Cross-regulation of viral kinases with cyclin A secures shutoff of host DNA synthesis

Boris Bogdanow1,5,9, Max Schmidt2,6,9, Henry Weisbach2,7, Iris Gruska2, Barbara Vetter2, Koshi Imami1,8, Eleonore Ostermann3, Wolfram Brune3, Matthias Selbach1,4, Christian Hagemeier2 & Lüder Wiebusch2

Herpesviruses encode conserved protein kinases (CHPKs) to stimulate phosphorylation-sensitive processes during infection. How CHPKs bind to cellular factors and how this impacts their regulatory functions is poorly understood. Here, we use quantitative proteomics to determine cellular interaction partners of human herpesvirus (HHV) CHPKs. We find that CHPKs can target key regulators of transcription and replication. The interaction with Cyclin A and associated factors is identified as a signature of β-herpesvirus kinases. Cyclin A is recruited via RXL motifs that overlap with nuclear localization signals (NLS) in the non-catalytic N termini. This architecture is conserved in HHV6, HHV7 and rodent cytomegaloviruses. Cyclin A binding competes with NLS function, enabling dynamic changes in CHPK localization and substrate phosphorylation. The cytomegalovirus kinase M97 sequesters Cyclin A in the cytosol, which is essential for viral inhibition of cellular replication. Our data highlight a fine-tuned and physiologically important interplay between a cellular cyclin and viral kinases.

1 Research group “Proteome Dynamics”, Max Delbrück Center for Molecular Medicine, 13125 Berlin, Germany. 2 Labor für Pädiatrische Molekularbiologie, Charité Universitätsmedizin Berlin, 13353 Berlin, Germany. 3 Heinrich Pette Institute, Leibniz Institute for Experimental Virology, 20251 Hamburg, Germany. 4 Charité-Universitätsmedizin Berlin, 10117 Berlin, Germany. 5 Present address: Research group “Structural Interactomics”, Leibniz Forschungsinstitut für Molekulare Pharmakologie, 13125 Berlin, Germany. 6 Present address: Medizinische Klinik m.S. Hämatologie, Onkologie und Tumorimmunologie, Charité Universitätsmedizin Berlin, 12200 Berlin, Germany. 7 Present address: PenCef Pharma GmbH, 13509 Berlin, Germany. 8 Present address: Laboratory of Molecular & Cellular BioAnalysis, Kyoto University, 606-8501 Kyoto, Japan. 9 These authors contributed equally: Boris Bogdanow, Max Schmidt.

✉email: lueder.wiebusch@charite.de
Herpesviruses are a wide-spread family of large, double-stranded DNA viruses replicating within the nuclei of their host cells. Generally, herpesviruses are highly adapted to their hosts and persist in a latent mode of infection unless immunodeficiency provokes viral reactivation and disease. They have diversified into three subfamilies: neurotropic α-herpesviruses, broadly infective β-herpesviruses, and lymphotropic γ-herpesviruses. Despite their widely different pathogenic properties and clinical manifestations, all herpesviruses share a common set of conserved core genes, mostly encoding essential structural components and replication factors. Among the few core genes with regulatory function are the conserved herpesvirus protein kinases (CHPKs), which are medically important both as drug targets and as produg activating enzymes.

CHPKs belong to the group of viral serine/threonine kinases. Although possessing a considerable sequence divergence, CHPKs have a number of common characteristics including autophosphorylation, nuclear localization, incorporation into the tegument layer of virus particles, and phosphorylation of other tegument proteins. CHPKs target regulators of the DNA damage checkpoint, phosphorylate the translational elongation factor EF-1γ, and counteract the IFR3-dependent type I interferon response. CHPKs were reported to preferentially target cellular cyclin-dependent kinase (CDK) phosphorylation sites. In particular, the kinases of human β- and γ-herpesviruses show significant structural and functional homology to CDKS-13-16. This has led to their designation as viral CDK-like kinases (v-CDKs). V-CDKs lack amino acids that are known to be essential for interaction of cellular CDKs with cyclins, CDK inhibitors (CKIs) and CDK activating kinases (CAKs). Accordingly, v-CDKs are considered immune to cellular control mechanisms, despite the fact that the UL97 kinase of human cytomegalovirus (human herpesvirus 5, HHV5) can physically interact with various cyclins. V-CDKs mimic CDK1 and 2 in phosphorylating Lamin A/C, and cellular replication factors for viral DNA replication.

