representing a CDK ortholog (Hume et al., 2008; Prichard et al., 2008; Marschall et al., 2005). pUL97 phosphorylates and inactivates retinoblastoma protein and stimulates cell cycle progression. However, pUL97 is insensitive to cellular CDK regulator proteins that normally attenuate CDK activity (Hume et al., 2008). In general, pUL97 is an important determinant of viral replication (Prichard et al., 2008; Michel and Mertens, 2004; Prichard et al., 1999) and is a key factor in prodrug activation (B-2). Deletion of the UL97 region from the viral genome or inhibition of pUL97 kinase activity pharmacologically severely reduce viral replication (Schleiss et al., 2008; Marschall et al., 2005; Herget et al., 2004; Biron et al., 2002; Marschall et al., 2002). Furthermore, a number of cellular and viral interacting proteins and substrates of pUL97 have been described, including HCMV pUL69 (Thomas et al., 2009), HCMV pUL44 (Marschall et al., 2003; Krosky et al., 2003b), autophosphorylated pUL97 (Schregel et al., 2007; He et al., 1997), p32 (Marschall et al., 2005), EF-1δ (Romaker et al., 2006; Kawaguchii et al., 1999), histone 2B (Marschall et al., 2001; He et al., 1997) and retinoblastoma protein (Hume et al., 2008; Prichard et al., 2008).

B-5 CDK inhibitors

Deregulation of CDKs in numerous diseases has stimulated intensive research for pharmacological inhibitors of these enzymes. Classical pharmacological CDK inhibitors (PCIs) are low molecular compounds (< 600 Da) consisting of flat hydrophobic heterocycles which bind mostly by hydrophobic interactions and hydrogen bonds to the target kinases (Hardcastle et al., 2002). Functionally, they bind mostly to the ATP-binding pocket of their target CDKs, thus inhibiting them commonly by competing with the ATP co-substrate. However, it seems as if the way of binding of PCIs to the kinase does not mimic ATP (Fischer and Lane, 2000). According to their specificity, the inhibitors can be classified as non-, pan-, oligo- and mono-specific (Schang, 2005a; de la Fuente et al., 2003; Fischer and Gianella-
Borradori, 2003; Schang, 2002; Schang, 2001). Non-specific PCIs act not only against CDKs but also against numerous unrelated protein kinases. Pan-specific PCIs such as flavopiridol, preferentially inhibit CDKs over other protein kinases, but fail to discriminate among different CDKs. On the other hand, oligo-specific PCIs such as roscovitine, olomoucine and purvalanol target only a subset of CDKs. In addition, several potential mono-specific compounds have been suggested (Chassagnole et al., 2006; Voigt et al., 2005; Misra et al., 2004; Schoepfer et al., 2002; Toogood, 2001; Soni et al., 2000), but there is no definite experimental evidence available proving the selectivity against exclusively one CDK.

The use of PCIs in therapy might be broadly diversified as CDKs are involved in multiple physiological pathways. For that reason, PCIs are currently evaluated for therapeutic use against cancer, alopecia, glomerulonephritis, protozoan diseases, neurodegenerative and cardiovascular disorders (Johansson and Persson, 2008; Grant, 2008; Benson et al., 2007; Geyer et al., 2005; Doerig, 2004; Andres, 2004). Especially in tumor therapy, PCIs appear to have a great potential. Some of these anticancer agents are already in clinical trials with encouraging results (Grant, 2008; O’Bien, 2008; Perez, I et al., 2008; Basso and Doll, 2006; Grant and Roberts, 2003; Patel et al., 1998; Senderowicz et al., 1998; Drees et al., 1997).

Furthermore, a broad-spectrum antiviral activity has been discovered in the last couple of years, thus PCIs are also in discussion as antiviral drugs (Herget and Marschall, 2006; Schang et al., 2006; Schang, 2001; Schang et al., 1998; Bresnahan et al., 1997; Mancebo et al., 1997). Originally, it was stated that CDKs are required for viruses exclusively replicating in dividing cells such as adeno- and papillomaviruses, however, it was shown recently that CDKs are also important determinants for the replication of viruses that can replicate in non-dividing cells such as HCMV, HSV-1, HSV-2, EBV and HIV-1 (Kapasi and Spector, 2008; Biglione et al., 2007; Sanchez and Spector, 2006; Dai-Ju et al., 2006; Pumfery et al., 2006; Tamrakar et al., 2005; Schang et al., 2005; Schang, 2005b; Sanchez et al., 2004; Diwan et al., 2004; Kudoh et al., 2004; Sanchez et al., 2003; Kudoh et al., 2003; Davido et al., 2003; Schang, 2002; Wang et al., 2001; Salvant et al., 1998; Schang et al., 1998; Jault et al., 1995).

In clinical terms, the use of PCIs as potential antiviral drugs has raised the crucial question of cytotoxicity as the target CDKs are ubiquitously expressed in all cell types. Worth mentioning, PCIs typically do not kill dividing cells but rather arrest cells in specific phases of the cell cycle. Clinical trials testing PCIs as antitumor agents have demonstrated PCIs such as flavopiridol and roscovitine revealed only minor adverse effects. This was a rather unexpected finding which supported the idea that PCIs may have a multitude of promising features as antiviral agents. The advantages over conventional drugs may include broad-spectrum antiviral activity, possibly a reduced frequency of induction of drug-resistant mutants, an activity against variants exhibiting resistance to conventional therapy and potential use in combination therapies with conventional drugs.
Researchers have made use of CDK inhibitors to investigate virus-host interactions in more detail. One well-characterized CDK inhibitor is the purine analog roscovitine which is classified as a oligo-specific PCI inhibiting CDK1, -2, -5, -7, and -9. Interestingly, it binds with high affinity only to the active form of the target CDKs (Knockaert et al., 2002; De Azevedo et al., 1997). For HCMV, studies using roscovitine demonstrated inhibition of viral replication at various levels. Thereby, the time of drug addition is highly relevant (Kapasi and Spector, 2008; Sanchez and Spector, 2006; Tamrakar et al., 2005; Sanchez et al., 2004). It can be distinguished between antiviral activity upon presence of roscovitine immediately after infection and addition of roscovitine 6-8 hpi. Instant inhibition of CDK activity impairs already the IE gene expression. Due to altered splicing of the major immediate early transcript, the synthesis of IE2p86 is increased at the expense of IE1p72. Given that HCMV replication proceeds in a temporal fashion where one event is required before the next can occur, the alterations in IE gene expression lead to impaired expression of E and L genes, inefficient viral DNA replication and in turn to a dramatic reduction in assembly and release of infectious viral particles. Additionally, CDK7 and -9, usually recruited to IE transcription sites very early after infection, do not accumulate to these sites in the presence of roscovitine but are localized evenly distributed throughout the nucleus. Moreover, the increased protein levels of CDK7 and -9 along with increased phosphorylation of the CTD of RNAPII are abolished. However, when the addition of the CDK inhibitor is delayed to 6-24 hpi, this type of roscovitine-induced effects is mostly abrogated. Viral gene expression proceeds quite normally, though production of IE2p86 as well as major tegument protein pp150 is decreased while expression of pUL69 is augmented and accumulates in its hyperphosphorylated form. Despite these minor effects on gene expression, viral titer is effectively reduced. Thus, late effects of roscovitine can have a similarly drastic inhibitory effect on HCMV replication as the early effects. Taken together, several lines of evidence indicate that CDK activity is required at both very early and late times of infection for viral gene expression, modification as well as localization of virus-encoded proteins and possibly virion assembly.

B-6 CDK inhibitor sensitivity of the regulatory HCMV protein pUL69

One HCMV-encoded protein described to be influenced by roscovitine is the regulatory protein pUL69. It is expressed with early/late kinetics from the viral genome and is subject to phosphorylation. As a result of differential phosphorylation, three isoforms of pUL69 can be detected and the 110 kDa hypophosphorylated isoform is a constituent of the viral tegument (Winkler and Stamminger, 1996). Just recently, a study performed by our research group (including experiments performed within this PhD thesis) demonstrated phosphorylation of pUL69 can be mediated by interaction with the viral protein kinase pUL97 (Thomas et al., 2009). Furthermore, pUL69 binds to viral RNA and exhibits nucleo-cytoplasmic shuttling activity (Toth et al., 2006; Lischka et al., 2001). Functionally, pUL69 is pleiotropic and
contributes to the HCMV-induced cycle arrest (Hayashi et al., 2000; Lu and Shenk, 1999), to viral transcriptional activation as well as to nuclear mRNA export (Lischka et al., 2006; Winkler and Stamminger, 1996; Winkler et al., 1994). Hereby, its abilities of binding RNA and shuttling between nucleus and cytoplasm along with pUL69-mediated recruitment of the cellular mRNA export machinery (Winkler et al., 2000) promotes cytoplasmic accumulation of unspliced mRNA. At late time points of infection, pUL69 is typically localized in the nucleus with accumulation in viral replication centers (Winkler et al., 1994). However, when CDK activity is inhibited by roscovitine, pUL69 accumulates in intranuclear speckled aggregates (Sanchez and Spector, 2006). This phenomenon, originally described for HCMV strain Towne, supports the concept of the requirement of CDK activity for an efficient cytomegaloviral infection. However, the underlying mechanisms as well as the individual CDK/cyclin complex(es) involved in the temporally ordered processes during productive replication were still to be defined.
C Objectives

Infection with human cytomegalovirus (HCMV), a human pathogen of high clinical importance, causes deregulation of multiple host cell pathways. Thereby, cyclin-dependent protein kinases (CDKs) are described as crucial determinants of efficient viral replication. Studies using CDK inhibitors showed that the activity of these kinases is required at various stages during the replication cycle for expression, modification and localization of viral proteins. In this regard, it was a highly relevant report that inhibition of CDK activity induces an intranuclear speckled aggregation of the viral regulator pUL69. So far, the mechanistic basis of this phenomenon was unclear and direct functional interactions between CDKs and viral proteins had not been identified.

The aim of this study was to define and functionally characterize direct interactions between CDK/cyclin complexes and HCMV-encoded proteins. For this, yeast two-hybrid analyses should be performed in order to determine potential viral interactors of CDK/cyclin complexes. CDK-associated viral proteins should then be analyzed with regard to CDK-mediated phosphorylation by the use of in vitro kinase assays and functional consequences of the interaction by the use of reporter assays. The generation of HCMV-permissive cell populations with an siRNA-mediated CDK knockdown should offer another possibility to study the interlinkage between CDKs and the virus. Particularly, the roscovitine-induced intranuclear speckled aggregates of pUL69 should be characterized in detail. The use of a panel of CDK inhibitors and inhibitors of the HCMV-encoded CDK ortholog pUL97 should provide information about the regulatory basis of pUL69 aggregate formation. Further analyses of the colocalization of CDK1, -2, -7, and -9 with CDK inhibitor-induced pUL69 aggregates should gain additional insight into the assumed requirement of CDK activity. These studies were thought to improve the understanding of CDK-cytomegalovirus interregulation and to provide input in the development of novel antiviral strategies.
D Results

D-1 Anti-cytomegalovirus potency of a panel of selected CDK inhibitors

Cyclin-dependent protein kinases (CDKs) represent a promising class of novel antiviral targets (Herget and Marschall, 2006; Schang et al., 2006; Schang, 2001; Schang et al., 1998). Numerous studies demonstrated CDK activity is required during the replication cycle of several herpesviruses and viruses of non-related families (Kapasi and Spector, 2008; Sanchez and Spector, 2006; Diwan et al., 2004; Kudoh et al., 2004; Sanchez et al., 2003; Kudoh et al., 2003; Wang et al., 2001; Schang et al., 1998; Jault et al., 1995). A number of CDK inhibitors are already commercially available and the possible therapeutic use of pharmacological CDK inhibitors (PCIs) supports the design of novel CDK inhibitory compounds. GPC Biotech AG, an industrial collaboration partner, had developed compounds belonging to the class of aminopyrimidines with selected inhibitory activity. The potency of these newly designed CDK inhibitors to suppress HCMV replication was investigated in this study.

In a first step, the antiviral activity of the commercially available, purine-containing PCIs roscovitine, olomoucine II, purvalanol A and CDK2 inhibitor was measured in a GFP-based HCMV replication assay in which GFP expression increases proportionally to HCMV replication in the course of several rounds of viral replication. Thus, GFP signals can be used as a sensitive reporter for replication efficiency (Marschall et al., 2000). Primary human foreskin fibroblasts (HFFs) were infected with HCMV AD169-GFP followed by addition of the inhibitors immediately after infection. Thereby, increasing concentrations (0.03 µM, 0.1 µM, 0.3 µM, 1.1 µM, 3.3 µM, 10 µM) were used. Untreated mock-infected cells and infected cells treated with 20 µM GCV served as controls. 7 days post infection, cells were analyzed for GFP levels. The control samples without inhibitor (no inhib.) were set to 100%. As shown in Fig. 3A, several CDK inhibitors reduced viral replication in a dose-dependent manner. Thereby, only olomoucine II and CDK2 inhibitor exhibited a microscopically detectable cytotoxicity at 10 µM. On the basis of the broad literature characterizing roscovitine, this compound was selected as a reference compound for further experiments.

In the following, a selection of novel CDK inhibitors was analyzed in the GFP-based replication assay. Compounds R10, R25, R58, R89, R372, and R972 were able to inhibit viral replication in a dose-dependent manner (Fig. 3B and C). As shown in Fig. 3C, R58 yielded the highest anti-HCMV index (15.9) and was chosen for further analyses described below. In respect of selectivity profiles, these compounds share a strong inhibitory potential against cell cycle-associated CDKs in vitro (in particular CDK1 and CDK2, IC50 < 1 µM) with additional individual CDK targets within the low micromolar range (i.e., CDK5 for R58; CDK5, CDK9 for R372). Moreover, so far unrecognized inhibitory effects cannot be excluded, as not all CDKs
Fig. 3: Determination of anti-HCMV activity of a variety of commercial (A) and novel (B, C) CDK inhibitors. HFFs were infected with HCMV AD169-GFP (MOI 0.25) and treated with CDK inhibitors as indicated. Cells were lysed 7 days post infection and subjected to GFP fluorometry. Determinations were performed in quadruplicate. (A) Antiviral activity of commercially available CDK inhibitors; *, cytotoxicity as observed by brightfield microscopy. (B) Antiviral activity of novel CDK inhibitors; ganciclovir (GCV, 20µM) and Rosco (10 µM) served as controls. (C) Anti-HCMV indices of CDK inhibitors: C50/IC50; CC50, concentration of 50% cytotoxicity, determined by means of a lactate dehydrogenase cytotoxicity assay; IC50, concentration of 50% reduction of HCMV replication. Highlighted in blue color are the most potent inhibitors used in this study.
have been evaluated for each inhibitor. Therefore, the exact pattern of CDK inhibition required for anti-HCMV activity is not to be clearly deduced from the experiments depicted in Fig. 3. However, taken together, the results indicate that the analyzed CDK inhibitors possess strong anti-HCMV activity and suggest that HCMV replication is dependent on the activity of several CDKs.

D-2 Effect of HCMV replication on the expression levels of individual CDKs and cyclins

Although studies using CDK inhibitors pointed to the fact that several CDK/cyclin complexes are involved in productive replication, it remained to be defined which are the key CDKs and/or cyclins. An experimental approach to narrow down the participating CDKs was the determination of expression levels of several CDKs and cyclins in the course of HCMV infection by Western blot analysis. CDK1, -2, -7, and -9 were selected for the analysis, based on the fact that several reports showed alterations in their expression and activity (Kapasi and Spector, 2008; Sanchez and Spector, 2006; Tamrakar et al., 2005; Sanchez et al., 2004; Sanchez et al., 2003; Salvant et al., 1998; Bresnahan et al., 1997; Jault et al., 1995). Mock-infected and HCMV AD169-infected HFFs were harvested between 0 hours post infection (hpi) and 72 hpi as indicated (Fig. 4). The subsequent analysis revealed an upregulation of each CDK, starting at approximately 48 hpi (Fig. 4A). This effect was most pronounced for CDK1 since its increased protein levels were maintained throughout the period analyzed while in mock-infected cells expression of CDK1 was downregulated at 48 hpi and 72 hpi. For CDK2, -7, and -9, the steady state protein levels were fairly constant in mock-infected control cells, however, enhanced following infection. In addition, the expression of a subset of cyclins was determined (Fig. 4B). Cyclin B1 and cyclin T1 were chosen since the upregulation of the corresponding CDKs, CDK1 and -9 was highly pronounced. Similar to the CDKs, synthesis of both cyclins was increased at early and late (cycB1; Fig. 4B) or at late times (cycT1; Fig. 4B) of infection. These findings are in line with published results (Kapasi and Spector, 2008; Tamrakar et al., 2005; Sanchez et al., 2003; Salvant et al., 1998; Jault et al., 1995) and further support the hypothesis that not only one but several CDKs and cyclins are regulated by HCMV. Furthermore, the results suggested CDK1, -2, -7, and -9 as well as cyclin B1 and cyclin T1 as potential direct interactors of HCMV-encoded proteins.
Fig. 4: Upregulation of expression levels of CDKs (A) and cyclins (B) during the course of HCMV infection. Samples of mock-infected or infected (HCMV AD169; MOI 1) HFFs were taken at various time points post infection as indicated and subjected to Western blot analysis. (A) Impact of viral replication on expression levels of CDK1, -2, -7, and -9 was determined via CDK-specific antibodies. β-actin, loading control. (B) Expression of cyclin B1 (cycB1) and cyclin T1 (cycT1) was detected by means of PAb-cycB1 and PAb-cycT1.

D-3 Impact of knockdown of CDK1, -2, -7, or -9 on replication efficiency of HCMV

In order to deepen the knowledge of the HCMV-CDK interregulation, the relevance of individual CDKs for HCMV infection was analyzed. An powerful approach was the generation of HFFs with a short interfering (si)RNA-mediated stable knockdown of CDK1, -2, -7, or -9. For
this, the retroviral short hairpin (sh)RNA expression vectors RetroQ-siNon (pSIREN RetroQ; pHM2237) and RetroQ-GFP-siNon (pSIREN IRES-EGFP-RetroQ; pHM2244) were used which enable the RNA interference (RNAi)-mediated knockdown.

D-3.1 Selection of siRNA sequences for specific knockdown of individual CDKs

Crucial for successful knockdown of CDK1, -2, -7, and -9 was the identification of suitable siRNA sequences. As the vectors RetroQ-siNon and RetroQ-GFP-siNon are destined to express shRNAs which are then processed into siRNAs by cellular enzymes, shRNA sequences were designed for each CDK and cloned into both vectors. The prediction of effective shRNA sequences was performed by the use of softwares provided by Dharmacon and BD Biosciences (http://www.dharmacon.com/DesignCenter/DesignCenterPage.aspx; http://bioinfo.clontech.com/sirnaSeqDesignInit.do). The chosen sequences

Fig. 5: Transient siRNA-mediated knockdown of individual CDKs. 293T cells were cotransfected with CDK-expressing plasmids and plasmids coexpressing GFP together with siRNAs that correspond to specific CDKs as indicated. Transfection efficiency was monitored by GFP fluorescence microscopy. Transfected cells were harvested 48 h post transfection before expression of CDK1-HA, CDK2-HA, CDK7-HA, and CDK9-HA was assessed via Western blot analysis. β-actin, loading control.
were directed to nucleotides (nt) 490-508 of CDK1, nt 689-707 of CDK2, nt 356-374 of CDK7 and nt 293-311 of CDK9. The obtained plasmids, designated as RetroQ-siCDK1, -2, -7, and -9 (pHM2414, pHM2416, pHM2417, pHM2419) or RetroQ-GFP-siCDK1, -2, -7, and -9 (pHM2985, pHM2986, pHM2987, pHM2988), were first analyzed for functionality. For this, the siRNA constructs were cotransfected with plasmids coding for CDK1, -2, -7, or -9. The empty vectors (RetroQ-siNon, RetroQ-GFP-siNon) as well as plasmids coding for a non-related siRNA directed against luciferase (RetroQ-siLuci, RetroQ-GFP-siLuci) served as controls. As illustrated in Fig. 5, intracellular production of siCDK1 and siCDK9 resulted in a complete loss of CDK1-HA or CDK9-HA, respectively (Fig. 5; upper panel, lane 6 and lower panel, lane 9). Expression of CDK2-HA and CDK7-HA were not completely blocked, but substantially downregulated after coexpression of siCDK2 or siCDK7 (Fig. 5; middle upper panel, lane 7 and middle lower panel, lane 8). In contrast, the protein level of each CDK was not affected by those siRNAs directed to the other three CDKs analyzed indicating a specific targeting. A similar pattern of CDK knockdown was detected when the identical set of siRNA constructs based on RetroQ-siNon were used (data not shown).

D-3.2 Generation and characterization of CDK-deficient human fibroblasts

After the identification of functional and specific siRNA sequences, HFFs with a stable siRNA-mediated knockdown of the CDKs of interest were generated. For this, recombinant retroviruses containing the sequences for the respective siRNAs were used for transduction of human fibroblasts. Subsequent selection with puromycin led to the enrichment of HFF subpopulations containing copies of stably integrated retroviral transgenes encoding the respective siRNAs. These subpopulations were termed HFF-siCDK1, -2, -7, and -9 or HFF-GFP-siCDK1, -2, -7, and -9, respectively. In parallel, control HFFs lacking expression of siRNA (HFF-siNon, HFF-GFP-siNon) or expressing siLuci (HFF-siLuci, HFF-GFP-siLuci) were produced. CDK-specific knockdown in these HFF-siRNA subpopulations was examined by Western blot analysis (Fig. 6). The data showed a substantial level of downregulation of endogenous CDK1, -2, and -9 in both types of subpopulations, either based on vector RetroQ-siRNA or on vector RetroQ-GFP-siRNA (Fig. 6A and B, lanes 3, 4 and 6), whereas no effect on CDK7 expression was detectable (Fig. 6A and B, lane 5). Thus, functionality and specificity of the selected siRNA sequences was confirmed in stably transduced HFFs with the exception of siCDK7.

In order to further characterize the HFF-siRNA subpopulations and monitor possible effects of the retroviral gene transfer onto cell metabolism, a flow cytometry analysis was applied (Fig. 7). Equal numbers of HFF-siRNA cells were cultivated for 2 days prior to staining with reagents of the LIVE/DEAD Viability/Cytotoxicity kit which enabled an evaluation of the cell viability.
Fig. 6: Verification of knockdown of endogenous CDKs in stably transduced human fibroblasts. HFFs were stably transduced using recombinant retroviral transfer particles carrying sequences for production of siRNAs specific for CDK1, -2, -7, or -9 (A, B) and additionally for GFP expression (B). Equal amounts of cells were harvested and subjected to Western blot analysis using CDK-specific antibodies. β-actin, loading control.

The cytometry histograms (Fig. 7A) clearly demonstrated most of the cells remained highly viable after retroviral gene transfer (lower right) while only a minor fraction contained dead cells (upper left). The percentages of the fractions (Fig. 7B) clarified this observation with up to 92% viable cells in both CDK knockdown subpopulations and control cells (lower right, LR). The outcome of the experiment indicates that neither transduction alone nor siRNA-mediated CDK knockdown changed cell viability to a significantly measureable extent. In addition, an examination of cell morphology under the microscope confirmed that CDK knockdown cells were equivalent to untransduced cells or transduced control cells (data not shown). These observations were supported by proliferation studies determining the number of cells on day 0, 1, and 2. Data showed that HFF-siCDK1, -7, and -9 propagated similar, while HFF-siCDK2 subpopulation proliferated slightly faster compared to controls (HFF-siNon, HFF-siLuci; data not shown). Thus, the stable expression of siRNAs in transduced primary
human fibroblasts exhibit a verifiable and specific knockdown of CDK1, -2, or -9 in the absence of negative effects on cell viability.

![Images of cell viability analysis](image)

**Fig. 7**: Determination of cell viability of CDK knockdown subpopulations. $5 \times 10^5$ cells of each HFF-siRNA subpopulation were seeded and harvested 2 days later to investigate cell viability via flow cytometry analysis. FL1-H, calcein-positive cells; FL2-H, ethidium-homodimer-positive cells. Histogram depiction (A) and distribution in percentage (B) is given. UL, upper left, represents non-viable cell population; LR, lower right; represents viable cell population; UR, upper right; LL, lower left.

### D-3.3 Effect of CDK knockdown on HCMV replication

CDK-deficient fibroblasts were used for infection experiments. Thereby, HFF-siRNA cells were infected with HCMV AD169-GFP and subjected to automated GFP fluorometry, whereas infection of HFF-GFP-siRNA cells was performed with HCMV AD169 and plaque formation was determined. For each experiment, the value for the control subpopulation, HFF-siNon or HFF-GFP-siNon, was set to 100% (Fig. 8). Remarkably, HCMV replication was reduced in each CDK knockdown subpopulation. The decline in efficiency of viral replication was more pronounced in AD169-infected HFF-GFP-siRNA cells than in AD169-GFP-infected HFF-
siRNA cells, but this can likely be attributed to a higher sensitivity of the HCMV GFP-based replication assay. Viral replication was decreased to a range between 30%-60% in HFFs deficient in CDK expression. This level was comparable to the inhibition by roscovitine treatment. Unexpectedly, a reduction in HCMV replication was also determined in HFF-siCDK7 cells in which no CDK7 knockdown was detectable (Fig. 6). As both assays yielded a similar result and controls indicated the reliability of the experiment, unspecific artefacts could mostly be excluded. It appeared more likely that CDK7 activity was influenced to some poorly detectable extent by siCDK7 expression and this slight but specific impairment had an inhibitory effect on HCMV replication. Taken together, these data indicate that knockdown of one of the chosen CDKs affects HCMV replication in a direct or indirect manner. Thus, each analyzed CDK, that is CDK1, -2, -7, and -9, are required for high efficiency of HCMV replication in fibroblasts.

**Fig. 8: Impact of CDK knockdown on HCMV replication efficiency.** (A) HFF-siRNA subpopulations were infected with HCMV AD169-GFP (MOI 0.25) and harvested 7 days post infection before GFP counts were measured in cell lysates. Value of HFF-siNon, no inhib., was set to 100%. (B) HFF-GFP-siRNA subpopulations were infected with HCMV AD169 (MOI 1) and viral replication was determined via plaque formation 9 days post infection. Number of plaques of HFF-GFP-siNon, no inhib. was set to 100%. In both experimental setups, replication efficiencies in the presence of GCV (20 µM) or roscovitine (Rosco, 10 µM) served as controls. Determinations were performed in quadruplicate.

**D-4 Yeast two-hybrid analysis of interactions between CDK/cyclin complexes and HCMV-encoded proteins**

Several lines of evidence indicated an involvement of several CDK/cyclin complexes at different stages of viral replication, though a direct interaction between HCMV-encoded proteins and CDKs or cyclins had not been determined yet. Therefore, the goal of this study was to identify viral interaction partners of CDK/cyclin complexes via yeast two-hybrid analyses. In a first step, plasmids coding for CDK1, -2, -7, or -9 fused to either GAL4 DNA binding domain (BD; pHM2563, pHM2564, pHM2565, pHM2566) or GAL4 activation domain
(AD; pHM2567, pHM2568, pHM2569, pHM2570) were cloned and cotransfected with a selection of viral protein-expressing plasmids which had previously been used successfully for other settings of yeast two-hybrid analyses. However, no interaction was detectable for neither of the CDKs (data not shown). A reasonable explanation is given by the fact that CDKs may require contact with sufficient amounts of the corresponding cyclin subunit to achieve activity in this assay.

Fig. 9: Yeast two-hybrid analysis of interactions between cyc::CDK fusion proteins and various HCMV proteins. (A) Plasmids expressing cycA::CDK1 or cycT1::CDK9 tagged to GAL4 activation domain (AD, left panel) or cycA::CDK1, cycH::CDK7 or cycT1::CDK9 tagged to GAL4 DNA binding domain (BD, right panel) were cotransfected with a series of HCMV protein-expressing constructs fused to BD (left panel) and AD (right panel), respectively. Direct interaction was determined by filter lift assay. Controls are given in the middle panel. The known interaction between p53 and SV40 T-antigen (SV40-T) was used as positive control. (B) Summary of the identified interaction between pUL69 and cycT1::CDK9 or cycT1 including relevant controls.

Hence, yeast plasmids containing GAL4 AD or GAL4 BD were used for cloning of cyclin::CDK fusion constructs. The following plasmids were generated: AD-cycA::CDK1, AD-cycT1::CDK9, BD-cycA::CDK1, BD-cycH::CDK7 and BD-cycT1::CDK9. Using these constructs along with the identical selection of plasmids encoding viral proteins in cotransfection experiments led to the detection of two positive signals. CycT1::CDK9 fused to
GAL4 AD interacted with pUL69 (Fig. 9A, left panel) and cycT1::CDK9 in fusion with GAL4 BD strongly interacted with IE2p86 (Fig. 9A, right panel). In this analysis, both interactions of cycT1::CDK9 were detectable only in one direction, but not in the reverse setting concerning tagged GAL4 BD and GAL4 AD, respectively. Nevertheless, the findings were reproducible in several independent experiments. Furthermore, it should be mentioned that negative results in the yeast two-hybrid system may generally be due to simple limitations such as low levels of expression or unfavorable protein folding. Specificity-controlled positive results, however, even at a low signal intensity, generally argue for direct protein-protein interaction. Selfactivation of the constructs was excluded, as expression of AD-cycT1::CDK9 (Fig. 9A, left panel), BD-cycT1::CDK9 (Fig. 9A, right panel), AD-IE2p86 or BD-pUL69 (Fig. 9A, middle panel) individually with vector controls yielded no signal. Cotransfection of vector controls (GBT9, GAD424) served as important negative control (Fig. 9A, middle panel). For pUL69, an additional analysis was performed to distinguish whether cyclin T1 or CDK9 represents the direct interaction partner. Both proteins were separately coexpressed with pUL69. The subsequent filter lift assay identified a clear signal for the combination of cyclin T1 and pUL69 (Fig. 9B), whereas no interaction between CDK9 and pUL69 was observed. Taken the interaction between CDK9 and cyclin T1 into account, which served as positive control, the limited signal intensity of the cyclin T1-pUL69 interaction implies a dynamic low-affinity interaction. The yeast two-hybrid study using well-characterized constructs for HCMV proteins is summarized in Table 3.

**Table 3: Yeast two-hybrid analysis based on selected HCMV clones.**

<table>
<thead>
<tr>
<th>Collection of well-characterized HCMV clones (GAL4 BD / AD)</th>
<th>( \text{GAL4 BD / AD} )</th>
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<tr>
<td>confirmation (FLA no.2)</td>
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<td>identified interactor proteins</td>
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</table>

* GAL4 BD only

A second approach with the yeast two-hybrid system was based on the HCMV gene library pPC86EmL-TB40E (Schierling et al., 2004). Plasmids encoding CDK1, -2, -7, -9 or cycT1::CDK9 fused to GAL4 BD were utilized as baits and the HCMV library was cotransfected as a pray for the subsequent screening. Although the large-scale transfection yielded approximately \(5 \times 10^5\) transformants and 2, 3, or 5 candidates were further analyzed for the individual CDK constructs, no viral interactor could finally be confirmed (Table 4).
Table 4: Yeast two-hybrid analysis based on a HCMV gene library.

<table>
<thead>
<tr>
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<tr>
<td>interactor proteins</td>
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*following plasmid isolation and retransformation into yeast

D-5 Investigation of the phosphorylation of HCMV-encoded IE2p86 by CDK/cyclin complexes

The question whether IE2p86 represents a phosphorylation substrate of CDKs was addressed by in vitro kinase assays. In a first approach, overexpressed IE2p86 and CDK1, -2, -7, or -9 immunoprecipitated from the lysates of transfected cells were used. Two independent experiments are depicted in Fig. 10.

Fig. 10: Direct phosphorylation of IE2p86 by overexpressed immunoprecipitated CDKs in vitro. FLAG-tagged IE2p86 and HA-tagged CDK1, -2, -7, -9 (A) or CDK7, -9 (B) were coexpressed in 293T cells followed by immunoprecipitation with PAb-IE2p86 and MAb-HA. Radioactive signals were detected by phosphoimager exposure after SDS-PAGE and Western blotting of samples (upper panels). Expression levels of overexpressed proteins was monitored by Western blots analysis (lower panels).

In the first experiment (Fig. 10A, upper panel), CDK-mediated phosphorylation was detected in the sample containing CDK7, whereas both CDK7 and -9 phosphorylated IE2p86 in the second setup (Fig. 10B, upper panel). As a limitation in these assays, it should be mentioned that for so far unknown reasons no or only traces of IE2p86 protein were
detectable when CDK9 was coexpressed (Fig. 10, expression controls). Furthermore, the overall kinase activity seemed higher in the experiment displayed in Fig. 10B. Thus, IE2p86 can act as an in vitro substrate of CDK7 and -9 but the reproducibility of this result is limited. The cell cycle-associated CDK1 and -2 never exhibited IE2p86-directed kinase activity in these experiments.

Fig. 11: Direct phosphorylation of IE2p86 by purified recombinant CDK/cyclin complexes in vitro. (A) Following expression of IE2p86 in 293T cells and immunoprecipitation with PAb-IE2p86, recombinant CDK/cyclin complexes and \([\gamma^{32P}]ATP\) were combined with the precipitated protein to allow for phosphorylation reaction. Level of phosphorylation was determined via phosphoimager detection after Western blot transfer of products. (B) Increase of CDK/cyclin-mediated phosphorylation of IE2p86 in relation to basal level was calculated via densitometry (data in quadruplicate). —, control without kinase.

Next, a second setting of in vitro kinase assay was applied. Hereby, purified recombinant CDK/cyclin complexes were used, as in their active form CDKs are associated with cyclins (commercially available from ProQinase, Freiburg). The activity of the CDK/cyclin complexes CDK1/cycB1, CDK2/cycE, CDK7/cycH/MAT1 and CDK9/cycT were controlled by phosphorylation of the reference substrate RB-CTF (C-terminal fragment of retinoblastoma protein; data not shown). In the subsequent in vitro kinase assay, CDK/cyclin complexes were exogenously added to precipitated IE2p86 from transfected 293T cells to allow for kinase reaction in the presence of \([\gamma^{32P}]ATP\). Samples were analyzed as described above. Additionally, levels of phosphorylation were measured by densitometry. Interestingly, direct IE2p86 phosphorylation was mediated by each complex (Fig. 11A). The baseline phosphorylation of IE2p86 detected in the control samples without CDK/cyclin complex can likely be ascribed to traces of coprecipitated endogenous cellular kinases. The fold increase in phosphorylation was highest for CDK1/cycB1 (3-fold) and CDK2/cycE (2.5-fold) while the levels mediated by CDK7/cycH/MAT1 or CDK9/cycT remained below 2-fold (Fig. 11B). Although the quantitative levels of phosphorylation of IE2p86 were limited, the results from the in vitro kinase assays suggest that CDK1/cycB1 and CDK2/cycE are kinases mediating
phosphorylation of IE2p86 and a contribution from other CDK/cyclin complexes seems suggestive.

D-6 Investigation of the interregulation between cellular CDK/cyclin complexes as well as HCMV-encoded CDK ortholog pUL97 and HCMV protein pUL69

The HCMV-encoded multifunctional protein pUL69 was considered as a potential CDK-regulated protein. Findings supporting this conclusions were (i) pUL69 directly interacts with the regulatory subunit cyclin T1 of the CDK9/cyclin T1 complex in the yeast two-hybrid system (D.4), (ii) pUL69 is phosphorylated by the viral CDK ortholog pUL97 (Thomas et al., 2009), and (iii) pUL69 localization changes from homogeneous nuclear distribution towards speckled aggregates in the presence of the CDK inhibitor roscovitine (Sanchez and Spector, 2006). The following study was therefore initiated to characterize CDK-dependent regulation of pUL69 in respect of localization, phosphorylation and functionality.

D-6.1 CDK inhibitors and inhibitors of the viral CDK ortholog pUL97 induce intranuclear speckled aggregates of pUL69

In a first step, human fibroblasts were infected with HCMV AD169 in order to verify the phenomenon of CDK inhibitor-induced aggregation of pUL69 reported for Towne by Sanchez and Spector (2006). As depicted in Fig. 12, pUL69 encoded by AD169 likewise accumulated in intranuclear speckled aggregates when cells were treated with roscovitine, while showing the typically nuclear localization with accumulation in replication centers in the absence of the inhibitor.

Fig. 12: Roscovitine-mediated accumulation of pUL69 in intranuclear speckled aggregates. HFFs, infected with HCMV AD169 (MOI 1) were treated with roscovitine (addition 24 hpi) and analyzed for localization of pUL69 at 72 hpi. No inhib, no inhibitor; Rosco, roscovitine 10 μM.

Besides roscovitine, a subset of CDK inhibitors along with inhibitors of the HCMV-encoded protein kinase pUL97 were examined for their ability to induce an aggregation of pUL69 into intranuclear speckles (Table 5). Interestingly, in addition to several CDK inhibitors with anti-cytomegaloviral activity (Rosc, R25, R58; Fig. 3) also pUL97 inhibitors (Gö6976, Ax7396) which inhibit pUL97 activity and HCMV replication in vitro (Schleiss et al., 2008;
Results

Herget et al., 2004) induced microspeckled accumulation of pUL69. In contrast, pUL69 remained in its typical even distribution when HCMV AD169-infected fibroblasts were treated with CDK inhibitor A50 exhibiting no anti-cytomegaloviral activity (data not shown) or chemically related control compounds Gö7874 and AG1478 or an unrelated tyrosine kinase inhibitor (AG490).

Table 5: Induction of pUL69 nuclear speckled aggregation by CDK and pUL97 inhibitors including controls. PKC, protein kinase C; EGFR, epidermal growth factor receptor; no inhib, no inhibitor; Rosco, roscovitine.