Significant knowledge has accumulated over the recent years about CHPK functions. It is known that CHPKs can recruit cellular proteins as substrates via conserved docking motifs. Further, CHPKs can be regulated by their interaction partners and even exert kinase independent functions. However, a lack of amino acids that are known to be essential for interaction of cellular CDKs with cyclins, CDK inhibitors (CKIs) and CDK activating kinases (CAKs). The CHPKs are considered immune to cellular control mechanisms, despite the fact that the UL97 kinase of human cytomegalovirus (human herpesvirus 5, HHV5) can physically interact with various cyclins. V-CDKs mimic CDK1 and 2 in phosphorylating Lamin A/C, and cellular replication factors for viral DNA replication.

Results

CHPKs target key regulators of transcription and replication. Systems-level approaches have provided important insights into the molecular functions of CHPKs. For example, yeast 2-hybrid screens revealed several binary binding partners of CHPKs and phosphoproteome profiling was successfully used to assess CHPK substrate specificity. However, comprehensive and comparative information about CHPK interaction partners at the proteome level is lacking.

To identify CHPK interaction partners, we transfected SILAC (stable isotope labeling of amino acids in cell culture) heavy and light labeled HEK-293T cells with HA-AP-tagged CHPKs of seven different human herpesviruses (HHVs) or vector controls. We performed these experiments in triplicates, including label-swaps (Fig. 1a, and subjected the samples to HA affinity purification (HA-AP) and shotgun proteomics. We specifically detected the baits in extracts of transfected cells and in eluates of HA-APs (Supplementary Fig. 1a, b). The baits were among the most abundant proteins in the eluates (Supplementary Fig. 1c), abundant proteins in the eluates (Supplementary Fig. 1a, b). The baits were among the most abundant proteins in the eluates (Supplementary Fig. 1c).

We next aimed to determine interaction partners that were common to all kinases, class-specific (i.e., restricted to either kinases of α, β, or γ-classes) or unique to individual kinases. Therefore, we clustered the SILAC fold-changes of candidate interaction partners across all tested kinases (Fig. 1c). We found the chaperonin containing TCP1 and the kinase maturation complex (CDC37, HSP90) to co-purify with all kinases analyzed. This confirms the previous observation that CHPKs interact with the same set of cellular proteins that assist in folding and maturation of cellular kinases.

Proteins that co-purified with BGLF4 (HHV4, Epstein–Barr-virus), but were not significantly enriched with other kinases, include factors involved in DNA replication (HERC2, E4F1, PCNA, SSBP1) and chromatin silencing (SMCHD1, CABIN1, HIRA, BEN3). Proteins that co-purified with pUL97 (HHV5) but not other kinases were functionally related to regulation of transcription (PHC2, RING1, CBX4, TRIM28, ZNF136, ZNF97). To validate our approach, we performed an additional SILAC AP–MS experiment directly comparing proteins enriched to BGLF4 and pUL97 (Supplementary Fig. 3a). Again, we found selective enrichment of the specific sets of host interactors described above to either BGLF4 or pUL97 (Supplementary Fig. 3a). Again, we found selective enrichment of the specific sets of host interactors described above to either BGLF4 or pUL97. By performing co-immunoprecipitation (co-IPs) experiments, we were able to validate the transcriptional regulators SPOP, CCAR2, BMI1, and TRIM28 as specific interactors of pUL97 (Supplementary Fig. 3c–f).

Importantly, we found that all human β-herpesvirus kinases co-purify with S/G2 phase-specific cyclins (Cyclin A, gene symbol: CCNA2, Cyclin B, gene symbol: CCNB1), CDKs (CDK1, CDK2), and associated proteins (SKP1, SKP2). While enrichment of Cyclin A–CDK2 was reproducibly strong, Cyclin B–CDK1 did not enrich with pUL97 in our transfection experiment (Fig. 1d). We were able to validate the interaction with Cyclin A–CDK2 by reverse co-IPs. Also, we observed this type of interaction for the homologous M97 kinase of MuHV-1, better known as MCMV (Fig. 1e). Collectively, our interactionome of human CHPKs provides a rich resource and suggests...
that these kinases are crucially involved in the regulation of transcription, epigenetic remodeling, and cell cycle control. Cyclin A–CDK2 complexes build a common subset of interactors for β-herpesvirus kinases, suggesting important functional implications.