<table>
<thead>
<tr>
<th>targets</th>
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<th>EGFR</th>
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<tr>
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</tbody>
</table>

*++, strong and defined speckled pUL69-aggregation (>50% of infected cells)
+ , speckled pUL69-aggregation clearly recognizable but quantitatively limited (<50%)
*-, no speckled pUL69-aggregation (<5%), homogenous distribution

D-6.2  HCMV variants show quantitative differences in the formation of CDK inhibitor-induced pUL69 aggregates

Although the inhibitor-induced pUL69 aggregation was observed for several HCMV strains such as AD169 (Fig. 12; Thomas et al., 2009), Towne (Sanchez and Spector, 2006), and using clinical isolates (data not shown), phenotypical variations were detected by analysis of AD169-derived variants GDGrP53, GDGrXbaF4 and 759rD100. These variants carry therapy-resistance mutations in the open reading frames (ORFs) of the viral protein kinase UL97 or the viral DNA-polymerase UL54 or both (Fig. 13C). GDGrXbaF4 and 759rD100 contain the GCV resistance-conferring deletion mutation in pUL97 (amino acids 590-593; Sullivan et al., 1992; Biron et al., 1986). GDGrP53 and 759rD100 contain the point mutation in pUL54 (A987G) resulting in cidofovir (CDV) resistance and high level GCV resistance (Sullivan et al., 1993; Biron et al., 1986). Thus, GDGrP53 and GDGrXbaF4 are single mutants whereas 759rD100 is a double mutant. These HCMV variants were used to study the localization of pUL69 in the context of lytic replication with mutated versions of pUL97 and/or pUL54 expressed. For this, infected HFFs were treated with CDK inhibitors roscovitine or R58 from 24 hpi followed by immunofluorescence analysis at a late time point of infection (Fig. 13A). In quantitative terms, the percentage of pUL69-positive cells showing intranuclear pUL69 aggregates was determined (Fig. 13B). Control samples without CDK inhibitors showed pUL69 in the typical intranuclear localization pattern (Fig. 13A, a-e). For strain AD169, roscovitine or R58 treatment produced pUL69 aggregation in 67.3% or 60.7% of virus-positive cells (Fig. 13A, g, I; Fig. 13B). When analyzing the HCMV variants, GDGrXbaF4 and GDGrP53 were similar to AD169 with pUL69 aggregate formation in response to both
inhibitors (Fig. 13A, h-i, m-n). However, in quantitative terms, pUL69 aggregation was significantly reduced in GDGrP53-infected cells (36.7% or 37.3% of virus-positive cells, p<0.01; Fig. 13B) compared to the parental strain AD169. Interestingly, both inhibitors failed to induce an efficient aggregation of pUL69 in nuclear speckles when cells were infected with the

![Image](https://example.com/figure13.png)

**Fig. 13: CDK inhibitor-induced aggregation of pUL69 in fibroblasts infected with variants of HCMV.** (A) HFFs were infected with HCMV AD169 or variants GDGrXbaF4, GDGrP53 and 759rD100 (MOI 0.5). Roscovitine (Rosco, 10µM) or R58 (10µM) were added 24 hpi. Detection of intranuclear localization of pUL69 in the presence or absence of CDK inhibitors was performed by indirect immunofluorescence analysis 72 hpi. (B) pUL69-positive cells showing pUL69 aggregates were quantified by counting 50 cells in triplicates. Statistical significance was calculated by student's t-test. (C) Variations in the amino acid sequence of pUL97 and pUL54 between HCMV AD169 and the drug-resistant HCMV variants are shown. Highlighted in light gray is wild-type HCMV AD169 sequence, black indicates mutations or deletion (-).
double mutant 759rD100 (Fig. 13A, j, o). Quantitative analysis revealed only 14% (roscovitine) or 2.7% (R58) of 759rD100-infected fibroblasts showing pUL69 aggregates under CDK inhibitor treatment which represented a highly significant reduction (p<0.0001; Fig. 13B). Taken together, the phenotype of pUL69 aggregation induced by CDK inhibition varied, particularly in quantitative terms, between virus variants GDGrP53 and 759rD100 compared to parental strain AD169. Although the HCMV variants showed alterations in their replication capacity (Zielke, 2008), the data cannot fully explain the differences in the aggregation of pUL69. As demonstrated by quantitative PCR measuring the kinetics of viral genome replication, GDGrP53 exhibited the lowest replication efficiency (Rechter et al., 2009). Despite this, 759rD100, but not GDGrP53, showed a clear lack of CDK inhibitor-induced pUL69 aggregates. Thus, the peculiarity of the phenotype produced by double mutant 759rD100 could not be explained yet and needs to be further analyzed on a mechanistic basis.

D-6.3 Induction of pUL69 aggregates is not necessarily a prerequisite of antiviral activity of CDK inhibitors

Since HCMV variant 759rD100 was insensitive in regard to roscovitine-mediated aggregation of pUL69, the question was raised whether roscovitine and further CDK inhibitors do not exert antiviral activity against double mutant 759rD100. To answer this issue, plaque reduction assay was performed with HCMV AD169- and 759rD100-infected cells and plaque numbers in samples without inhibitor were set to 100% for each virus. (Fig. 14).

![Graph showing plaque reduction assay results](image)

**Fig. 14: Sensitivity of HCMV AD169 and HCMV 759rD100 towards CDK inhibitors.** HFFs, infected with AD169 or 759rD100, were treated with GCV (20 µM), roscovitine (Rosco, 10 µM) or R58 (10 µM) and plaque formation was determined 10 days post infection (data in quadruplicate).

The double mutant 759rD100 showed reduced replicative sensitivity towards roscovitine,
Results

whereas a strong sensitivity towards the second CDK inhibitor, R58, was detected. This indicates that R58, although inefficient in inducing pUL69 aggregates in 759rD100-infected cells, mediated an inhibitory effect on viral replication of 759rD100, albeit through a mode of action which seemed independent from pUL69. In contrast, the parental virus AD169 was sensitive to roscovitine, R58 (Fig. 14) and a number of other CDK inhibitors (data not shown). Thus, the potential of CDK inhibitors to induce intranuclear speckles of pUL69 does not necessarily correlate in full terms with the antiviral activity of the inhibitors.

D-6.4 Activity of pUL97 is also required for the typical intranuclear localization of pUL69

Besides roscovitine and R58, inhibitors of further protein kinases, particularly those inhibiting the viral protein kinase pUL97, also known as viral CDK ortholog, were investigated for their ability to induce formation of pUL69 aggregates in HFFs infected with HCMV AD169 or the HCMV variants GDGrP53, GDGrXbaF4 or 759rD100. Both proteins, pUL69 and pUL97, were visualized and the localization was qualitatively as well as quantitatively determined. Two inhibitors of pUL97, Gö6976 (inhibitor of pUL97 and PKC) and Ax7396 (inhibitor of pUL97 and EGFR), which inhibit HCMV replication in vitro (Schleiss et al., 2008; Herget et al., 2004) induced nuclear pUL69 speckled aggregates very similar to roscovitine or R58 (Fig. 15A, t, v, x; data not shown for Ax7396). In contrast, treatment of infected cells with inhibitors of PKC (Gö7874), EGFR (AG1478, PD153035; Thomas et al., 2009) or an unrelated tyrosine kinase inhibitor, AG490, did not have a similar effect on pUL69 localization (Fig. 15A, j-r). The quantitative analysis revealed that parental strain AD169 along with variants GDGrXbaF4 and GDGrP53 showed pronounced Gö6976-induced aggregation of pUL69 (Fig. 15A, t, v, x and Fig. 15B), whereas very little aggregate formation was observed for 759rD100 (Fig. 15A, z and Fig. 15B). The difference between AD169 and 759rD100 was statistically significant (p<0.01). Interestingly, pUL97 was never observed in colocalization with pUL69 aggregates but remained in a homogeneous nuclear distribution. Of note, treatment with Gö6976 led to some exclusion of pUL97 from viral replication centers (Fig. 15, c, u), an effect that had been reported before (Marschall et al., 2003). The data suggest that besides the activity of CDKs also pUL97 activity influences the intranuclear localization of pUL69 in fibroblasts infected with AD169 or several HCMV variants. This conclusion was verified by a study using a recombinant HCMV AD169-GFP-based virus with a deletion of the UL97 gene, AD169-delUL97-GFP, also termed BAC213 (Fig. 15C). Immunofluorescence analysis showed that pUL69 was localized in speckled aggregates already in the absence of an inhibitor (Fig. 15C, d). Treatment of the BAC213-infected cells with CDK inhibitor roscovitine, or pUL97 inhibitor Gö6976 did not further affect pUL69 in its localization (Fig. 15C, f, h). Overall, the data presented in Fig. 15, Fig. 13 and Table 5 indicate that both CDK activity as well as activity of
the viral CDK ortholog pUL97 contribute to mechanisms regulating pUL69 localization.

D-6.5 CDK inhibitor-induced speckled aggregates of pUL69 accumulate within replication centers

In order to define the CDK inhibitor-mediated pUL69 aggregates more precisely, HCMV AD169-infected fibroblasts under roscovitine treatment were analyzed by costaining of pUL69
Results with a selection of viral and cellular nuclear proteins (Fig. 16).

Fig. 16: Examination of speckled aggregates of pUL69 in respect of viral and cellular markers of nuclear compartments. Subsequent to infection with HCMV AD169 (MOI 1) and addition of roscovitine (Rosco, 10 µM) 24 hpi, the localization of pUL69 along with pUL44, SC-35 or nuclear pore complex (NPC) was detected 72 hpi by indirect immunofluorescence analysis.

For virus-associated nuclear structures, DNA polymerase pUL54 and its processivity factor pUL44 were employed as prominent markers of viral replication centers in which the proteins usually colocalize with pUL69. In the presence of roscovitine, pUL44 (Fig. 16, c and g) and pUL54 (data not shown) remained in their typical localization pattern but remarkably, the speckles aggregates of pUL69 fully localized within the area of replication centers (Fig. 16, e-h). In a next step, prominent cellular nuclear structures were examined, including splicing compartments (visualized by splicosome assembly factor SC-35), the nuclear pore complex (NPC, visualized by NUP62/152/90) or aggresome structures (visualized by heat shock cognate protein 70, HSC70; Fortun et al., 2003). Neither SC-35 (Fig. 16, k and o), NUP62/152/90 (Fig. 16, s and w) nor HSC70 (data not shown) altered their localization under conditions of CDK inhibition and never colocalized with pUL69. The analysis implies not only
Results

that the roscovitine-induced aggregates of pUL69 are localized within viral replication centers, but also that only the replication center-associated fraction of pUL69 accumulates into these subnuclear speckled structures.

D-6.6 CDK9 and cyclin T1 colocalize with pUL69 in replication centers and in speckled aggregates

In a next step, it was crucial to analyze whether protein kinase inhibitors of CDKs and the viral CDK ortholog pUL97 influence the localization of CDK1, -2, -7, and -9. Human fibroblasts infected with HCMV AD169 and treated with roscovitine, R58 or Gö6976 were studied for distribution patterns of the different CDKs in relation to pUL69. For CDK1, a previously described HCMV infection-induced translocation of CDK1 from the nucleus to the cytoplasm (Sanchez et al., 2003) was observed, which was unaffected by inhibitors (data not shown). Hence, a colocalization between CDK1 and pUL69 was never detected. For CDK2, neither infection nor treatment with inhibitors impaired its localization. CDK2 remained in its typically nuclear pattern (data not shown). On the other hand, CDK7 accumulated in replication centers together with pUL69 (Fig. 17, e-h) and a number of viral proteins known to be contained within these compartments (Alvisi et al., 2006; Park et al., 2006; Xu et al., 2002; Ahn et al., 1999; Michel et al., 1996). However, both types of inhibitors did not induce a speckled aggregation of CDK7 (Fig. 17, k, o and s).

Fig. 17: Investigation of intranuclear localization patterns of CDK7 and pUL69. HFFs were infected with HCMV AD169 (MOI 1) and treated with CDK inhibitors roscovitine (Rosco, 10 µM), R58 (10 µM) or pUL97 inhibitor Gö6976 (2 µM) from 24 hpi. Intranuclear distribution of pUL69 and CDK7 was visualized via indirect immunofluorescence analysis at 72 hpi.
Results

Strikingly, localization of CDK9 was altered in a pattern very similar to pUL69. Firstly, HCMV infection led to an incorporation of CDK9 into viral replication centers as shown by colocalization with pUL69 (Fig. 18A, d-f). Secondly, in the presence of roscovitine or R58, CDK9 was recruited to aggregates in form of nuclear speckles (Fig. 18A, h and k) which perfectly colocalized with pUL69 (Fig. 18A, g-l). By contrast, CDK9 aggregation was only marginally detectable when infected cells were treated with pUL97 inhibitor Gö6976 (Fig. 18, n).

![Colocalization between pUL69 and CDK9 (A) or cyclin T1 (B).](image)

Subsequent to infection with HCMV AD169 (MOI 1), protein kinase inhibitors roscovitine (Rosco, 10 µM), R58 (10 µM) or Gö6976 (2 µM) were added 24 hpi. Colocalization between pUL69 and CDK9 (A) or pUL69 and cyclin T1 (B) was detected via indirect immunofluorescence analysis at 72 hpi.

These findings suggested to perform an immunofluorescence analysis of the regulatory subunit of CDK9, cyclin T1, to address the question of colocalization between pUL69 and cyclin T1. Like CDK9, cyclin T1 was recruited to replication centers upon infection (Fig. 18B, e) and the colocalization between cyclin T1 and pUL69 was further developed in subnuclear speckles aggregates under treatment with roscovitine or R58 (Fig. 18B, h and k). However, when pUL97 activity was inhibited by Gö6976, cyclin T1 neither accumulated in replication centers nor localized in speckles (Fig. 18B, n). Gö7874, which neither inhibit pUL97 nor CDK activity, served as specificity control (data not shown). Further immunofluorescence analysis using uninfected roscovitine-treated fibroblasts revealed slight intranuclear structural changes for CDK7, CDK9 and cyclin T1 but these were clearly not identical with the fine-speckled
nuclear aggregates observed in HCMV-infected cells (data not shown). Thus, the presence of pUL69 appears decisive for the induction of aggregation of CDK9 and/or cyclin T1 under roscovitine treatment. Overall, the data led to the conclusion that aggregates constituted by pUL69, CDK9 and cyclin T1 are induced by CDK inhibitors but not pUL97 inhibitors. Moreover, although both classes of inhibitors induce a very similar subnuclear structure of pUL69, the underlying regulatory mechanisms appear to differ: roscovitine- and R58-mediated formation of these aggregates are likely associated with CDK9/cycT1 activity, while Gö6976-mediated aggregation appears to be CDK-independent.

D-6.7 Cyclin T1 but not CDK9 directly interacts with pUL69 in mammalian cells

On the basis of immunofluorescence and yeast two-hybrid data, coimmunoprecipitation (CoIP) experiments were performed in order to assess direct interaction between pUL69 and CDK9 or cyclin T1. As shown in Fig. 19, immunoprecipitation of pUL69 from cell lysates resulted in a distinct coprecipitation of cyclin T1. The CoIP was positive for cyclin T1 when pUL69 was cotransfected with cyclin T1 or with cyclin T1 and CDK9 (Fig. 19A, lanes 8-9). Conversely, CDK9 was neither detectable after coexpression with pUL69 nor after coexpression with pUL69 and cyclin T1 (Fig. 19A, lanes 7-8). The known self-interaction of pUL69 served as positive control (Fig. 19A, lane 2; Lischka et al., 2007) and expression levels of the proteins were monitored by lysate controls taken prior to the addition of the CoIP antibody (Fig. 19B). It was apparent that expression levels of cyclin T1 and pUL69 varied. On the one hand, cyclin T1 was higher expressed when pUL69 was coexpressed (Fig. 19B, lane 9) and, on the other hand, pUL69 was less produced when CDK9 and cyclin T1 were coexpressed. This may provide an explanation for the slight difference in the cyclin T1 signal in lanes 8 and 9 of the CoIP detection blot (Fig. 19A, lanes 8-9). Although the signal intensity obtained for coimmunoprecipitated cyclin T1 was weak compared to precipitation control for pUL69 (Fig. 19A, lanes 2-3 and 7-9), the lack of any background in samples referring to proteins expressed separately (Fig. 19A, lanes 3-6) or samples immunoprecipitated with an unrelated MAb-UL44 antibody (Fig. 19A, lanes 11-13) demonstrated a high specificity. Moreover, another FLAG-tagged HCMV-encoded protein (pUL26) did not coimmunoprecipitate cyclin T1 (Fig. 19A, lane 10) underlining the reliability of the experiment. Besides these controls, the data were confirmed with a similar experimental setup using Myc-tagged cyclin T1 (data not shown). Together, these data provide evidence for an interaction between cyclin T1 and pUL69.
Fig. 19: Interaction analysis of CDK9 and cyclin T1 with pUL69. 293T cells were cotransfected with CDK9-HA, cyclin T1 (cycT1) and FLAG-pUL69 in different combinations as indicated and lysed 48 h post transfection. (A) FLAG-pUL69 was immunoprecipitated using MAb-FLAG and immunoprecipitates were subjected to Western blot analysis using indicated detection antibodies. (B) Expression of proteins was controlled via tag- or protein-specific antibodies. RFP, red fluorescent protein.

D-6.8 Direct phosphorylation of pUL69 by CDK/cyclin complexes

In order to investigate whether pUL69 serves as a phosphorylation substrate of CDK/cyclin complexes, pUL69 was overexpressed and immunoprecipitated from cell lysates before purified recombinant CDK/cyclin complexes were added along with radioactive labeled [γ-33P]ATP to allow for kinase reaction. A pronounced phosphorylation of pUL69 was demonstrated for CDK1/cycB1, CDK7/cyclH/MAT1, CDK9/cycT (Fig. 20A, upper panel, lanes 1, 3 and 4) and, at a lower level, for CDK2/cycE (Fig. 20A, lane 2). In the sample without
CDK/cyclin complex (---), a slight level of baseline phosphorylation of pUL69 was observed (Fig. 20A, upper panel, lane 5) which can possibly be attributed to traces of endogenous CDKs in the immunoprecipitates. Interestingly, pUL69 phosphorylation was mediated by all CDK/cyclin complexes, but to different levels (Fig. 20B). The fold increase in pUL69 phosphorylation was therefore quantified by densitometry. The strongest phosphorylation of pUL69 was measured in the presence of CDK1/cycB1 (12.4-fold; Fig. 20B). CDK7/cycH/MAT1 and CDK9/cycT also mediated pronounced levels of phosphorylation with 6.4-fold or 7.2-fold increase, respectively. A nonspecific phosphorylation activity of the CDK/cyclin complexes was excluded as a further HCMV-encoded protein, pUL26, was not phosphorylated (Fig. 20A, lower panel). These in vitro findings identified several CDK/cyclin complexes to phosphorylate pUL69. It should be mentioned that further analyses of the phosphorylation of pUL69 in in vitro kinase assays (in cooperation with Marco Thomas and further colleagues of the research group) demonstrated an additional important role of pUL97 since the viral CDK ortholog also proved to be an efficient pUL69-phosphorylating kinase (Thomas et al., 2009). Thus, more than one kinase is responsible for the regulatory phosphorylation status of pUL69.

Fig. 20: Direct phosphorylation of pUL69 by CDK/cyclin complexes in vitro (A, B) and detection of phosphorylated pUL69 in HCMV-infected fibroblasts (C). (A) Recombinant pUL69 and pUL26 (FLAG-tagged) were expressed in 293T cells and immunoprecipitated with indicated antibodies following lysis at 48 h post transfection. Phosphorylation reaction was started by adding recombinant CDK/cyclin complexes in the presence of radiolabelled [γ-32P]ATP to the precipitated proteins. The samples were subjected to Western blotting followed by detection of labeled phosphorylation products via phosphoimager exposure. (B) Increase in CDK/cyclin-mediated phosphorylation of pUL69 in relation to basal levels was determined via densitometry (data in quadruplicate). —, control w/o kinase. (C) HFFs were infected with HCMV AD169 (MOI 0.5) for 2 days followed by an overnight in vivo labeling of proteins. pUL69 was immunoprecipitated by PAb-UL69 followed by SDS-PAGE and Western blotting of the immunoprecipitates. Phosphorylated pUL69 was detected via phosphoimager exposure (upper panel) and total pUL69 was detected by subsequent immunostaining of the blot (lower panel).
Next, pUL69 was immunoprecipitated from HCMV AD169-infected HFFs which were treated with [γ-33P]orthophosphate to allow for in vivo labeling of proteins. Investigation of the samples revealed a strong pUL69 phosphorylation signal (Fig. 20C) which could be reduced by treatment of the cells with roscovitine (15 µM; reduction of 66% ± 13%; data not shown). However, this inhibition of phosphorylation did not occur in a CDK-specific manner and was also observed for other protein kinase inhibitors. This results suggest that CDK activity is required for in vivo phosphorylation of pUL69, with contribution of further cellular and viral protein kinases such as viral pUL97 (Thomas et al., 2009). Taken together, the data presented in Fig. 20 specify CDKs as a novel class of pUL69-phosphorylating protein kinases.

D-6.9 Kinetic study of the nucleo-cytoplasmic translocation of CDK1 in HCMV-infected cells

The finding that CDK1/cycB1 mediated a particularly strong phosphorylation of pUL69 in vitro stood in a certain contrast to the translocation of CDK1 from the nucleus into the cytoplasm in HCMV-infected cells. Therefore, the question of any possibility of direct interaction and interregulation between CDK1 and nuclear pUL69 was addressed. To this end, a kinetic study of the HCMV-induced nucleo-cytoplasmic translocation of CDK1 was performed. HCMV AD169-infected HFFs were stained simultaneously for CDK1 and pUL69 at various time points post infection, representing immediate early and early stages of the replication cycle. First, the expression kinetics of pUL69 was determined (Fig. 21A). Starting from 4 hpi, nuclear pUL69 was observed and over the time the percentage of pUL69-positive cells increased continuously. Under roscovitine treatment, pUL69 expression was transiently retarded. In addition, reduced signal intensities of pUL69 per cell were noted under roscovitine treatment. Second, the localization of CDK1 in pUL69-positive cells was analyzed. Here, localization patterns of CDK1, i.e. CDK1 only in the nucleus, concurrently in the nucleus plus cytoplasm or only in the cytoplasm, were distinguished (Fig. 21B). Up to 6 hpi, both CDK1 and pUL69 localized within the nucleus. At 8 hpi the nucleo-cytoplasmic translocation of CDK1 started in untreated as well as roscovitine-treated cells and the fraction of cells showing CDK1 in a nucleo-cytoplasmic or cytoplasmic localization progressively increased with a completion of the translocation of CDK1 to the cytoplasm at about 24 hpi. Of note, inhibition of CDK activity by roscovitine produced a slight delay of this HCMV-induced alteration of CDK1 localization. Despite a lack of direct colocalization of both proteins, these findings suggest that the nuclear localization of both CDK1 and pUL69 within the first hours of infection may allow an interregulation at early stages of the replication cycle.
Results

Fig. 21: Kinetics of pUL69 production (A) and nucleo-cytoplasmic translocation of CDK1 (B) in HCMV-infected cells. HFFs were infected with HCMV AD169 (MOI 1) and cultivated in the presence or absence of roscovitine (Rosco). Cells were harvested at the indicated time points and immunofluorescence staining of CDK1 and pUL69 was performed by use of PAb-CDK1 and MAb-UL69. A mean ± SD of determination in quadruplicate is given. (A) Percentage of pUL69-positive cells and impact of roscovitine on expression level of pUL69 within the first 24 h of infection. (B) The distribution pattern of CDK1 in pUL69-positive, roscovitine-treated or untreated cells was distinguish: nuclear, nucleo-cytoplasmic or cytoplasmic localization.

D-6.10 The mRNA export activity of pUL69 requires CDK activity

Since the hitherto data provide evidence for pUL69-CDK interaction and a CDK-mediated phosphorylation of pUL69, it was important to investigate whether CDK activity is required for the mRNA export activity of pUL69. For this, a reporter assay was employed (Lischka et al., 2006) to determine pUL69-mediated nuclear export of an intron-containing mRNA coding for chloramphenicol acetyl transferase (CAT) in the absence or presence of CDK inhibitors (Fig. 22). The pUL69 activity obtained in the absence of inhibitor was set to 100%. Roscovitine was most effective and reduced CAT activity, i.e. pUL69-mediated mRNA export, to 43%
Results (statistically significant, p<0.01). Treatment with a second CDK inhibitor, R58, resulted in a decline to 63%. Thus, pUL69 requires CDK activity to achieve full functionality in mRNA export.

![Graph showing contribution of CDK activity to mRNA export activity of pUL69.]

Fig. 22: Contribution of CDK activity to the mRNA export activity of pUL69. HeLa cells were transfected with a pUL69-expressing plasmid along with a reporter plasmid (unspliced mRNA encodes chloramphenicol acetyl transferase, CAT). CDK inhibitors were added 16 h post transfection. 48 h post transfection, the mRNA activity of pUL69 was determined using the CAT enzyme-linked immunosorbent assay. Determinations were performed in triplicate. Statistical significance was calculated by student’s t-test.

This finding was underlined by additional data obtained in this experimental system when using inhibitors of the viral CDK ortholog pUL97. Inhibition of pUL97 activity similarly reduced the nuclear mRNA export activity of pUL69 to a significant extent (Thomas et al., 2009). This result is consistent with the results described above for the induction of intranuclear speckled aggregates of pUL69 by both, inhibitors of CDKs and pUL97. Combined, these pUL69-phosphorylating protein kinases appear to exert a major regulatory impact on pUL69.
E Discussion

The human cytomegalovirus (HCMV) has developed a replication strategy that involves multiple interregulations with cellular factors. As a consequence, viral replication is closely connected with host cell pathways such as cell cycle control, transcription and nucleo-cytoplasmic transport. This strategy modifies cellular proteins towards virus-specific functions, thus ensuring an environment efficiently supporting viral reproduction. Several studies demonstrated a functional integration of CDK activity with virus-specific gene and protein regulation (Kapasi and Spector, 2008; Bain and Sinclair, 2007; Sanchez and Spector, 2006; Tamrakar *et al.*, 2005; Sanchez *et al.*, 2004; Sanchez *et al.*, 2003; Bresnahan *et al.*, 1997; Bresnahan *et al.*, 1996; Jault *et al.*, 1995). Since the discovery of these aspects of HCMV-CDK interregulation, the debate has been going on which CDKs are particularly important for viral replication.

Elucidating the role of CDKs for HCMV replication, pharmacological CDK inhibitors (PCIs) have been proven as useful tools. In this work, several recently designed PCIs showing individual selectivity profiles with a strong anti-cytomegalovirus activity were determined. These data further strengthened the importance of CDKs for HCMV infection. However, as the compounds possessed partly different selectivity profiles towards CDKs, it remained speculative which CDKs are the key determinants for HCMV replication. Limitations in using CDK inhibitors are given by the determination of their selectivity profiles *in vitro* which were restricted to an available range of possible CDK targets. Although the information from inhibitory profiles do not allow a simple assortment of CDKs required for HCMV replication, the data from the inhibitor studies suggest that not only one but a combination of several CDKs are subject to regulation by HCMV. Further indications in support of this conclusion are the findings of Western blot analyses showing a HCMV-induced upregulation of CDK1, -2, -7, and -9 along with cyclins B1 and T1. This upregulation is not a general observation for all CDKs and cyclins as, for instance, protein levels of cyclin A as well as CDK4 and CDK6 remain unchanged or are downregulated during HCMV replication (Bresnahan *et al.*, 1996; Jault *et al.*, 1995). Interestingly, although CDK activity is required for numerous and also distantly related viruses, the role of individual CDKs for the respective viruses seem to differ. While CDK1 activity is induced and maintained during HCMV replicative cycle (Sanchez *et al.*, 2003; Jault *et al.*, 1995), the same kinase is indirectly inhibited by the viral protein vpr during HIV-1 replication (Planelles *et al.*, 1996). On the other hand, CDK9, for instance, is considered as an important determinant for the replication of a variety of viruses including HCMV, HSV-1, HIV-1 as well as human T-lymphotropic virus 1 (HTLV-1; Kapasi and Spector, 2008; Wang and Fischer, 2008; Durand and Roizman, 2008; Biglione *et al.*, 2007; Salerno *et al.*, 2007; Cho *et al.*, 2007; Pumfery *et al.*, 2006; Zhou *et al.*, 2006; Tamrakar *et al.*, 2005; Chiu *et al.*, 2004; Jault *et al.*, 1995). Overall, CDK inhibitors with antiviral activity like roscovitine possess diverse
underlying modes of action against different viruses. Thus, when used as a pharmacological tool for the functional investigation of a particular CDK in viral infection, selectivity of CDK inhibitors is a key issue.

In order to assure a specific suppression of a single CDK, the technique of siRNA-mediated knockdown was applied. This method has become a very powerful and widely used strategy for genetic analysis, based on the highly specific and efficient silencing of target genes. For HCMV, several studies using siRNA-mediated RNA interference (RNAi) were performed. So far, mostly viral targets were chosen to investigate mechanisms regulating HCMV replication and to determine their therapeutic potential (Tao et al., 2008; Wang et al., 2008; Dittmer and Bogner, 2006; Poole et al., 2006; Wiebusch et al., 2004), although a couple of studies targeting cellular proteins have also been published (Fraile-Ramos et al., 2007; Poole et al., 2006; DeFilippis et al., 2006). However, despite the known importance of CDKs for HCMV replication, an experimental approach with CDK-directed knockdown had been missing. Crucial challenges for achieving an effective knockdown by RNAi is the siRNA sequence and its delivery to each desired target cell. For HCMV, primary human fibroblasts (HFFs) represent the most suitable cell culture infection system as they are fully permissive for HCMV infection. Since it is difficult to achieve efficient transient transfection of these primary cells, HFFs were transduced with recombinant retroviral transfer particles to generate HFF subpopulations stably expressing siRNA directed against CDK1, -2, -7, or -9. For the generation of these constructs, the siRNA sequences were aligned for specificity and position within the ORF. Where possible, sequences of the kinase domain were preferred as mutations are less likely to occur within these conserved regions. Detailed analysis of the generated CDK knockdown subpopulations showed that retroviral transduction of the sequences encoding the respective siRNAs provides an effective means to substantially reduce expression of the CDKs in human fibroblasts. Interestingly, HCMV infection of CDK-depleted cells revealed that already knockdown of a single CDK negatively impairs viral replication. This could not be easily expected since CDK and cyclin knockout experiments have indicated that CDKs are functionally redundant (Santamaria and Ortega, 2006; Malumbres et al., 2004; Berthet et al., 2003; Ortega et al., 2003; Rane et al., 1999). That is, suppression of a single CDK has been considered as possibly insufficient for exerting major effects on viral replication. However, secondary effects appeared rather proable as each CDK participates in various cellular pathways. Hence, inhibition of a single CDK activity likely entails diverse changes in cellular regulatory mechanisms which may consequently inhibit viral replication. The RNAi approach in this work confirms the strong requirement of CDK1, -2, -7, and -9 for an efficient HCMV replication and demonstrates for the first time the usefulness of a siRNA approach in this context.

In this work, first evidence for a direct interaction between CDKs and viral proteins was
obtained by yeast two-hybrid analyses identifying the multifunctional regulator pUL69 and the immediate early protein IE2p86 as interaction partners of the CDK9/cyclin T1 complex. Further experimentation using a variety of different methods characterized these protein interactions. In summary, these investigations provided novel insights into the link between CDKs and regulation of cytomegaloviral proteins.

IE2p86, expressed shortly after infection, is involved in the regulation of the transcriptional activation of viral and cellular promoters, thus in the control of the temporal expression of HCMV genes (Caswell et al., 1996; Lukac et al., 1994; Kerry et al., 1994; Hagemeier et al., 1992; Monick et al., 1992; Stenberg et al., 1990; Hermiston et al., 1987). Additionally, this transactivator plays a role in the modulation of the host cell cycle, both in stimulating and inhibiting individual steps of the process eventually contributing to the pseudo-G1 arrest (Wiebusch and Hagemeier, 2001; Castillo et al., 2000; Murphy et al., 2000; Wiebusch and Hagemeier, 1999). IE2p86 is described as a SUMO-modified phosphoprotein, with major phosphorylation sites having been mapped (Berndt, PhD thesis, 2008; Lee and Ahn, 2004; Ahn et al., 2001; Hofmann et al., 2000; Harel and Alwine, 1998). In this work, results from in vitro kinase assays particularly identified CDK1/cycB1 and CDK2/cycE as IE2p86-phosphorylating kinases. This is consistent with reports from Lukac et al. (1997) and Barrasa et al. (2005) which showed that purified IE2p86 domains fused to GST (Lukac et al., 1997) were phosphorylated by a subset of purified kinases including extracellular regulated kinase 2 (ERK2), PKA, PKC and CDK1 (Barrasa et al., 2005). As further demonstrated, GST-IE2p86 phosphorylation by CDK1 was inhibited by kinase site mutations (Barrasa et al., 2005; Harel and Alwine, 1998). Interestingly, several cases of overlapping kinase specificities were detected for specific regions within IE2p86 (Barrasa et al., 2005), supporting a concept of differential phosphorylation of IE2p86 in the course of HCMV replication cycle. It is likely that phosphorylation of IE2p86 influences its ability to control the temporal viral gene expression as was suggested for the serine-rich putative phosphorylation region between amino acids 258 and 275 by Barrasa and colleagues (2005). Moreover, a model of the C-terminus of IE2p86 indicates a dynamic tertiary structure in which changes caused by phosphorylation or SUMOylation may induce alterations in the functions of the protein (Barrasa et al., 2003). Since differences in IE2p86 SUMOylation pattern was observed when its phosphorylation status was changed due to mutations (Barrasa et al., 2003), and defects in SUMOylation significantly reduced transcriptional activation of the viral promoters of UL84 and UL112-113 (Hofmann et al., 2000), CDK-mediated phosphorylation of IE2p86 may effect its SUMO modification and consequently its conformation. This may change functional properties of IE2p86 concerning cell cycle arrest as well as transactivation capacity of early and late viral promoters, thus impairing progression of viral growth.

In respect of a second cytomegaloviral protein, pUL69, the study revealed a direct
association of CDK activity with the localization, phosphorylation and functionality of pUL69. The data indicate: (i) activities of CDKs and the viral CDK ortholog pUL97 are linked with the intranuclear localization of pUL69; (ii) CDK inhibitor-induced aggregates of pUL69 are localized within viral replication centers; (iii) CDK9 and cyclin T1 strictly colocalize with pUL69 in replication centers as well as in CDK inhibitor-induced speckled aggregates at late times of infection; (iv) cyclin T1 directly interacts with pUL69; (v) the HCMV-triggered nucleocytoplasmic translocation of CDK1 does not exclude a putative nuclear interaction with pUL69 within the first hours of infection; (vi) CDKs, prevalently CDK1 and -9, are novel pUL69-phosphorylating kinases \textit{in vitro} and \textit{in vivo}; and (vii) CDK activity is required for a high level of nuclear mRNA export activity of pUL69.

The pleiotropic viral regulator pUL69 contributes to the HCMV-triggered cell cycle arrest shortly after infection which may involve interaction with CDKs and/or cyclins. Yet, the molecular events are poorly understood so far (Hayashi et al., 2000; Lu and Shenk, 1999). Furthermore, pUL69 is capable of transactivating multiple promoters via interaction with the cellular transcription elongation factor hSPT6 (Lischka et al., 2007; Winkler et al., 2000) and possesses properties of a mRNA export factor: pUL69 binds RNA, shuttles between the nucleus and the cytoplasm and interacts with the cellular mRNA export factor UAP56/URH49 to facilitate the cytoplasmic accumulation of unspliced mRNA (Lischka et al., 2006; Toth et al., 2006; Lischka et al., 2001). Interestingly, a report by Sanchez and Spector (2006) showed the typical alteration in pUL69 localization when HCMV-infected cells were treated with CDK inhibitor roscovitine which was the basis for the detailed analysis of this phenomenon in this study. Findings with inhibitors of CDKs as well as inhibitors of the HCMV-encoded protein kinase pUL97 suggest that both CDK and pUL97 activities contribute to the regulation of pUL69 localization since suppression of the activity of CDKs or pUL97 induced a similar fine-speckled pattern of intranuclear pUL69 aggregates. This conclusion was compatible with reports describing pUL97 as a viral CDK ortholog possessing similar functional properties but being insensitive towards normal CDK control mechanisms (Hume et al., 2008). Thus, HCMV may have envolved a strategy to bypass cellular regulatory mechanisms and, at the same time, use functional properties of cellular proteins. Despite this strategy, pUL97 activity is not sufficient to fully substitute CDK activity. Rather a combination of both pUL97 and CDK activity appears to fulfill important regulatory steps for the functionality of pUL69 in the course of productive viral replication. Furthermore, it was reported that pUL97 affects the formation of complex structures termed aggresomes which represent large nuclear aggregates of proteins (Prichard et al., 2008). However, in contrast to morphologically large aggresomes, the inhibitor-induced nuclear pUL69 showed a microspeckled distribution and did not colocalize with HSC70, a marker for aggresome structures. In contrast, CDK inhibitor-induced pUL69 aggregates were detected within the area of viral replication centers, suggesting that only
pUL69 localized in these domains accumulates into speckles. The replication center-associated fraction of pUL69 may differ in its secondary modifications from pUL69 localized outside of the areas of replication. Thus, particularly pUL69 which has been recruited to replication centers may be able to interact with CDKs, while pUL69 distributed throughout the nucleus may remain mostly unaffected. The evolving concept of a regulatory impact of CDK activity on pUL69 localization was further supported by the finding that exclusively CDK9 along with cyclin T1 showed perfect colocalization with pUL69 in replication centers and aggregates at later times of infection, whereas CDK1, -2, and -7 behaved differently. Moreover, a physical interaction between pUL69 and cyclin T1 rather than pUL69 and CDK9 was determined by CoIP using lysates from transiently transfected cells, implying that the regulatory cyclin subunit may be the crucial component of the direct HCMV-CDK interregulation. Interestingly, immunofluorescence analysis indicate that pUL69 recruits the CDK9/cycT1 to the aggregates since in uninfected roscovitine-treated fibroblasts CDK9, cyclin T1 along with CDK7 were only slightly affected in their localization. Overall, these data partly define the composition of the CDK inhibitor-induced aggregates and additionally, may provide an explanation for the formation of these aggregates as well as for the reported increase in pUL69 protein levels after roscovitine treatment (Sanchez and Spector, 2006). Perhaps binding of roscovitine to the ATP pocket site of CDK9 confers a conformational change of the kinase which alters the binding dynamics between CDK9, cyclin T1 and pUL69. Thus, the proteins may remain associated in a tight complex resulting in the formation of the observed aggregates while preventing protein turnover of pUL69.