β-herpesvirus kinases bind cyclin–CDKs via NLS-RXL modules. β-herpesvirus kinases lack most of the residues of CDKs that directly interact with cyclins, including a conserved PSTAIRE helix. Instead, we found RXL/Cy motifs in the N-terminal, non-catalytic parts of β-herpesvirus kinases (Fig. 1f–g). Such motifs...
are typically used for substrate and inhibitor recruitment to cyclin–CDKs. Importantly, the positions of the RXL-type sequences within the largely divergent N termini are well conserved (Supplementary Fig. 4). These putative Cyclin A binding elements in β-herpesvirus kinases of the roseolovirus (HHV6, HHV7) and muromegalovirus (MuHV1, MuHV2, MuHV8) genera are in close proximity to clusters of positively charged residues (Supplementary Fig. 4), which are predicted, and in the case of HHV6 validated, classical bipartite nuclear localization signals (NLS). In fact, the C-terminal part of the NLS sequences directly overlaps with the N-terminal part of the RXL motifs (Fig. 2a). Thus, when we set out to test the contribution of RXL motifs in U69 and M97 to Cyclin A binding, we had to consider the possibility that RXL mutations may negatively affect NLS function. We therefore designed two mutant versions of each kinase: one disrupting the core of the RXL motif (RXL→→AXA) and one changing only the hydrophobic part (LF→→AA, LXF→→AXA), leaving the basic residues of the NLS intact (Fig. 2a). Both mutations abolished binding of M97 and U69 kinases to Cyclin A (Fig. 2b). Thus, RXL sequence motifs found in β-herpesvirus kinases act as Cyclin A docking sites. RXL mutation not only prevents Cyclin A binding but also interaction with other cyclin-associated factors found in the interactome analysis (Fig. 1c), as exemplarily shown for HHV6-U69 (Supplementary Fig. 5). This indicates that those factors are not direct v-CDK interactors but instead co-recruited with Cyclin A. Thus, the RXL motif triggers the formation of higher-order v-CDK–cyclin–CDK complexes.

We then assessed the consequences of RXL mutations for NLS function. To this end, we cloned 32–37 amino acid segments encompassing the overlapping NLS and RXL elements of M97 and U69 kinases into an NLS reporter construct. Integration of wild-type (WT) NLS-RXL regions into the chimeric reporter induced nuclear accumulation of the otherwise cytoplasmic GFP signal (Fig. 2d, e), indicative of a functional NLS. RXL to AXA mutations weakened this activity in HHV6-U69 and even disrupted it in M97. By contrast, the NLS function remains intact when only the hydrophobic residues of the RXL/Cy motifs are mutated (Fig. 2d, e). These results demonstrate that cyclin binding and NLS sequences are integrated into a composite motif that can be functionally separated by mutations.

M97 assembles cyclin–CDK complexes in infected cells. We then aimed to analyze the NLS-RXL module in a representative infection system and chose MCMV for ease of manipulation. We first assessed the time-resolved protein interactome of M97 using SILAC and AP–MS (Supplementary Fig. 6a, Supplementary Data 3). Early during infection (12 h), the M97 interactome consisted almost exclusively of cyclins, CDKs, and associated proteins (Supplementary Fig. 4b). In the late phase (36 h), additional viral and cellular factors co-purified with M97 (Supplementary Fig. 4d), including M50–M53, the nuclear egress complex of MCMV, and Lm54, the DNA-binding subunit of the cell cycle regulatory MuvB complex, which is known to be regulated by pUL97 (ref. 39). Taken together, this indicates that M97 functions in cell cycle regulation, viral egress, and control of gene expression.