Importantly, this study demonstrated an extensive phosphorylation of pUL69 in infected fibroblasts and identified CDK/cyclin complexes to phosphorylate pUL69 in vitro. pUL69 acted as a specific substrate for CDK1, -2, -7, and -9, whereby CDK1 and CDK9 exhibited the highest pUL69-directed phosphorylation activity. This was somewhat surprising, since CDK1 and -9 are only moderately conserved (32% amino acid identity on the total protein sequence; 56% on kinase domain), while CDK1 and -2 are comparatively close-related with 65% and 84% homology on total protein sequence or the kinase domain, respectively (B-5). Moreover, alignments of the protein sequences of CDKs (Canduri et al., 2008), provide no distinct regions of high homology between CDK1 and -9 which could serve as a distinct substrate binding domain for pUL69. However, the CoIP experiments showed that cyclin T1 acts as the direct binding partner of pUL69, indicating that the protein sequence of the cyclin subunits needs to be taken into account. When comparing the sequences of the corresponding cyclins of CDK1 and -9, cyclin B1 and cyclin T1, the overall amino acid homology is only 6.6%. On the other hand, despite structural and functional resemblance, differences in interactions with peptides derived from the substrate or inhibitor proteins can occur, as shown for cyclin E and cyclin B1 (Lee et al., 2007). This suggests that homology does not necessarily result in
binding similar proteins, thus, in return, structural homology may not be a prerequisite for binding the same interactor. Furthermore, from emerging structural data it becomes apparent that many protein kinases also utilize docking interactions in grooves outside the active site to recognize substrates (Goldsmith et al., 2007). Therefore, it is conceivable, that homologous regions outside as well as within the kinase domain, even between minor-related CDKs such as CDK1 and -9 (Canduri et al., 2008), could serve as additional docking and/or substrate binding domains for the same substrate. Even distantly related protein kinases are able to phosphorylate identical proteins as demonstrated for the viral CDK ortholog pUL97 which only shows a low level of sequence homology with CDKs (5.4% on total protein sequence of CDK2; 11.7% on kinase domain; Romaker et al., 2006), but nevertheless was identified as a further pUL69-phosphorylating kinase (Thomas et al., 2009). Together, this points to a combined impact of several CDKs along with pUL97 on the phosphorylation status of pUL69.

The functional integration of the CDKs in the regulation of HCMV replication may be an ordered sequence of events with pUL69-directed activity of CDK1 as an early event. Consistent with this, both CDK1 and pUL69 were detected in the nucleus prior to the nucleo-cytoplasmic translocation of CDK1. Although not colocalizing with pUL69, CDK1 may contribute to a regulatory phosphorylation of pUL69 within the first hours of infection. This appears relevant since only the hypophosphorylated form of pUL69 is incorporated into the tegument of viral particles (Winkler and Stamminger, 1996). Hence, this tegument-coupled pUL69 may require an early phosphorylation step in order to effect HCMV-induced cell cycle arrest and further activities. CDK9, on the other hand, may be required at late stages of infection, as shown by the colocalization of CDK9, cyclin T1 and pUL69 in late-phase replication centers. It is tempting to speculate that in HCMV-infected cells, a complex constituted by pUL69, cyclin T1 and CDK9 forms and thereby enables a pUL69-directed phosphorylation through CDK9. This phosphorylation may subsequently contribute to the regulation of pUL69 activity (Fig. 23). It is known, that especially the N-terminus of pUL69 carries several overlapping important regulatory domains such as the RNA binding site, the motifs for interaction with the cellular mRNA export factor UAP56/URH49 or pUL97 and the nuclear localization signal (Thomas et al., 2009; Toth and Stamminger, 2008; Lischka et al., 2007; Lischka et al., 2006; Toth et al., 2006). Along with an in silico analysis predicting multiple putative phosphorylation sites within pUL69 (Thomas et al., 2009), a phosphorylation-dependent fine-regulation of pUL69 may enable a discrimination between the distinct functional properties in the course of viral replication. As demonstrated with an established reporter assay for the pUL69-mediated export of an unspliced, intron-containing mRNA (Lischka et al., 2006), activity was significantly reduced in the presence of CDK inhibitors. Interestingly, findings of Marco Thomas and further colleagues from the research group indicate an enhancement of pUL69 mRNA export activity by pUL97 coexpression in this
reporter system (Thomas et al., 2009). To summarize the findings of this study along with cooperation studies, an interregulation model is presented (Fig. 23). The data strongly argue that both CDK9/cycT1 and pUL97 dynamically interact with pUL69 and thereby differentially phosphorylate pUL69 as required for its intranuclear localization, its nucleo-cytoplasmic transport of unspliced mRNA and possibly for further pUL69-regulated processes.

The investigations of the functional interplay between CDK/cyclin complexes and pUL97 provide novel insights into the recently discovered cross-talk between cellular CDK and protein kinases of herpesviruses. For HSV-1, a sequentially ordered interregulation between cellular CDK1 along with cellular phosphatase cdc25C and HSV-1-encoded protein kinases UL13 and US3 has been reported (Smith-Donald and Roizman, 2008; Smith-Donald et al., 2008; Advani et al., 2001). In uninfected cells, cdc25C typically activates CDK1 by dephosphorylation, whereas in infected cells, this activation is reduced, despite an activation of cdc25C by the viral kinases UL13 and US3. While it seems as if cdc25C is diverted to perform functions other than those performed in uninfected cells, CDK1 acquires a new binding partner, the HSV1-encoded DNA polymerase processivity factor UL42. This complex recruits and phosphorylates topoisomerase II alpha resulting in a stimulated expression of viral late genes.

In conclusion, the data imply that a combined as well as timely ordered CDK-mediated phosphorylation acts as an important determinant for the functionality of the cytomegaloviral proteins pUL69 and IE2p86 in the course of HCMV infection. Particularly for pUL69, this study postulates a phosphorylation-dependent fine-regulation through CDK/cyclin complexes. Along with the viral CDK ortholog pUL97, CDKs exert a major impact on the intranuclear localization and activity of pUL69. Future investigation of the molecular events in CDK-pUL69 interaction may help to learn more about this specific contact point of coregulation between HCMV and the host cell.

Fig. 23: Postulated model of the interregulation between the cellular CDK9/cyclin T1 complex as well as the viral CDK ortholog and HCMV-encoded pUL69. Timely ordered and synergistically acting phosphorylation of pUL69 by CDK9/cycT1 and pUL97 may influence the intranuclear localization, the mRNA export activity and possibly further functions of pUL69.
Material and Methods

F-1 Biological materials

F-1.1 Bacteria

*Escherichia coli DH10B*: F° araD139 Δ(ara, leu) 7649 ΔlacX74 galU galK rpsL deOR Φ80 lacM15 endA1 nupG recA1 mcrA Δ(mrr hsdRMS mcrBC) (Grant et al., 1990).

F-1.2 Yeast

*Saccharomyces cerevisiae Y153*: MATa leu2-3, 112 ura3-52 trp1-901 his3-Δ200 ade2-101 lys2-801 gal4-542 gal80-538 URA3::GAL-lacZ LYS2::GAL-HIS3 can1’ (Durfee et al., 1993).

F-1.3 Eukaryotic cell cultures

HFF: primary human foreskin fibroblasts.

HEK293T: human embryonic kidney epithelial cell line transformed by adenovirus type 5 (Ad5) that contains a genomic integrate encoding the simian virus 40 (SV40) large tumor antigen (Pear et al., 1993).


F-1.4 Virus strains

HCMV AD169: laboratory strain of the human cytomegalovirus originally isolated by Rowe and colleagues (Rowe et al., 1956).

HCMV AD169-GFP: AD169-derived recombinant virus additionally containing an expression cassette for the green fluorescent protein (GFP) within nonessential stretches of the US region (Marschall et al., 2000).

BAC213 (HCMV AD169delUL97-GFP): HCMV AD169-GFP mutant with a deletion of the UL97 gene (Marschall et al., 2005).

HCMV GDGrXbaF4: GCV-resistant HCMV mutant, derived from AD169. This variant shows a deletion mutation in the UL97 gene, leading to a loss of amino acids 590-593 (Sullivan et al., 1993).

HCMV GDGrP53: GCV- and CDV-resistant HCMV mutant, derived from AD169. This variant contains a point mutation in the UL54 gene, which results in the amino acid exchange A987G (Sullivan et al., 1992).

HCMV 759rD100: GCV- and CDV-resistant HCMV mutant, derived from AD169. This double-mutant exhibits the amino acid (aa) deletion mutation in the UL97 gene (Δ aa 590-593) and the amino acid exchange in the UL54 gene (A987G; Biron et al., 1986).
F-1.5 Antibodies

F-1.5.1 Monoclonal antibodies

**MAb-CDK1 (sc-54):** mouse monoclonal antibody for the detection of CDK1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

**MAb-CDK7 (sc-56284):** mouse monoclonal antibody for the detection of CDK7 (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

**MAb-CDK9 (sc-13130):** mouse monoclonal antibody for the detection of CDK9 (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

**MAb-HSC70 (sc-7298):** mouse monoclonal antibody for the detection of the heat shock cognate protein 70 (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

**MAb-SC-35:** mouse monoclonal antibody for the detection of SC-35 (Fu and Maniatis, 1990).

**MAb-414:** mouse monoclonal antibody for the detection of nuclear pore complex proteins NUP62/152/90 (Hiss Diagnostics, Freiburg, Germany).

**MAb-UL69 (69-66):** mouse monoclonal antibody for the detection of pUL69 (Winkler et al., 1994).

**MAb-UL44 (BS 510):** mouse monoclonal antibody for the detection of pUL44 (kindly provided by B. Plachter, Mainz, Germany).

**MAb-FLAG (M2):** mouse monoclonal antibody directed against the FLAG epitope (Sigma-Aldrich, Deisenhofen, Germany).

**MAb-HA (12CA5):** mouse monoclonal antibody directed against the HA epitope (Roche, Mannheim, Germany).

**MAb-Myc (1-9E10.2; CRL-1729):** mouse monoclonal antibody directed against the Myc epitope (ATCC, LGC Standards GmbH, Wesel, Germany).

**MAb-β-actin (AC-15):** mouse monoclonal antibody for the detection of β-actin (Sigma-Aldrich, Deisenhofen, Germany).

F-1.5.2 Polyclonal antibodies

**PAb-CDK1 (sc-954):** rabbit polyclonal antibody for the detection of CDK1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

**PAb-CDK2 (sc-6248):** rabbit polyclonal antibody for the detection of CDK2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

**PAb-CDK7 (sc-56284):** rabbit polyclonal antibody for the detection of CDK7 (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

**PAb-CDK9 (sc-484):** rabbit polyclonal antibody for the detection of CDK9 (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

**PAb-cycB1 (sc-752):** rabbit polyclonal antibody for the detection of cyclin B1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA).
Material and Methods

Biotechnology, Santa Cruz, CA, USA).

**PAb-cyclT1 (sc-10750):** rabbit polyclonal antibody for the detection of cyclin T1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

**PAb-UL69:** rabbit polyclonal antibody for the detection of pUL69 (Winkler et al., 1994).

**PAb-UL97:** rabbit polyclonal antibody for the detection of pUL97 (kindly provided by D. Michels, Ulm, Germany).

**PAb-UL54:** human polyclonal antibody for the detection of pUL54; recovered from human antisera (Zielke, diploma thesis, 2008).

**PAb-IE2p86 (anti pHM178):** rabbit polyclonal antibody for the detection of IE2p86 (Hofmann et al., 2000).

### F-1.5.3 Secondary antibodies

All secondary antibodies coupled to horseradish peroxidase (HRP) were purchased from Dianova (Hamburg, Germany).

- HRP-coupled goat anti-mouse IgG
- HRP-coupled goat anti-rabbit IgG

All secondary antibodies coupled with fluorescent dyes were purchased from Molecular Probes (Invitrogen, Karlsruhe, Germany).

- Alexa 488- or Alexa 555-conjugated goat anti-mouse IgG
- Alexa 488- or Alexa 555-conjugated goat anti-rabbit IgG

### F-2 Nucleic acids

#### F-2.1 Oligonucleotides

All nucleotides were purchased from Biomers.net GmbH (Ulm, Germany). The sequences of oligonucleotides (annotated from 5’ to 3’) used for nucleotide sequencing, PCR cloning or the generation of short hairpin (sh) RNAs are listed below.

**Sequencing**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>T7</td>
<td>TTAATACGACTCACTATAGGG</td>
</tr>
<tr>
<td>Sp6</td>
<td>GGGGCAAACAAACAGATG</td>
</tr>
<tr>
<td>U6Forward</td>
<td>GGGCAGGAAGAGGGCCTAT</td>
</tr>
<tr>
<td>U6-tet-Forward</td>
<td>CTTGAACCTCCTCGTTCGACCCCGCCTC</td>
</tr>
<tr>
<td>CDK1-for-368</td>
<td>GAGTTCTTCAAGACAGAGAC</td>
</tr>
<tr>
<td>CDK1-rev-517</td>
<td>CTGGAGATCTGTACCAG</td>
</tr>
</tbody>
</table>
CDK2-for-419  CCATCAAGCTAGCAGAC
CDK2-rev-558  GATGTCCACAGCTGTGG
CDK7-for-500G AGCTTATACACATCAGG
CDK7-rev-664  GCTGATCAAGGTCTGAATC
CDK9-for-486  CCTGAAGCTGCGACAG
CDK9-rev-659  GCCATGATGCACCAGC
CycT-for-580  CCTGTGGTGCCCTGTCTGC
CycT-for-917  CCACAAGTGCAGTGCCTTCCC
CycT-for-1230 GGAAGCCAATGTGAAGTCAC
CycT-for-1581 GCACTTCATTCCAACTTCCC
CycT-for-1910 CGAAACTTAAAGGGCC
Gal119 TCTAACATTGAGACACAGCATAG
pGBT9-rev-1051 GCAACCTGACCTACAGG
Gal843 GCGTTTGGAATCACTACAGGG
pGAD424-3' TGGTGCACGATGCACAGTTGAAGTG

**PCR cloning**
The sequence corresponding to the gene of interest is underlined. Restriction enzyme cleavage sites are highlighted by bold and the sequence for the FLAG or Myc epitope are in italics.

CDK1-for
TACGGATCCATGAATTCATGGAAGATTATACCAAAAATAGAG
CDK1-rev
TACCTGCAGGTGACCTTTACTTGTGCTCATTCTTTTGTAGTCCATCTTCTTAATCTGATTGTC
CDK2-for
TACGGATCCATGAATTCATGGGAAACTTCCAAAGGTGG
CDK2-rev
TACCTGCAGGTGACCTTTACTTGTGCTCATTCTTTTGTAGTCCAGAAGATGGGGTACTGG
CDK7-for
TACGGATCCATGAATTCATGGGAAACTTCCAAAGGTGG
CDK7-rev
TACCTGCAGGTGACCTTTACTTGTGCTCATTCTTTTGTAGTCCAAAATTAGTTTCTTGGAATCC
CDK9-for
TACGGATCCATGAATTCATGGGAAACTTCCAAAGGTGG
CDK9-rev
TACCTGCAGGTGACCTTTACTTGTGCTCATTCTTTTGTAGTCAAGACGCCTCAAACCTCCG
5-cycT-BamHI
TACGGATCCATGGAGGGAGAGGAAGAAC

3-cycT-EcoRI
TACGAATTCTTAGGAAGGGGTGGAAGTGG

3-CycT-Myc-XbaI
TACTCTAGAAGATCTCTCTGAGATGATTTTTTTTTGATCCATCTTACGGAGGATGGAAGTGG

5-CDK1-BamHI-EcoRI
TACGGATCCGATGGAAGATTATACCAAAATAGAG

3-CDK1-Flag-SalI-PstI
TACCTGCACTGATGGAAGTAGATGGAAGTGG

5-CDK2-Sall-EcoRI
TACGTGACGAATTCATGGGAAACTTCAAAGGTGG

3-CDK2-Flag-PstI
TACCTGCACTGATGGAAGTAGATGGAAGTGG

5-CDK7-Sall-EcoRI
TACGTGACGAATTCATGGGAAACTTCAAAGGTGG

3-CDK7-Flag-PstI
TACCTGCACTGATGGAAGTAGATGGAAGTGG

5-CDK9-BamHI-EcoRI
TACGGATCCGATGGAAGATTATACCAAAATAGAG

3-CycA-Linker-BamHI
TACGGATCCCTGGCCTGGCCCCAGATTATAGTGCTCTCTGGG

5-CycH-BamHI-Smal
TACGGATCCATCCGGGATGTACCAACAGTTAGTCAG

3-CycH-Linker-SalI
TACGTGACCTGGCCTGGCCCCAGATTATAGTCAG

5-CycT-EcoRI-Smal
TACGGATCCCTGGCCTGGCCCCAGATTATAGTCAG

3-CycT-Linker-BamHI
TACGGATCCCTGGCCTGGCCCCAGATTATAGTCAG
Material and Methods

Oligonucleotides for shRNAs

Target sequences for RNA interference (RNAi) were selected using siRNA Designer software (BD Biosciences, Heidelberg, Germany; Dharmacon, Bonn, Germany). For the generation of the corresponding short hairpin (sh)RNA-expressing plasmids, oligonucleotides were designed with the siRNA Hairpin Oligonucleotide Sequence Designer Tool (BD Biosciences) and contained the following features: restriction enzyme cleavage sites \textit{Bam}HI and \textit{Eco}RI (broken underline) for cloning and \textit{Mlu}I or \textit{Nhe}I (bold) for control of successful insertion; the complementary siRNA sequence (underlined); the sequence for the formation of a loop (in between the siRNA-representing sequence); an RNA polymerase III termination sequence (TTTTTT or AAAAAA).

\begin{verbatim}
siLuci-5
GATCCGTCGGTGGCTAGTACCAACTTCAAGAGAAGTTGGTACTAGCAACGACCTTTTTTACGCGTG

siLuci-3
AAATTCGCTAGCAAAAAGTGGCGGGCTAGTACCAACTTCAAGAGAAGTTGGTACTAGCAACGACGCG

siCDK1-5
GATCCGTTACTTCATCGGTACATTCAAGAGAGATGACCAGAGTTACTACCTTTTTACGCGTG

siCDK1-3
AAATTCGCGGTAAAAAGTAAGACCTTCAAGAGAGATGACCAGAGTTACTACCTTTTTACGCGTG

siCDK2-5
GATCCGTATTTCTATGCCTGATTATTTCAAGAGAGAATCAGGCATAGAAGTTTTTTACGCGTG

siCDK2-3
AAATTCGCGGTAAAAATTACTTTACGTCTAGTTATTTAATCAGGCATAGAAGTTTTTTACGCGTG

siCDK7-5
GATCCGTAGTACCTTCAAGAGATGAGCTCATCTTTTTACGCGTG

siCDK7-3
AAATTCGCGGTAAAAATGARGACTTTCAAGAGATGAGCTCATCTTTTTACGCGTG

siCDK9-5
GATCCGTATATACCTGGTGTTCAGATCGAACACCAGGTATATACCTTTTTACGCGTG

siCDK9-3
AAATTCGCGGTAAAAAGTATACTCCTGGTGTTGCGATCTTTGAATCAGGACACCAGGTATATACCG
\end{verbatim}

F-2.2 Vectors and expression plasmids

F-2.2.1 Eukaryotic cloning vectors

pHM2237 (pSIREN RetroQ; RetroQ-siNon): self-inactivating retroviral vector containing restriction enzyme cleavage sites \textit{Bam}HI and \textit{Eco}RI for cloning of shRNA-encoding ds


Material and Methods

oligonucleotides. The expression of the shRNAs is under control of the RNA polymerase III-dependent human U6 promoter (BD Biosciences Clontech, Heidelberg, Germany). This vector was employed as a cloning vector for shRNA-expression plasmids, which were used for transient expression experiments and for the generation of viral genomic transcripts (retroviral gene transfer) after transfection into an appropriate packaging cell line.

**pHM2244 (pSIREN IRES-EGFP-RetroQ; RetroQ-GFP-siNon)**: pSIREN RetroQ-derived vector that additionally encodes for green fluorescent protein (GFP). The expression of GFP is mediated by an internal ribosomal entry site (IRES) so that the levels of expression between GFP and shRNAs correlate to each other (kindly provided by Effi Wies and PD Dr. Frank Neipel, Institute for Clinical and Molecular Virology, Erlangen, Germany).

**pHM1580 (pcDNA3-Myc)**: eukaryotic expression plasmid encoding the Myc epitope (Hofmann et al., 2000).

**pGBT9**: yeast vector containing the GAL4 DNA binding domain (BD) under the control of the ADH promoter and the tryptophan (TRP) 1 gene for selection in yeast (BD Biosciences Clontech, Mountain View, CA, USA).

**pGAD424**: yeast vector containing the GAL4 activation domain (AD) under the control of the ADH promoter and the leucin (LEU) gene for selection in yeast (BD Biosciences Clontech, Mountain View, CA, USA).

**F-2.2.2 Ready-to-use plasmids**

**Mammalian expression plasmids**

**pVSV-G**: eukaryotic expression plasmid encoding the vesicular-stomatitis-virus (VSV) glycoprotein G (Invitrogen, Karlsruhe, Germany).

**pHIT60**: eukaryotic expression plasmid coding for the gag and pol proteins of the murine leukemia virus (MLV) under the control of the HCMV promoter (kindly provided by Prof. K. Überla, Bochum, Germany).

**pHM134 (pBS-IE2p86)**: eukaryotic expression plasmid encoding IE2p86 (Lang et al., 1995).

**pHM160 (pCB6-pUL69)**: eukaryotic expression plasmid encoding pUL69 (Winkler et al., 1994).

**pHM1808 (pcDNA-pSC-FLAG-pUL26\textsubscript{1-223})**: eukaryotic expression plasmid encoding amino acids 1-223 of pUL26, N-terminally fused to the FLAG epitope (Lorz et al., 2006; Marschall et al., 2003).

**pHM2098 (pcDNA3-FLAG-pUL69)**: eukaryotic expression plasmid encoding pUL69, N-terminally fused to the FLAG epitope (Lischka et al., 2006).

**pHM2235 (Myc-pUL69)**: eukaryotic expression plasmid encoding pUL69, N-terminally fused to the Myc epitope (Lischka et al., 2007).

**pHM2239 (RetroQ-siLuc)**: retroviral expression plasmid encoding shRNA complementary to
luciferase, which was used as a specificity control in comparison to the expression of shRNAs specific for individual CDKs (Rechter, diploma thesis, 2004).

**pHM2246 (RetroQ-GFP-siLuci):** retroviral expression plasmid encoding shRNA complementary to luciferase and GFP, which was used as a specificity control in comparison to shRNAs specific for individual CDKs (Rechter, diploma thesis, 2004).

**pCMV-CDK1-HA:** eukaryotic expression plasmid encoding CDK1, C-terminally fused to the HA epitope (van den Heuvel and Harlow, 1993). It was obtained via Addgene Inc. (Cambridge, MA, USA; Addgene plasmid 1888).

**pCMV-CDK2-HA:** eukaryotic expression plasmid encoding CDK2, C-terminally fused to the HA epitope (van den Heuvel and Harlow, 1993). It was obtained via Addgene Inc. (Cambridge, MA, USA; Addgene plasmid 1884).

**SRα-CDK7-HA:** eukaryotic expression plasmid encoding CDK7, C-terminally fused to the HA epitope (Fisher and Morgan, 1994).

**pRc/CMV-PITALRE-HA:** eukaryotic expression plasmid encoding CDK2, C-terminally fused to the HA epitope (Garriga et al., 1996).

**pPM7tet_CyclinA:** eukaryotic expression plasmid encoding cyclin A (kindly provided by GPC Biotech AG, Martinsried, Germany).

**pcDNA-UL44-FLAG:** eukaryotic expression plasmid encoding pUL44, N-terminally fused to the FLAG epitope (Marschall et al., 2003).

**phCMV/Xi-UL54-FLAG:** eukaryotic expression plasmid encoding pUL54 fused to the FLAG epitope (kindly provided by Prof. G. Pari, Reno, Nevada, USA).

**pDsRed1-N1:** eukaryotic expression plasmid encoding the red fluorescent protein (RFP) under the control of the HCMV MIEP (BD Clontech, Heidelberg, Germany).

**pDM128/CMV/RRE:** eukaryotic expression plasmid containing the intronic CAT reporter gene along with the HIF-1 Rev-responsive element (Cullen, 2004).

**Yeast expression plasmids**

**pTD1:** yeast expression plasmid encoding the SV40 large T antigen fused to the the GAL4 activation domain (AD) in pGAD3F with the LEU2 gene for selection (Chien et al., 1991).

**pVA3:** yeast expression plasmid encoding the murine p53 protein fused to the the GAL4 DNA-binding domain (BD) in pGBT9 with the TRP1 gene for selection (Iwabuchi et al., 1993).

**pACTII-cycT1:** yeast expression plasmid encoding cyclin T1 fused to the GAL4 AD (Fraldi et al., 2001).

**pGBK7-CycH3-323:** yeast expression plasmid encoding amino acid 3-323 of cyclin H, fused to the GAL4 BD (kindly provided by Prof. M. Montenarh, Homburg, Germany).

**pACT-pp65:** yeast expression plasmid encoding pp65 fused to the GAL4 AD (kindly provided by Prof. B. Plachter, Mainz, Germany; Marschall et al., 2003).
**pACT-pp150:** yeast expression plasmid encoding pp150 fused to the GAL4 AD (kindly provided by Prof. B. Plachter, Mainz, Germany; Marschall et al., 2003).

**pHM300 (pGBT9-UL69):** yeast expression plasmid encoding pUL69 fused to the GAL4 BD (Winkler et al., 2000).

**pHM301 (pGAD424-UL69):** yeast expression plasmid encoding pUL69 fused to the GAL4 AD (Winkler et al., 2000).

**pHM342 (pGAD424-UL57):** yeast expression plasmid encoding pUL57 fused to the GAL4 AD (kindly provided by Prof. T. Stamminger, Erlangen, Germany; Marschall et al., 2003).

**pHM344 (pGBT9-UL57):** yeast expression plasmid encoding pUL57 fused to the GAL4 BD (kindly provided by Prof. T. Stamminger, Erlangen, Germany; Marschall et al., 2003).

**pHM379 (pGAD424-pUL84):** yeast expression plasmid encoding pUL84 fused to the GAL4 AD (Lischka et al., 2003).

**pHM479 (pGBT9-UL84):** yeast expression plasmid encoding pUL84 fused to the GAL4 BD (Lischka et al., 2003).

**pHM704 (pACT-IE2p86\textsuperscript{145-579}):** yeast expression plasmid encoding amino acids 145-579 of IE2p86 fused to the GAL4 AD (Hofmann et al., 2000).

**pHM720 (pGBT9-IE2p86):** yeast expression plasmid encoding IE2p86 fused to the GAL4 BD (Hofmann et al., 2000).

**pHM870 (pAS-UL97):** yeast expression plasmid encoding pUL97 fused to the GAL4 BD (Marschall et al., 2005; Marschall et al., 2003).

**pHM892 (pACT-UL97):** yeast expression plasmid encoding pUL97 fused to the GAL4 AD (Marschall et al., 2005; Marschall et al., 2003).

**pHM1797 (pGBT9-UL26):** yeast expression plasmid encoding pUL26 fused to the GAL4 BD (Lorz et al., 2006; Marschall et al., 2003).

**pHM2593 (pGBT9-UL50\textsuperscript{1-358}):** yeast expression plasmid encoding amino acids 1-358 of pUL50 fused to the GAL4 BD (Milbradt et al., 2007).

**pHM2595 (pGBT9-UL53):** yeast expression plasmid encoding pUL53 fused to the GAL4 BD (Milbradt et al., 2007).

**pHM2597 (pGAD424-UL50\textsuperscript{1-358}):** yeast expression plasmid encoding amino acids 1-358 of pUL50 fused to the GAL4 AD (Milbradt et al., 2007).

**pHM2599 (pGAD424-UL53):** yeast expression plasmid encoding pUL53 fused to the GAL4 AD (Milbradt et al., 2007).

### F-2.2.3 Newly generated plasmids

#### Mammalian expression plasmids

The following RetroQ-siNon-derived plasmids were generated for the RNAi-mediated knockdown of CDKs via the expression of short hairpin RNAs (shRNAs) as indicated. These
plasmids were used for the transient or stable knockdown of the individual CDK targets. The listed oligonucleotides were annealed and introduced into the vector pHM2244 (pHM2414 to pHM2419) or pHM2239 (pHM2985 to pHM2988) using restriction sites BamHI and EcoRI.

<table>
<thead>
<tr>
<th>pHM number</th>
<th>designation</th>
<th>oligonucleotides</th>
<th>knockdown</th>
<th>GFP expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>pHM2414</td>
<td>RetroQ-GFP-siCDK1</td>
<td>siCDK1-5, siCDK1-3</td>
<td>CDK1</td>
<td>+</td>
</tr>
<tr>
<td>pHM2416</td>
<td>RetroQ-GFP-siCDK2</td>
<td>siCDK2-5, siCDK2-3</td>
<td>CDK2</td>
<td>+</td>
</tr>
<tr>
<td>pHM2417</td>
<td>RetroQ-GFP-siCDK7</td>
<td>siCDK7-5, siCDK7-3</td>
<td>CDK7</td>
<td>+</td>
</tr>
<tr>
<td>pHM2419</td>
<td>RetroQ-GFP-siCDK9</td>
<td>siCDK9-5, siCDK9-3</td>
<td>CDK9</td>
<td>+</td>
</tr>
<tr>
<td>pHM2985</td>
<td>RetroQ-siCDK1</td>
<td>siCDK1-5, siCDK1-3</td>
<td>CDK1</td>
<td>-</td>
</tr>
<tr>
<td>pHM2986</td>
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<td>siCDK2-5, siCDK2-3</td>
<td>CDK2</td>
<td>-</td>
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<td>pHM2987</td>
<td>RetroQ-siCDK7</td>
<td>siCDK7-5, siCDK7-3</td>
<td>CDK7</td>
<td>-</td>
</tr>
<tr>
<td>pHM2988</td>
<td>RetroQ-siCDK9</td>
<td>siCDK9-5, siCDK9-3</td>
<td>CDK9</td>
<td>-</td>
</tr>
</tbody>
</table>

**pHM3000 (pcDNA3.1-cycT1):** eukaryotic expression plasmid encoding cyclin T1. Following PCR amplification using oligonucleotides 5-cycT-BamHI and 3-cycT-EcoRI along with pACTII-cycT1 as template the PCR product was inserted into pcDNA3.1 via BamHI and EcoRI.

**pHM3001 (pcDNA3-Myc-cycT1):** eukaryotic expression plasmid coding for cyclin T1 in fusion with the Myc epitope at the N-terminus. The plasmid was constructed as described for pHM3000 using pHM1580 as vector.

**Yeast expression plasmids**
The following yeast expression plasmids code for different CDKs fused to the N-terminus of either the GAL4 DNA binding domain (BD) or the GAL4 activation domain (AD) as indicated. All constructs also express the FLAG epitope, fused C-terminally to the CDKs. The CDK-FLAG fragments were generated by PCR amplification of the CDK coding sequences using oligonucleotides and templates as indicated. Then, the fragments were inserted into pGBT9 or pGAD424 via EcoRI and SalI.

<table>
<thead>
<tr>
<th>pHM number</th>
<th>designation</th>
<th>oligonucleotides</th>
<th>template</th>
<th>BD/AD</th>
</tr>
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<tr>
<td>pHM2563</td>
<td>pGBT9-CDK1-FLAG</td>
<td>CDK1-for, CDK1-rev</td>
<td>pCMV-CDK1-HA</td>
<td>BD</td>
</tr>
<tr>
<td>pHM2564</td>
<td>pGBT9-CDK2-FLAG</td>
<td>CDK2-for, CDK2-rev</td>
<td>pCMV-CDK2-HA</td>
<td>BD</td>
</tr>
<tr>
<td>pHM2565</td>
<td>pGBT9-CDK7-FLAG</td>
<td>CDK7-for, CDK7-rev</td>
<td>SRα-CDK7-HA</td>
<td>BD</td>
</tr>
<tr>
<td>pHM2566</td>
<td>pGBT9-CDK9-FLAG</td>
<td>CDK9-for, CDK9-rev</td>
<td>pRc/CMV-PITALRE-HA</td>
<td>BD</td>
</tr>
<tr>
<td>Plasmid Code</td>
<td>Expression Plasmid</td>
<td>Protein Fusion</td>
<td>Vector</td>
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<td>pHM2567</td>
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<td>pCMV-CDK1-HA</td>
<td>AD</td>
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<tr>
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<td>SRα-CDK7-HA</td>
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</tr>
<tr>
<td>pHM2570</td>
<td>pGAD424-CDK9-FLAG</td>
<td>CDK9-for, CDK9-rev</td>
<td>pRc/CMV-PITALRE-HA</td>
<td>AD</td>
</tr>
</tbody>
</table>

**pHM2995 (pGAD424-cycA-CDK1-FLAG):** yeast expression plasmid coding for the cyclinA-CDK1 fusion protein in fusion to the GAL4 AD at the N-terminus and with the FLAG epitope at the C-terminus. First, the coding sequences of cyclin A and CDK1 were independently amplified with pCMV-CDK1-HA and pPM7tet_CyclinA as templates and using oligonucleotides as follows: cyclin A, 5-CycA-EcoRI and 3-CycA-Linker-BamHI; CDK1, 5-CDK1-BamHI-EcoRI and 3-CDK1-Flag-Sall-PstI. Both fragments were subjected to restriction enzymes digestions (cyclin A, EcoRI and BamHI; CDK1, BamHI and Sall) and were then simultaneously ligated into pGAD424, which had been linearized with EcoRI and Sall.

**pHM2996 (pGAD424-cycT1-CDK9-FLAG):** yeast expression plasmid coding for the cyclinT1-CDK9 fusion protein in fusion to the GAL4 AD at the N-terminus and with the FLAG epitope at the C-terminus. The plasmid was generated as described for pHM2995 using pACTII-cycT1 and pRc/CMV-PITALRE-HA as templates. Oligonucleotides and restriction enzymes were used as follows: cyclin T1, 5-CycT-EcoRI-Smal and 3-CycT-Linker-BamHI, EcoRI and BamHI; CDK9, 5-CDK9-BamHI-EcoRI and 3-CDK9-Flag-Sall-PstI, BamHI and Sall. pGAD424 was linearized with EcoRI and Sall.

**pHM2997 (pGBT9-cycA-CDK1-FLAG):** yeast expression plasmid coding for the cyclinA-CDK1 fusion protein in fusion to the GAL4 BD at the N-terminus and with the FLAG epitope at the C-terminus. The plasmid was generated as described for pHM2995 using the same templates, oligonucleotides and restriction enzymes but vector pGBT9.

**pHM2998 (pGBT9-cycH-CDK7-FLAG):** yeast expression plasmid coding for the cyclinH-CDK7 fusion protein in fusion to the GAL4 BD at the N-terminus and with the FLAG epitope at the C-terminus. The plasmid was generated as described for pHM2995 using pGBK7-CycH323 and SRα-CDK7-HA as templates. Oligonucleotides and restriction enzymes were used as follows: cyclin H, 5-CycH-BamHI-Smal and 3-CycH-Linker-Sall, BamHI and Sall; CDK7, 5-CDK7-Sall-EcoRI and 3-CDK7-Flag-PstI, Sall and PstI. pGAD424 was linearized with BamHI and PstI.

**pHM2999 (pGBT9-cycT1-CDK9-FLAG):** yeast expression plasmid coding for the cyclinT1-CDK9 fusion protein in fusion to the GAL4 BD at the N-terminus and with the FLAG epitope at the C-terminus. The plasmid was generated as described for pHM2996 using the same templates, oligonucleotides and restriction enzymes but vector pGBT9.
F-3 Enzymes, media and buffers

F-3.1 Enzymes
Restriction enzymes were purchased from Roche (Mannheim, Germany), Gibco/BRL (Eggenstein, Germany), New England Biolabs (Schwalbach, Germany) or Pharmacia (Freiburg, Germany) and used with provided buffers according to the manufacturer’s protocol. T4 DNA ligase (Gibco/BRL, Eggenstein, Germany), and Vent-Polymerase (New England Biolabs, Schwalbach, Germany) were utilized with buffers recommended by the manufacturers.

F-3.2 Media

F-3.2.1 Bacterial media
LB medium (Luria-Bertani medium): 10 g of bactotryptone, 5 g of bacto yeast, 8 g of NaCl and 1 g of glucose were dissolved in 1 liter of H2O and adjusted to pH 7.2 using NaOH followed by autoclaving. Optionally, ampicillin (100 µg/ml) was added to the media.

LB agar (Luria-Bertani agar): 15 g of agar were dissolved in 1 liter of LB medium followed by autoclaving. After cooling of the solution to about 55°C, 1 ml of ampicillin (50 mg/ml), was added.