In consistency with the data from transfected cells, M97 interacted with Cyclin A/B–CDK complexes throughout infection (Fig. 3a, Supplementary Fig. 6b,d). In particular, Cyclin A–CDK2 was present in similar molar amounts as M97 itself in HA-M97 MS samples (Supplementary Fig. 6c, e), suggesting a strong and stoichiometric interaction in infected cells. Mutation of the RXL/Cy motif (Supplementary Fig. 5) disrupted the interaction of Cyclin A with M97 (Fig. 3c, d). However, these mutations did not influence Cyclin A protein levels (Fig. 3b) or Cyclin A-associated kinase activity (Fig. 3f). Moreover, RXL/Cy mutations, in contrast to the “kinase dead” K290Q mutation, did not compromise M97 levels and activity (Fig. 3b, e). Thus, M97–Cyclin A binding has no influence on abundance and enzymatic activity of the involved interaction partners. This is consistent with the view that β-herpesviral CHPKs are cyclin-independent kinases.
We globally assessed substrate phosphorylation by performing a proteomic analysis of phosphopeptides and whole cell lysates of SILAC-labeled cells at 24 h post infection (Supplementary Fig. 8a, Supplementary Data 4).

First, we corrected phosphosite ratios for protein level changes (Supplementary Fig. 8b, c). Next, we categorized proteins and corresponding phosphosites based on their GO annotations as nuclear, cytoplasmic or unclear (“no category”) (Supplementary Fig. 8d). Then, we specifically analyzed phosphosites that match known v-CDK target motifs, such as pS/P, pSXXK, LXPSP (p denotes the phosphorylated residue). When cells were infected with M97R45A/L47A mutant, pS/TP and pSXXK sites residing in the cytosol were significantly stronger phosphorylated than nuclear sites (Fig. 4c). By contrast, when cells were infected with the M97L47A/F49A mutant, the same set of sites were stronger phosphorylated when they belonged to nuclear proteins. The most pronounced differences in the target spectrum were observed when phospho-serines followed by prolines were positioned between hydrophobic amino acids and lysines (Supplementary Fig. 8e). Collectively, these data argue that Cyclin A binding enables switch-like changes in the substrate spectrum and subcellular distribution of M97.

M97 inhibits host DNA synthesis by Cyclin A sequestration.

The binding of M97 to Cyclin A may have functional consequences not only for M97 but also for Cyclin A. Cyclin A is essential for cellular DNA replication and cell cycle progression from S phase to mitosis. Importantly, Cyclin A function depends on its nuclear localization. Therefore, we analyzed whether M97 binding impacts the subcellular distribution of Cyclin A. We found Cyclin A, and to a lesser extent CDK2, to be depleted from the nucleus in MCMV-WT-infected cells (Fig. 5a). This effect was Cyclin A specific as Cyclin E was evenly distributed between nuclear and cytoplasmic fractions. Mutation of the M97 translation start site prevented the cytoplasmic enrichment of Cyclin A. Mutations of the RXL/Cy motif in M97 even led to a predominant nuclear localization of Cyclin A. Thus, Cyclin A is sequestered within the cytosol dependent on its binding to M97.

This observation prompted us to investigate whether the nuclear depletion of Cyclin A by M97 affects cell cycle
progression. Therefore, we measured the DNA content of quiescent fibroblasts infected with WT and M97 mutant viruses by flow cytometry (Fig. 5b, c, Supplementary Fig. 9). We found a ganciclovir-sensitive increase of viral DNA in MCMV-WT-infected cells, consistent with previous reports. By contrast, M97 mutant viruses caused a rapid and ganciclovir-resistant accumulation of cells with a G2/M DNA content. The latter phenotype was reversed by stable and ganciclovir-resistant accumulation of cells with a G2/M DNA content. The latter phenotype was reversed by stable and ganciclovir-resistant accumulation of cells with a G2/M DNA content.

We aimed to confirm the crucial role of M97–Cyclin A interaction for inhibition of cellular DNA synthesis by an orthogonal approach. We chose to combine EdU pulse labeling and fluorescence microscopy to spatially discriminate sites of active viral or cellular DNA synthesis. In MCMV-WT-infected cells, DNA synthesis is confined to nuclear replication compartments that stain positive for the viral single-stranded DNA-binding protein M57 (Fig. 5d, e). By contrast, cells infected with M97-RXL/Cy mutants incorporated EdU in foci distributed over the whole nucleus, indicating that the restriction of cellular DNA replication was lost. Thus, M97–Cyclin A complex formation serves as a mechanism to shut off competing host DNA synthesis during productive infection.