SOC medium: 20 g of bactotryptone, 5 g of bacto yeast, 2.5 mM NaCl, 10 mM MgCl2, 10 mM MgSO4 and 20 mM glucose were dissolved in 1 liter of H2O followed by filter sterilization.

F-3.2.2 Yeast media
10x Yeast Nitrogen Base (YNB): 33.5 g YNB lacking amino acids and ammonium sulfate were dissolved in 500 ml H2O followed by filter sterilization.

20x amino acid mix: 0.9 g adenine, 0.43g arginine, 2.16 g aspartic acid, 2.16 g glutamic acid, 0.65 g isoleucine, 0.65 g lysine, 0.43 g methionine, 1.08 g phenylalanine, 7.92 g serine, 4.32 g threonine, 0.65 g tyrosine and 3.24 g valine were dissolved in 900 ml H2O followed by filter sterilization.

100x uracil: 0.24 g uracil were dissolved in 100 ml H2O followed by filter sterilization.

100x histidine: 0.48 g histidine were dissolved in 100 ml H2O followed by filter sterilization.

100x leucine: 1.44 g leucine were dissolved in 100 ml H2O followed by filter sterilization.

100x tryptophan: 0.96 g tryptophan were dissolved in 100 ml H2O followed by filter sterilization.

YAPD (full medium): 20 g bacto yeast and 40 g bacto peptone were dissolved in 900 ml H2O and autoclaved followed by addition of 100 ml 20% glucose.

Minimal medium: 50 ml 10x YNB, 50 ml 20% glucose, 25 ml 20x amino acid mix, 5 ml 100x
uracil and according to requirements 2.5 ml 200x histidine, 2.5 ml 200x tryptophan and 2.5 ml 200x leucine were dissolved in 500 ml H₂O.

**Minimal agar:** 10 g bacto agar were dissolved in 500 ml H₂O and autoclaved. Following cooling to about 55°C supplements used for minimal medium were added according to requirements.

**F-3.2.3 Cell culture media**

**MEM** (Eagle’s minimal essential medium) was obtained from Gibco/BRL (Eggenstein, Germany), dissolved in sterile H₂O and adjusted to pH 7.

**DMEM** (Dulbecco’s modified Eagle medium) was obtained from Gibco/BRL (Eggenstein, Germany), dissolved in sterile H₂O and adjusted to pH 7.

**FBS** (fetal bovine serum) was obtained from Cambrex (Verviers, Belgium).

**Trypsin/EDTA:** 0.25% trypsin, 140 mM NaCl, 5 mM KCl, 0.56 mM Na₂HPO₄, 5 mM D(+) glucose, 25 mM Tris/HC1, 0.01% EDTA, pH 7.0.

**F-3.3 Standard buffers and solutions**

**PBS0** (Phosphate-buffered saline without CaCl₂ and MgCl₂): 138 mM NaCl, 2.7 mM KCl, 6.5 mM Na₂HPO₄, 1.5 mM KH₂PO₄.

**6x DNA loading buffer:** 30% glycerol, 0.25% bromphenol blue, 0.25% xylene cyanol.

**1x TAE buffer:** 24.2 g Tris base, 1.7 g EDTA, 5.7 ml glacial acetic acid were dissolved H₂O adjusting the volume to 5 liters.

**1x TE buffer:** 10 mM Tris/HCl (pH 6.8), 1 mM EDTA.

**4x SDS sample buffer:** 125 mM Tris/HCl (pH 6.8), 2 mM EDTA, 20% glycerol, 4 %SDS, 10% β-mercaptoethanol, 0.01% bromphenol blue.

**10x SDS-PAGE buffer:** 286 g of glycine, 60.6 g of Tris base and 20 g of SDS were dissolved in H₂O adjusting the volume to 2 liters.

**Western blotting buffer:** 15.1 g of Tris base, 75 g of glycine and 1 liter of ethanol were dissolved in H₂O adjusting the volume to 5 liters.

**ECL solution A:** 50 mg luminol (Sigma-Aldrich, Deisenhofen, Germany) were dissolved in 200 ml 0.1 M Tris/HCl (pH 8.6).

**ECL solution B:** 11 mg p-hydroxycoumarin acid (Sigma-Aldrich, Deisenhofen, Germany) were dissolved in 10 ml DMSO.

**4% paraformaldehyde solution:** 4% paraformaldehyde were dissolved in 50 ml H₂O including some drops of a 1 N NaOH solution at 60°C and after cooling to Rt the solution was mixed with 50 ml 2x PBS0.

**0.2% Triton X-100:** 0.2 % Triton X-100 were dissolved in PBS0.

**1% crystal violet solution:** 1% crystal violet dissolved in 20% ethanol.
**RIPA lysis buffer**: 0.1% SDS, 1% Na-desoxycholate, 10 mM Tris/Cl pH 7.5, 150 mM NaCl were dissolved in sterile H$_2$O followed by filter sterilization. Shortly before usage 2 mM PMSF was added.

**Lysis buffer**: 25 mM Tris/HCl (pH 7.8), 2 mM dithiothreitol (DTT), 2 mM trans-1,2-diaminocyclohexane-N,N',N'-tetraacetic acid, 1% Triton X-100, 10% glycerol.

**HBS solution** (HEPES-buffered saline): 4.4 g NaCl and 2.4 g HEPES were dissolved in 500 ml H$_2$O and adjusted to pH 7.4 followed by filter sterilization.

**PEI2000**: 9 mg polyethylene imine MW 2000 (Sigma-Aldrich, Deisenhofen, Germany) were dissolved in 10 ml H$_2$O and adjusted to pH 7.0 followed by filter sterilization.

**PEI25000**: 9 mg polyethylene imine MW 25000 (Sigma-Aldrich, Deisenhofen, Germany) were dissolved in 10 ml H$_2$O and adjusted to pH 7.0 followed by filter sterilization.

**CoIP buffer**: 25 ml 1 M Tris/HCl pH 8.0, 15 ml 5 M NaCl, 5 ml 0.5 M EDTA and 25 ml 10% NP40 were dissolved in H$_2$O adjusting volume to 500 ml followed by filter sterilization. Shortly before usage 100 µl 100 mM PMSF, 20 µl 1 mg/ml aproptin, 20 µl 1 mg/ml leupeptin and 20 µl 1 mg/ml pepstatin were added per 10 ml CoIP stock solution.

**HNTG buffer**: 50 mM HEPES pH 7.5, 150 mM NaCl, 1 mM EDTA, 10% glycerine and 0.1% Triton X-100.

**Standard kinase assay buffer**: 125 mM HEPES-NaOH pH 7.5, 7.5 mM MgCl$_2$, 7.5 mM MnCl$_2$, 7.5 µM Na-orthovanadate and 2.5 mM DTT.

**Kinase dilution buffer**: 500 mM HEPES-NaOH pH 7.5, 2.5 mg/ml PEG20000, 10 mM DTT.

**CDK IVKA buffer**: 8 mM MOPS pH 7.0, 0.2 mM EDTA, 10 mM Mg-acetate. Before kinase reaction was started Mg-ATP mix was added (10 µM ATP).

**Buffer Z**: 16.1 g/l Na$_2$HPO$_4$·7xH$_2$O, 5.8 g/l NaH$_2$PO$_4$·H$_2$O, 0.75 g/l KCl and 0.246 g/l MgSO$_4$·7xH$_2$O were dissolved in H$_2$O and adjusted to pH 7.0 followed by autoclaving.

**Xgal**: 20 mg Xgal were dissolved in 20 ml dimethylformamide.

### F-4 CDK inhibitors and reference compounds

The CDK inhibitors used can be classified into two groups: commercially available compounds (roscovitine, purvalanol, olomoucine II and CDK2 inhibitor; purchased from Calbiochem, Germany) and recently designed, novel compounds belonging to the chemical class of aminopyrimidines (R10, R12, R17, R25, R37, R49, R58, R80, R89, R372, R972, provided by GPC Biotech AG, Martinsried, Germany). Additionally, inhibitors directed to other protein kinases were purchased from Sigma-Aldrich, Deisenhofen, Germany as follows: Gö6976 (inhibitor of pUL97 and protein kinase C, PKC), Gö7874 (inhibitor of PKC), Ax7396 (inhibitor of pUL97 and epidermal growth factor receptor, EGFR), AG490 (tyrphostin; active against Janus kinases). Ganciclovir (GCV) was used as reference compound (Sigma-Aldrich,
Deisenhofen, Germany). All compounds were prepared in DMSO or aqueous solution (GCV) and aliquots were stored at -20°C or -80°C.

F-5  Standard molecular biology techniques
- PCR (polymerase chain reaction) for amplification of DNA fragments according to Sambrook et al. (1989). If required, DMSO, formamide or MgSO4 were added for optimization.
- Restriction enzyme digestion of DNA, ligation with T4 DNA ligase and agarose gel electrophoresis (Sambrook et al., 1989).
- Elution of DNA fragments from agarose gels using PureLink™ Quick Gel Extraction Kit from Invitrogen (Karlsruhe, Germany).
- Transformation of plasmid DNA into bacteria by electropulsing (Sambrook et al., 1989).
- Mini-preparation of DNA plasmid via standard alkaline lysis procedure (Zagursky and Berman, 1984).
- Midi- or large-scale preparation of plasmid by PureLink™ HiPure Plasmid Midiprep Kit or PureLink™ HiPure Plasmid Maxiprep Kit obtained from Invitrogen (Karlsruhe, Germany).
- SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Laemmli, 1970).
- Enhanced chemiluminescence (ECL) immunodetection of proteins (Amersham, Braunschweig, Germany).
- Photometric determination of DNA concentrations (Sambrook and Russel, 2001).
- Automated nucleotide sequencing of DNA using fluorescence-based ABI-Prism 2000 sequencing detector (ABI, Weiterstadt, Germany).

F-6  Cell culture techniques

F-6.1  Maintenance of cell cultures
Eukaryotic cell cultures were maintained at 37°C, 5 % CO₂ and 80% humidity in culture media as follows:
HFF: MEM supplemented with 7.5% FBS, 350 µg/ml l-glutamine and 10 µg/ml gentamicin.
HeLa: MEM supplemented with 5% FBS, 350 µg/ml l-glutamine and 10 µg/ml gentamicin.
HEK293T: DMEM supplemented with 10% FBS, 350 µg/ml l-glutamine and 10 µg/ml gentamicin.
F-6.2 Transfection of cultured cells

F-6.2.1 TransPEI transfection of 293T cells

The day before transfection, 293T cells were seeded into wells of 6-well plates in 2 ml of medium \((5.5 \times 10^5 \text{ cells per well})\). Transfection was performed by using the polyethylenimine reagent (PEI; Sigma-Aldrich, Deisenhofen, Germany; Schregel et al., 2007). For this, 6 µg of total DNA were mixed with 250 µl HBS solution and incubated with 5 µl PEI2000 in 250 µl HBS solution for 20 min at Rt. Thereafter, the solution was mixed with 9 µl PEI25000 in 250 µl HBS solution and incubated for 20 min at Rt. Within this time period, cell monolayers were washed twice with DMEM supplemented with l-glutamine and cultured in 1 ml of the medium in the absence of antibiotics. Then, the DNA/PEI mixture was added dropwise to the cells. After incubation for 3.5 h at 37 °C, the transfection solution was replaced by fresh medium. At about 48 h post transfection, cells were harvested for Western blot analysis, coimmunoprecipitation, in vitro kinase assay or in vivo labeling.

F-6.3 Cytotoxicity assay

HFFs were seeded in 48-well plates \((4.2 \times 10^4 \text{ cells per well})\), grown to subconfluent layers and cultivated with the CDK inhibitors in various concentrations in culture media without phenol red. 7 days later, medium samples (150 µl each) were taken and assayed using the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega GmbH, Mannheim, Germany) to determine the lactate dehydrogenase (LDH) released from nonviable cells via colour substrate conversion. All settings were performed in duplicates. Cell debris was removed by centrifugation of the medium samples (3000 rpm, 15 min) before supernatants from each well was divided into two samples \((2 \times 50 \mu l)\). Each sample was incubated with 50 µl of the substrate mix for 30 min in the dark. Thereafter, 50 µl of stopping solution was added. The photometric determination was performed via ELISA Reader (OD 490 nm).

F-7 Virus infection

F-7.1 Virus stocks

For preparation of virus stocks, HFFs were infected with a chosen multiplicity of infection (MOI 0.1 to 1) and incubated at 37°C until the cultures showed a pronounced cytopathic effect (7 to 10 days). Supernatants were then collected, cleared from cell debris by centrifugation (2000 rpm, 10 min) and stored in aliquots at -80°C. Titer s of virus stocks were determined by standard plaque titration.
F-7.2 Plaque reduction assay
For plaque reduction assays, 2.25 x 10^5 HFFs were seeded into wells of 12-well plates. The next day, infection with AD169 and 759rD100 was performed at a MOI of 0.5 in 400 µl. After incubation of 1.5 h, virus inoculi were replaced by overlay medium (0.6% agarose and 2x MEM which was supplemented with 15% FBS, 700 µg/ml l-glutamine and 20 µg/ml gentamicin were mixed at a ratio of 1 : 2 v/v), supplemented with CDK inhibitors or reference compounds. Incubation was performed at 37°C, 5% CO_2 and 80% humidity until plaques formed (7 to 14 days). To determine the number of plaques, overlays were removed and the cells were stained with 1% crystal violet solution.

F-7.3 HCMV GFP-based replication assay
HFFs were cultivated in 12-well plates (2.25 x 10^5 cells per well) and used for infection with HCMV AD169-GFP, at a MOI of 0.25 (that is a 25% GFP-forming infectious dose at 7 days post infection). After incubation of 1.5 h, viral inoculi were replaced by fresh medium supplemented with CDK inhibitors or reference compounds. All infections were performed in duplicates. 7 days post infection, culture media were removed and cells were lysed by addition of 200 µl lysis buffer onto the cells and incubation for 10 min at 37°C and 30 min at Rt under constant movement. Afterwards, cell suspensions were mixed and transferred to a 96-well plate. The subsequent centrifugation was performed at 3000 rpm for 15 min. Supernatant from each well was divided into two samples (2 x 80 µl) and transferred to a black 96-well plate before plates were subjected to an automated GFP quantification in a Victor 1420 Multilabel Counter (PerkinElmer Wallac GmbH, Freiburg, Germany; Marschall et al., 2000).

F-8 Generation and characteriziation of retrovirally transduced cell subpopulations
F-8.1 Generation of retroviral transfer particles
293T cells served as the producer cell line for the generation of retroviral transfer particles and were seeded in 10 cm dishes (5 x 10^6 cells per dish). The following day and 4 h prior to transfection, cell medium was replaced by medium without antibiotics (DMEM, 10% FBS, 350 µg/ml l-glutamine). For the generation of murine leukemia virus-based retroviruses, 3 µg of the plasmids coding for shRNAs under control of the cellular RNA-polymerase III-dependent U6-promotor were mixed with 4.5 µg of each of the packaging plasmids pHIT60 and pVSV-G in 1.5 ml medium without FBS and antibiotics (DMEM, 350 µg/ml l-glutamine). pHIT60 carries genes for the gag/pol proteins and pVSV-G carries the gene for the envelope protein VSV-G. The incorporation of VSV-G envelope protein enables the generation of retroviruses with a
broad host spectrum as well as an enhanced stability (Burns et al., 1993). In parallel, 36 µl of lipofectamine 2000 reagent (Invitrogen, Karlsruhe, Germany) was diluted in 1.5 ml FBS- and antibiotics-free medium per sample and incubated for 5 min at Rt. Then, both solutions were mixed and following an incubation period of 20 min at Rt to allow the formation of DNA-lipfectamin complexes, the transfection samples were added dropwise to the cells. After incubation overnight at 37°C, medium was replaced by fresh medium (DMEM, 10% FBS, 350 µg/ml l-glutamine, 10 µg/ml gentamicin). The viral supernatant was harvested 48 h post transfection, cleared from cell debris by centrifugation (3000 rpm, 15 min) and by an additional filtration using a 0.45 µm filter (Millipore, Schwabach, Germany) and stored in aliquots of 1 ml at -80°C.

F-8.2 Retroviral transduction and selection of stably transduced cell subpopulations

For the retroviral transduction, low-passage HFFs were seeded at low density in 6-well plates (8 x 10^4 cells per well) in 2 ml medium. The following day, 1 ml of the medium was replaced by 1 ml of the various retroviral supernatants along with 3 µl of polybrene (5 mg/ml; Sigma-Aldrich, Deisenhofen, Germany). The transduction media were substituted 24 h later with 2 ml of DMEM supplemented with 10% FBS, 350 µg/ml l-glutamine and 10 µg/ml gentamicin. Finally, stably transduced HFF subpopulations (HFF-siRNA) were selected by culturing the cells in media containing 5 µg/ml puromycin (Sigma-Aldrich, Deisenhofen, Germany) starting from 48 h post transduction.

F-8.3 Flow cytometry analysis

Stably transduced HFF subpopulation showing a knockdown of the individual CDKs were analyzed for cell proliferation and viability by using the LIVE/DEAD Viability/Cytotoxicity kit for mammalian cells (Molecular Probes, Invitrogen, Karlsruhe, Germany) followed by flow cytometry analysis. The kit uses a two-color fluorescence to determine the presence of live and dead cells simultaneously. In living cells, the nonfluorescent, cell-penetrating calcein acetoxyethyl (AM) dye is internalized and converted by intracellular esterase to calcein, which then emits a green fluorescence. Ethidium homodimer-1 (EthD-1) uptake is restricted to cells with damaged membranes and undergoes a 40-fold enhancement of red fluorescence upon binding to nucleic acids. For the analysis, HFF-siRNA subpopulations (5 x 10^5 cells per sample or 10 cm dish) were fixed immediately (day 0) or were cultivated for two days before fixation (day 2). Hereby, cells were detached with Trypsin/EDTA, washed in 1 ml PBS and sedimented by centrifugation (5000 rpm, 3 min). Fixation was performed by incubation of cell pellets in 500 µl 4% paraformaldehyde. HFF-siRNAs from day 0 were kept in paraformaldehyde solution at 4°C until fixation of samples at day 2. Finally, all samples were washed (1 ml; 5000 rpm, 3 min) and resuspended (1 ml) in PBS. In order to distinguish
between viable and non-viable cells, cells were stained as follows: 2 µl of calcein AM working solution (50 µM, diluted in DMSO) and 4 µl of EthD-1 solution (2 mM, undiluted) were added to the 1 ml samples and incubated for 20 min at Rt protected from light. Within 1 h post staining analysis was performed using the FACSCalibur flow cytometer (Becton Dickinson GmbH, Heidelberg, Germany). Viable cells were recognized by green fluorescence, while non-viable cells produced red fluorescence. Treatment of fixed cells with Triton X-100 prior to staining served as control for maximum staining of EthD-1.