Very similar results were obtained in primary, non-immortalized mouse embryonic fibroblasts (MEF), the only difference being that here the RXL/Cy mutation leads to a cellular DNA content that exceeds the normal 4n DNA content of diploid G2/M cells (Supplementary Fig. 11). This indicates that the M97 mutant virus bypasses cellular control mechanisms protecting primary cells from DNA over-replication.
Although M97 is not essential for MCMV in vitro, its deletion negatively affects virus growth. When we tested our set of M97 mutants on MEFs, a virus growth curve analysis revealed that the NLS-RXL module has a larger impact on virus replication than the kinase activity itself (Fig. 5f). This underlines the importance of nonenzymatic protein–protein interactions for the proper function of conserved β-herpesvirus kinases during the virus life cycle.

**Discussion**

Here, we present the first comparative analysis of CHPK interactomes. Our analysis led to the identification of host–protein interaction signatures that are common, class-specific or unique to CHPKs (Fig. 1c). The interaction of β-herpesvirus-CHPKs with Cyclin A orchestrates higher-order complex formation of cell cycle regulatory factors (Fig. 3). Notably, the interaction is
regulatory and has functional consequences for both, the CHPK and Cyclin A. For MCMV, it results in the dynamic relocalization of the viral kinase (Fig. 4a) and consequently an altered substrate spectrum (Fig. 4b). Further, the interaction leads to cytosolic sequestration of Cyclin A, which is essential for viral arrest of DNA replication (Fig. 5).

V-CDKs are a subset of CHPKs that share key aspects of host CDK-function and the ability to complement for CDK activity in yeast cells. V-CDKs have lost sequence features enabling control by cellular factors, such as CAK, CKI, and cyclins. Instead, we show that some v-CDKs acquired RXL motifs (Fig. 1), which are typically used by cellular CDK substrates and inhibitors for recognition by cyclins. These motifs enable a regulatory cross-talk of Cyclin A and v-CDKs, which is characterized by cell cycle dependent regulation of the viral kinase on the one hand (Fig. 4) and neutralization of Cyclin A in stoichiometric protein assemblies on the other hand (Fig. 5, Supplementary Figs. 6 and 10). The latter effect of v-CDK–Cyclin A interaction is reminiscent of CKI function. Thus, we propose that β-herpesviruses integrate the antipodal activities of CDKs and CKIs on one gene product. This combination allows a β-herpesvirus kinase like M97 to activate S/G2 metabolism while inhibiting cellular DNA synthesis (Fig. 5).

An exception among human β-herpesviruses is HCMV, which has evolved a different gene product for neutralization of Cyclin A. HCMV produces the small protein pUL21a, which targets Cyclin A for proteasomal degradation. Therefore, it seems that HCMV has shifted its Cyclin A-antagonistic, CKI function from its kinase to pUL21a. In that context it is interesting that the RXL motif in HCMV-pUL97 does not overlap with an NLS, suggesting an alternative function of pUL97–Cyclin A interaction (Fig. 1).

Short linear motifs (SLiMs) can be rapidly acquired by viruses and other pathogens to target host proteins. Here, we found that within HHV6, HHV7, and rodent CMV kinases two such SLiMs are fused into one regulatory sequence element (Fig. 2). The physical overlap of RXL/Cy and NLS motifs is facilitated by their sequence composition as both contain contiguous stretches of basic amino acids. NLS motifs function as docking sites for nuclear import factors, mainly importin-β. Accordingly, a docking competition mechanism makes binding to Cyclin A and nuclear localization of M97 mutually exclusive, enabling switch-like changes in viral kinase function (Fig. 6a). This puts β-herpesvirus kinases in a row with a number of cellular and viral proteins known to control nucleo-cytoplasmic localization via intermolecular NLS-masking, with NF-xB as the best understood example.

Remarkably, composite NLS-RXL/Cy elements are apparent in a number of key regulatory proteins of the host cell cycle. Although independently described, NLS and RXL/Cy motifs are overlapping in RB1 (refs. 53,54), CDKN1A (also known as p21) (refs. 55,56), E2F1 (refs. 57,58), and CDT1 (refs. 59,60) (Fig. 6b). This feature is highly conserved in vertebrate orthologues (Supplementary Fig. 12). For these cellular factors, it could be important to consider a docking competition between Cyclin A and importins, a possibility which is so far unexplored. Specifically, cyclin binding may be essential for the cell cycle dependent localization of these proteins. In other cases, such as CDC6, RXL motifs are adjacent to but not overlapping with NLS. Thus, Cyclin A recruits CDKs to neutralize the NLS by phosphorylation.

In addition to the functionally important interaction of β-herpesvirus kinases with Cyclin/CDKs, our unbiased proteomic survey indicates that CHPKs interact with many more cellular proteins (Fig. 1c, Supplementary Data 1). Consistent with previous reports, many CHPK interaction partners are functionally related to DNA repair and cell cycle control. Remarkably, we found that CHPKs also interact with a variety of prominent transcriptional repressor complexes. For example, pUL97 interacts with TRIM28-ZNF complexes (Fig. 1c, Supplementary Fig. 3e), known to silence CMV gene expression in stem cells depending on the phosphorylation status of TRIM28 (ref. 63). In addition, pUL97 co-purifies with PHC2, RING1, CXB4, and BMI1 (Fig. 1c, Supplementary Fig. 3f), all members of the polycomb repressive complex 1, which was recently linked to control of HCMV replication. Likewise, we found BGLF4 to interact with core subunits of the HIRA histone chaperone complex (HIRA, CABBIN1, and UB2N). This complex restricts lytic infection by depositing histone H3.3 on incoming herpesvirus genomes. Collectively, these data indicate that CHPKs can target host-derived master regulators of viral transcription, governing the decision between lytic and latent infection programs. This could allow tegument-delivered CHPKs to actively influence the outcome of herpesvirus infections.

Our interactome analysis indicates that CHPKs target master regulators involved in host replication, DNA repair, and transcription. It is important to note that this type of experiment cannot resolve protein interactions that depend on the environment of an infected cell. For example, CHPKs may be differentially abundant, differently modified, differently folded, or differently localized in transfected compared to infected cells. It is thus critical to interpret our findings in the broader context of kinase-associated functions that dynamically change during infection. For instance, phosphorylation of the nuclear protein SAMHD1 could be maintained by the M97 kinase early during infection when M97 localizes nuclear (Fig. 4a). During later stages of infection, the nuclear lamina is locally disassembled and egress of viral capsids to the cytoplasm occurs. The shift from nuclear replication to cytosolic assembly is accomplished by the concurrent relocalization of the viral kinase. Thus, Cyclin A interaction helps the kinase to exert infection stage-specific functions.

**Methods**

**Cells.** HEK-293T cells and NIH-3T3 fibroblasts were cultivated in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal (293T) or newborn (3T3) bovine serum, 2 mM L-alanyl-L-glutamine, 100 U mL⁻¹ penicillin, and 100 μg mL⁻¹ streptomycin. The indicated cell lines were infected with the indicated viruses at a multiplicity of infection of 10 when indicated.
100 µg mL

-1 streptomycin. Where indicated, cells were synchronized in G0/G1 phase by 48 h growth factor deprivation (0.05% serum). In preparation for proteomic analysis, cells were SILAC-labeled for at least five passages using lysine and arginine-deprived DMEM, supplemented with 10% dialyzed serum (cut-off: 10 kDa), 200 mg/L L-proline (only cells destined for phosphoproteome analysis), heavy (L-[13C6,15N2]-lysine (Lys8), L-[13C6,15N4]-arginine (Arg10)), medium (L-[2H4]-lysine (Lys4), L-[13C6]-arginine (Arg6)) or light (natural lysine (Lys0) and arginine (Arg0)) amino acids. Labeling efficiency and arginine–proline conversion was checked using LC-MS/MS.