F-9 Western blot analysis
For the detection of proteins derived from infection, transfection, coimmunoprecipitation, in vitro kinase assay and in vivo labeling procedures, protein samples were separated by SDS-PAGE and transferred onto nitrocellulose membranes (Whatman GmbH, Dassel, Germany) by electroblotting under buffer at 100 V for 60 min. Thereafter, the membranes were reversibly stained with Ponceau S solution (Salinovich and Montelaro, 1986) to highlight bands of a standard molecular weight marker (SDS6H2, Sigma-Aldrich, Deisenhofen, Germany). In order to avoid unspecific binding of antibodies, the membranes were saturated in 5% skim milk powder solution (Humana Milchunion, Herfold, Germany; dissolved in PBSo/0.1% Tween) for at least 2 h at Rt. Then, incubation with the primary antibody, dissolved in 3 ml 2.5% skim milk powder solution, was performed overnight at Rt or 4°C. After washing of the membrane in PBSo/0.1% Tween (app. 4 x 10 min), incubation with the respective horseradish-peroxidase-conjugated secondary antibody occurred for 1 h at Rt. After further washing steps and incubation of the membrane in freshly prepared ECL solution (10 ml ECL solution A, 100 µl ECL solution B and 3.1 µl hydrogen peroxide) for 30 sec, detection of the proteins was achieved by the use of the FUJIFILM Luminescent Image Analyzer LAS-1000 (FUJIFILM Europe GmbH, Düsseldorf, Germany). Hereby, the luminol of the ECL solution is converted by the horseradish-peroxidase under release of light, which could be detected by the camera (chemiluminescence reaction). If required, the antibodies were removed from the membranes via incubation in a Roti®-Free stripping buffer (Roth, Karlsruhe, Germany) at 55°C for 15 min. After extensive washing in PBSo/0.1% Tween, the membrane was stained a second time as described above.

F-10 Indirect immunofluorescence analysis
HFFs cultivated on coverslips in 6-well plates (4 x 10^5 cells per well) were infected with HCMV AD169, GDGrXbaF4, GDGrP53 or 759rD100 at MOI 0.5 or 1.0. Viral inoculi were removed 1.5 h post infection and replaced by fresh medium, optionally supplemented with reference compounds of protein kinase inhibitors (added 24 hpi if not indicated otherwise). At various
time points post infection, infected cells were washed with PBSo. Then, cells were fixed by the use of 4% paraformaldehyde solution (10 min, Rt) and, after a further washing step with PBSo, cells were permeabilized by incubation with 0.2% Triton-X100 solution for 20 min at 4°C. Cells were washed again before the incubation with blocking solution (2 mg/ml Cohn II in PBSo) was performed (30 min, 37°C). Thereafter, the cells were incubated with 150 µl of the appropriate primary antibody diluted in PBSo for 60 min at 37°C followed by incubation with 150 µl of the corresponding fluorescent dye-conjugated secondary antibody (diluted in PBSo; 30 min, 37°C). Cells were mounted using the DAPI-containing Vectashild mounting medium (Alexis, Grünberg, Germany) and analyzed with the Zeiss Axiovert-135 inverted fluorescence microscope (Zeiss, Jena, Germany) or the DMI6000 B microscope using the HCX PL APO 63x oil object lens (Leica GmbH, Wetzlar, Germany). Confocal laser-scanning microscopy was performed with a TCS SP5 microscope (Leica GmbH, Wetzlar, Germany). The Meta-Imaging series (MetaVue; Universial Imaging Cop., Downington, PA, USA), LAS AF SP5 software (Leica GmbH, Wetzlar, Germany) and Adobe Photoshop package (version 8.0.1) were used for processing of the images.

F-11 Analysis of protein-protein interaction

F-11.1 Yeast two hybrid analysis

For yeast two-hybrid analysis, yeast strain Y153 was transfected by the lithium acetate method (Gietz et al., 1992). Hereby, 5 ml of YAPD medium were inoculated with 50 µl of Y153 and grown overnight at 30°C and 180 rpm. Reared salmon sperm, which was used as the carrier substance, was denatured (95°C, 10 min) and quenched on ice. The Y153 culture was centrifuged and washed with 5 ml of H₂O (2200 rpm, 5 min, Rt) followed by resuspending of the cell pellet in 500 µl of a lithium acetate solution (LiAc, 0.2 M) and incubation for 60 min at 30°C. In the meantime, total DNA of 2 µg was prepared with 10 µl of the denatured carrier substance before 140 µl of the yeast-LiAc suspension was added. After incubation for 30 min at 30°C, 350 µl of a 50% PEG solution was added and the mixture was incubated for further 60 min at 30°C. Then, the heat shock (42°C, 5 min) was performed and subsequent to cooling to Rt, the transfection reaction was mixed with 500 µl H₂O. Then, the cells were pelleted by centrifugation (2000 rpm, 2 min) and washed in 1 ml H₂O (2000 rpm, 2 min). In a final step, the transfected cells were resuspended in 100 µl H₂O and plated on WL-plates. Incubation was conducted at 30°C for 3 days to allow transfected yeast cells to grow.

For large-scale of yeast two-hybrid screening, yeast strain Y153 was transfected by the lithium acetate method with bait plasmids as described above. These bait-positive Y153 cells were then used for large-scale transfection. Maintenance of the transfected bait plasmid was assured by selection for tryptophan prototrophy. For transfection, the genomic library of
HCMV pPC86EmL-TB40E 0.5 (Schierling et al., 2004) was used. Overnight culture of Y153-bait cells was diluted 1 : 100 in 300 ml YAPD medium and grown to $OD_{600} = 0.8$. After centrifugation (2500 rpm, 5 min, Rt), the cell pellet was washed twice in 10 ml H$_2$O and once with 20 ml 100 mM LiAc solution before resuspension in 1.5 ml 100 mM LiAc solution. 100 µl of the prepared Y153-bait cells were mixed with 4 µg HCMV library, 10 µl denaturated carrier substance and 600 µl PEG/LiAc solution (1 ml 1 M LiAc, 1 ml H$_2$O, 8 ml 50% PEG) followed by incubation at 30°C for 45 min. Then, heat shock was conducted at 42°C for 15 min. Transfected yeast cells were centrifuged (4000 rpm, 5 min, Rt) and resuspended in 500 µl H$_2$O. Primary transformants were selected for growth on HWL-deficient plates containing 30 mM 3-aminotriazole.

Protein interactions were analyzed using GAL4 fusion proteins. Thereby, protein 1 fused to the GAL4 activation domain (AD) and protein 2 fused to the GAL4 binding domain (BD) were coexpressed. In the case of a direct interaction, the AD and BD of the GAL4 transcription factor were brought into proximity resulting in the activation of the GAL4-responsive promoter. Thus transcription of the reporter enzyme $\beta$-galactosidase was induced. The activity of $\beta$-galactosidase was then determined via filter lift assays (Breeden and Nasmyth, 1985). Pieces of a nitrocellulose membrane (Hybond-C extra, GE Healthcare, Freiburg, Germany) were put onto the transformed colonies or freshly spotted transformants for about 1 min. The colonies adhered onto the membrane were then frozen in liquid nitrogen and directly thawed afterwards before incubated on a layer of Whatman paper which was soaked in Xgal solution (buffer Z, 1 mM $\beta$-mercaptoethanol, 1,5 mM Xgal). In the case of protein-protein interactions, $\beta$-galactosidase was expressed and able to cleave the Xgal substrate. Thus, yeast cells stained blue. Depending on the grade of interaction, the membrane was dried and analyzed after 8 to 24 h of incubation.

For a yeast two-hybrid screening, HWL-selected colonies from large-scale yeast transfection (F-6.2.1) were analyzed for $\beta$-galactosidase activity by filter lift assay. Interaction identified by blue staining due to $\beta$-galactosidase cleavage of Xgal substrate were further characterized as described before (Marschall et al., 2005; Schierling et al., 2004; Hofmann et al., 2002). In brief, by isolation of yeast DNA from positive clones, interactor plasmids were rescued by transformation of E. coli strain KC8. Then, isolated plasmids were retransformed into yeast to confirm interaction before sequences of the cDNA inserts were determined by automated sequence analysis.

**F-11.2 Coimmunoprecipitation**

The day before coimmunoprecipitation, 50 mg/ml protein A sepharose was soaked in CoIP buffer (without proteinase inhibitors) at 4°C for at least 2 h. The protein A sepharose beads were then incubated overnight at 4°C with the respective antibody as follows: 50 µl protein A
sepharose beads, 1 µl antibody and 500 µl CoIP buffer. The next day, transfected 293T cells were harvested and washed in 5 ml of cold PBSO (2000 rpm, 5 min) before cells were lysed in 800 µl of CoIP buffer supplemented with protease inhibitors, incubated for 20 min on ice and centrifuged (14000 rpm, 10 min, 4°C). Thereafter, 40 µl of the supernatant was boiled with 40 µl 2x SDS sample buffer (95°C, 10 min) as expression controls and the remaining volume of the supernatant (~ 450 µl) was incubated for 2 h at 4°C with the antibody-loaded protein A sepharose beads, which were washed three times in CoIP buffer (10000 rpm, 1 min, 4°C). After the incubation, the samples were centrifuged (10000 rpm, 1 min, 4°C) and washed five times in CoIP buffer (10000 rpm, 1 min, 4°C). Finally, the immunoprecipitates were recovered by boiling in 25 µl 4x SDS sample buffer (95°C, 10 min) and were subjected to SDS-PAGE and Western blot analysis. In parallel, the expression control samples were analyzed to assure a reliable expression level of all proteins.

F-12 Analysis of protein phosphorylation

F-12.1 In vitro kinase assay (IVKA)
For the putative phosphorylation of IE2p86 by CDKs, both, IE2p86 and CDK1, -2, -7, or -9 were recombinantly expressed in 293T cells. One day post transfection, protein A sepharose was soaked and incubated with the respective antibody in RIPA buffer as described for coimmunoprecipitation. Two days post transfection, cells were lysed in 500 µl RIPA buffer, incubated (20 min, ice) and centrifuged (14000 rpm, 30 min, 4°C). The supernatant was incubated for 2 h at 4°C with the antibody-loaded protein A sepharose beads (washed three times in RIPA buffer; 10000 rpm, 1 min, 4°C). Thereafter, samples were washed with 500 µl HNTG buffer and 500 µl CDK IVKA buffer before phosphorylation reaction was allowed by incubation in 40 µl CDK IVKA buffer including MgATP mix and 2.5 µCi [γ-33P]ATP for 30 min at 30°C. Phosphorylation reaction was stopped with 15 µl 4x SDS sample buffer (95°C, 10 min) and samples were analyzed by SDS-PAGE and Western blot procedure. Finally, radioactive signals were detected by exposure of the membranes to phosphoimager plates and quantified by the AIDA software.

For IVKAs with commercially available purified CDK/cyclin complexes, the following complexes were used (ProQinase, Freiburg, Germany): CDK1/cycB1 (0134-0135-1), CDK2/cycE (0050-0055-1), CDK7/cycH/MAT1 (0366-0360-4), CDK9/cycT (0371-0345-1). As putative substrate proteins, pUL69 and IE2p86 were expressed in 293T cells and recovered by immunoprecipitation as described above (F-11.2). After washing with 500 µl HNTG buffer and 500 µl standard assay buffer, immunoprecipitates were incubated with 20 µl standard assay buffer, 1 µM ATP, 2.5 µCi [γ-33P]ATP and 10 µl recombinant CDK/cylin complex (50 ng
of CDK2/cycE, 200 ng of CDK1/cycB1, 200 ng of CDK7/cycH-MAT1 or 200 ng of CDK9/cycT; each diluted in kinase-dilution-buffer) for 20 min at 30°C. As a positive control for substrate phosphorylation, purified RB-CTF (C-terminal fragment of the human retinoblastoma protein; 1 µg) was added to specific reactions. Phosphorylation signals were analyzed as described above.

F-12.2  In vivo labeling
In order to label the phosphorylation of proteins with a radioactive signal in living cells, HFFs were infected with HCMV AD169 (MOI 0.5). 1.5 hpi, virus inoculi were replaced by media containing various inhibitors. The in vivo labeling was performed by the addition of [γ-\(^{33}\)P]orthophosphate (150 µCi/4.5x10^5 cells) two days post infection. After overnight incubation, cells were lysed in RIPA buffer and subjected to immunoprecipitation and analysis as described for in vitro kinase assay (F-12.1).

F-13  CAT mRNA export assay
HeLa cells were transfected one day after seeding (3.5 x 10^5 cells in 6-well plates) by applying the standard calcium phosphate precipitation method (Ausubel et al., 1989). CAT reporter assays were performed as described by Farjot and colleagues (Farjot et al., 2000). For the assay, 150 ng of the reporter plasmid pDM128/CMV/RRE, encoding an intron-containing mRNA with the coding sequence of chloramphenicol acetyl transferase (CAT), and 500 ng of pHM160 for the expression of pUL69 were cotransfected and CDK inhibitors were added 16 h post transfection. To quantify CAT protein expression, which was used as a reporter of nuclear export activity, cells were lysed and analyzed 48 h post transfection by a CAT enzyme-linked immunosorbent assay, according to the instructions of the manufacturer (Roche Molecular Biochemicals, Mannheim, Germany). Determinations were performed in triplicate.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>aa</td>
<td>amino acid</td>
</tr>
<tr>
<td>AD</td>
<td>activation domain</td>
</tr>
<tr>
<td>AIDS</td>
<td>acquired immunodeficiency syndrome</td>
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<tr>
<td>BAC</td>
<td>bacterial artificial chromosome</td>
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<td>BD</td>
<td>binding domain</td>
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<td>CDV</td>
<td>cidofovir</td>
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<td>CDK</td>
<td>cyclin-dependent protein kinase</td>
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<td>CPE</td>
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<td>CoIP</td>
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<td>C-terminal domain</td>
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<td>Dulbecco’s modified Eagle medium</td>
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<td>ds</td>
<td>double-stranded</td>
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<td>EBV</td>
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<td>ECL</td>
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<td>ethylenediaminetetraacetic acid</td>
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<td>hour(s)</td>
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<td>nt</td>
<td>nucleotide</td>
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<td>Abbreviation</td>
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</tr>
<tr>
<td>OD</td>
<td>optical density</td>
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<td>ORF</td>
<td>open reading frame</td>
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<tr>
<td>PAb</td>
<td>polyclonal antibody</td>
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<td>phosphate-buffered saline without Ca$^{2+}$ and Mg$^{2+}$ ions</td>
</tr>
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<td>PCR</td>
<td>polymerase chain reaction</td>
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<td>short interfering RNA</td>
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<td>X-gal</td>
<td>5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside</td>
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References


**Plachter, B., Sinzger, C. and Jahn, G.** (1996). Cell types involved in replication and distribution of...


Roizman, B., Carmichael, L. E., Deinhardt, F., de-The, G., Nahmias, A. J., Plowright, W., Rapp, F.,


I Appendix

Own Publications


Contributions to national and international conferences

(1) 19th Annual Meeting of the Society for Virology (GfV), Leipzig, Germany, March 2009
CDK activity is required for specific intranuclear localization, phosphorylation and functionality of the cytomegalovirus regulatory protein pUL69

(2) 33rd International Herpesvirus Workshop (IHW2008), Estoril, Portugal, July 2008
Inhibition of cellular CDK or cytomegaloviral pUL97 activity alters the localization of pUL69 to nuclear speckled aggregates
(3) 18th Annual Meeting of the Society for Virology (GfV), Heidelberg, Germany, March 2008
The intranuclear localization of the regulator protein pUL69 of HCMV is influenced by cellular cyclin-dependent and viral protein kinase activity

(4) 3rd European Congress of Virology, Nuremberg, Germany, September 2007
Replication of strains and mutants of HCMV is differentially dependent on CDK activity

(5) 11th International Cytomegalovirus & Beta Herpesvirus Workshop, Toulouse, France, May 2007
The dependence of HCMV replication to CDK activity is variable between viral strains and mutants

(6) 16th Annual Meeting of the Society for Virology (GfV), Munich, Germany, March 2006
Spirulan-like polysaccharides derived from the microalga Arthrospira possess a broad spectrum of antiviral activity

Scholarships

(1) Doctoral Fellowship
German National Academic Foundation (Studienstiftung des deutschen Volkes), Bonn, Germany

(2) Doctoral Fellowship; 2006 Endeavour Europe Award
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               “Characterization of the interregulation between cyclin-dependent protein kinases and human cytomegalovirus regulatory proteins”
               Advisors:  Prof. Dr. Wolfgang Hillen, Microbiology
               Prof. Dr. Manfred Marschall, Virology
Acknowledgements

I would like to acknowledge Prof. Dr. Bernhard Fleckenstein for the opportunity to perform my PhD thesis at the Institute for Clinical and Molecular Virology, University Hospital Erlangen.

I wish to thank Prof. Dr. Wolfgang Hillen who kindly agreed to supervise and review my PhD thesis for the School of Sciences, Friedrich-Alexander-University Erlangen-Nuremberg.

I would like to express my sincere thanks to my advisor, Prof. Dr. Manfred Marschall, for giving me the great possibility to work on this project. Thank you so much for invaluable advice & support here in Erlangen and in Sydney, for awesome teamwork and encouragement throughout my doctoral thesis.

I am very grateful to Prof Dr. Thomas Stamminger for scientific support and reviewing my thesis. Furthermore, I would like to thank Prof. Dr. Thomas Mertens (Institute for Virology, University Hospital, Ulm) for taking the time to review my thesis.

I would like to extend my gratitude to Prof. Dr. Bill Rawlinson, Dr. Gillian Scott (Virology Division, University of New South Wales, Prince of Wales Hospital, Sydney, Australia) and all colleagues from the Rawlinson lab for scientific very interesting and overall incredible six months down under. Thank’s for your support, discussions, chats and the yummy lunches.

A huge “thank’s a lot” to all present and former members of the Stamminger/Marschall lab. It was just marvelous to work, discuss and laugh with all of you!

Jens(i), the confocal microscope chief & co-bakery-goer; lucky us you joined our group.

Sabrina, the heart and soul of our lab – thank’s for a great time.

Katrin, my first diploma student – it was fantastic, thank’s.

Anja, for working and laughing next, behind as well as opposite of and with me.

Naina, for shared Olympics-enthusiasm, laughter, chats and scientific discussions.

Marco – pUL69, fun, hypotheses, hilarious, shared writing-phase & certainly Dr. Max Munzl.

Antje, for chats, discussions, fun and hey, we actually managed to go climbing!

I owe immense gratitude to my friends for continous support, necessary distractions and sharing unforgettable moments. Special thank’s to:

Anke – one word says it all: MIP.

Andrea, for so many memorable times full of laughter, talks, wine, discussions & dancing.

Claudi, for a priceless friendship and the offer of the great loftbed whenever I’m in Munich.

Simone, the sunshine and enchanting fairy; happy you crossed my path.

Netsche, for the best neighborhood since childhood.

I sincerely thank a couple of people who helped me so much in different ways often without being aware of it. Thank you: Beate H., Micha R., Jo B., Ralph M., Tante Frieda, Tante Rosa & Onkel Kurt.

My sincere gratitude to my family who have given me deep roots to take along. I deeply thank you for your unwavering love, always being there and believing in me.

Very special thank’s to Christian, my tower of strength. I say “thank you” for your unquestioning love, your patience and never giving up in teaching me sereneness 😊