Viruses. Viruses were derived from the m129-repaired MCMV strain Smith bacterial artificial chromosome (BAC) pSM3fr-MCK-2

66. Infections were carried out at 37 °C under conditions of centrifugal enhancement. In brief, after a virus

PNATURE COMMUNICATIONS | https://doi.org/10.1038/s41467-020-18542-1
ARTICLE

9
Fig. 5 M97 causes shutoff of host DNA synthesis by Cyclin A sequestration. Serum-starved 3T3 cells were infected with the indicated recombinant viruses and subjected to subcellular fractionation (a), cell cycle analysis (b, c), or confocal microscopy (d). a The levels of Cyclin A, E, and Cdk2 proteins was determined at 24 h post infection in nuclear and cytosolic fractions by immunoblotting. The soluble viral nuclear protein IE1 and the cytosolic marker GAPDH served as controls. The immunoblots are representative of three independent experiments with similar results. b The DNA content of infected cells was analyzed by propidium iodide staining followed by flow cytometry and plotted as DNA histograms. b The accumulation of viral and cellular DNA was monitored over the time course of infection. c To discriminate viral from cellular DNA replication, infected cells were treated with ganciclovir (GCV) or left untreated. d, e At 30 hpi, infected cells were pulse-labeled with EdU. EdU staining via click-chemistry (green fluorescence) served to determine sites of cellular and viral DNA synthesis. EdU was combined with immunofluorescence detection of the viral replication factor M57 (red fluorescence) that marks sites of viral DNA synthesis. DAPI was used for nuclear counterstaining (blue fluorescence). Scale bars: 10 µm. e The indicated number of cells were categorized based on the (co-)localization of EdU and M57 fluorescence within the nucleus. f Mouse embryonic fibroblasts were infected with the indicated viruses at an MOI of 0.2. At the indicated days post infection, the infectious supernatant was harvested and subjected to virus titration. Means (center of the error bars) and standard errors of the mean of n = 3 (3, 5, 7 days post infection) biological replicates are depicted. For 0 days post infection: n = 2. Two-sided t-tests without multiple hypothesis correction were performed comparing the indicated viruses at 5 days post infection.

Fig. 6 The NLS-RXL module is conserved across several cell cycle regulators. a A model summarizing the function of the NLS-RXL/Cy module in infected cells. In the absence of Cyclin A (GO/G1, early), the NLS of M97 is functional and M97 is imported into the nucleus. MCMV induces Cyclin A and drives the cell cycle towards an S-phase environment (G1/S, late). Cyclin A binds to the RXL/Cy motif on M97 and masks the NLS, leading to cytosolic localization of M97. b Conservation of RXL/Cy-NLS modules across several cellular cell cycle regulatory proteins. The depicted sequences are of human origin.
second and 25 mM Tris-HCl (pH 7.4) for the final washing step. Samples were eluted in a total volume of 0.2 mL 8 M guanidine hydrochloride at 95°C. Proteins were precipitated by adding 1.8 mL LiChrosorb ethyl acetate and 1 µL Glycolco. After incubation at 4°C overnight, samples were centrifuged for 1 h at 4°C and ethanol was decanted before samples were resolved in 6 M urea–2 M thiourea buffer. Finally, samples were reduced, alkylated, digested, and desalted as described above (phosphoproteomics).

**NanolC-MS/MS analysis.** Phosphopeptides and peptides from whole cell lysates were separated on a MonoCep C18 High Resolution 2000 column (GL Sciences) at a flow rate of 300 nL/min. 6 and 4 h gradient MS scans were performed for whole cell peptides and phosphopeptides, respectively. Peptides from HA-AP samples were separated on 45 min, 2 or 4 h gradients with a 250 nL/min flow rate on a 15 cm column (inner diameter: 75 µm), packed in-house with ReproSil-Pur C18-AQ material (Dr. Maisch GmbH). A Q Exactive Plus instrument (Thermo Fisher) was operated in the data-dependent mode with a full scan in the Orbitrap followed by MS/MS scans of M97 interactomes, full scans were performed with a resolution of 70,000, a target value of 5 × 10^6 ions, a maximum injection time of 20 ms and a 2 m/z isolation window. The MS/MS scans were performed with a 17,500 resolution, a 1 × 10^6 target value, and a maximum injection time of 120 ms. MS/MS scans were performed with a 35,000 resolution, a 5 × 10^5 target value, 160 ms maximum injection time and a 2 m/z isolation window. Data analysis was performed using MaxQuant 1.5.28 (M97 interactomes) or 1.6.0.1 (phosphoproteomics, whole cell lysates, and interactomes of HHV-CHPKs) software. Search parameters included two missed cleavage sites, fixed cysteine carbamidomethyl modification, and variable modifications including methionine oxidation, N-terminal protein acetylation, asparagine–glutamine deamidation. In addition, serine, threonine, and tyrosine phosphorylations were searched as variable modifications for phosphoproteome analysis. Arg10 and Lys10 and Arg6 and Lys4 were set as labels where appropriate. The peptide mass accuracy of mass spectra were collected with 0.20 ppm for Mascot (MacX) and 20 ppm for PMF (McCyc). The “match between runs” option was disabled and “re-quantify”, “iBAQ” (intensity-based absolute quantification) and “second peptide” options were enabled. Database search was performed using Andromeda, the integrated MaxQuant search engine, against a protein database of MCMV strain Smith and a Uniprot database of human interactome protein complexes (updated July 2015) with common contaminants. Raw data from AP–MS samples of HEK-293T cells were searched against a Uniprot database of human proteins (downloaded August 2018 or October 2016) and the sequences of transgenic HHV kinetics including common contaminants. FDR was estimated based on target-decoy competition and set to 1% at PSM, protein and modification site level.

**Bioinformatic analysis of phosphoproteome profiles.** Phosphosite data and whole proteome data were filtered to exclude contaminant, reverse hits and proteins only identified by site (that is, only identified by a modified peptide). Phosphorylation sites were ranked according to their phosphorylation localization probabilities (P) as class I (P > 0.75), class II (0.75 > P > 0.5) and class III sites (P < 0.5). Class I sites (in at least one of the replicates) were used with a multiplicity of one (that is, only one phosphorylation site on a peptide). MaxQuant normalized site ratios (from Phospho(STAT)Sites.txt file) were used and corrected by the ratio of the corresponding protein (from ProteinGroups.txt file) for the respective replicate. SILAC ratios of replicates were log2 transformed, averaged and sites were considered that were quantified in at least one of the replicates. Sites were then categorized as belonging to nuclear or cytosolic proteins, based on the GO annotation of the source protein. Source proteins and corresponding sites with no clear nuclear or cytosolic annotation were classified as “no category”. To assess differences in subcellular phosphoproteome profiles we used the average SILAC ratio of cells infected with R45A/L47A and L47A/F49A mutant viruses and compared phosphorylated sites in M97 with any possible amino acid in the region +4 to +4 (0 refers to the phosphorylated amino acid). Comparisons were considered when there were at least 19 phosphosites from both cytosolic and nuclear proteins for an amino acid at a given position quantified.

**Bioinformatic analysis of HHV-CHPK interactions.** AP–MS data were filtered as described above, ratios were log2 transformed and replicates were averaged (mean) when they were quantified in all three replicates. Two-sided one sample t-tests (null hypothesis µ = 0) were performed on the experimental data and a set of simulated data where enrichment ratios were permuted for the individual replicates (9999 permutations). The t-test p-values were then adjusted according to the permuted data. The p-values in Supplementary Data 1, Fig.1 and Supplementary Fig. 1 were adjusted in this way. Candidate interactors were selected based on a combination of adjusted p-value and means of the three replicates. To harmonize the data obtained from the different CHPK-IPs, we discriminated between candidate interactors and binding partners based on volcano plots. For all APs, we used a fixed p-value cut-off of 0.05 and a flexible SILAC fold-change cut-off according to an FDR estimation. For this, we used the simulated data as a false positive set and accepted candidate interactors above a SILAC fold-change that recalled maximum 1% false positives.

FDR calculations were based on the simulated data as false positives, as previously suggested. This yielded a set of high-confidence candidate interactors for APs with individual HHV kinases. To compare individual prey proteins across the APs with different kinases we imputed missing values with random values from a normal distribution (with mean 0 and standard deviation 0.25). Enrichment profiles were clustered using Euclidean distance and assembled into a heatmap using R. GO enrichment of the prey proteins in selected sets of clusters were performed using Metascape.

**Bioinformatic analysis of M97 interactomes.** AP–MS data were filtered as described above, ratios were log2 transformed and replicates were averaged (mean) when they were quantified in at least two of the replicates. The two-sided one sample t-tests (null hypothesis µ = 0) were performed on the experimental data and proteins were considered as interactors when they were below a t-test p-value of 0.05 and above a log2 SILAC fold-change of 1.5. Additionally, the molar amount of bait and prey proteins in MS samples was estimated by iBAQ values. Therefore, for samples where M97 was purified, the iBAQ values were summed up, sorted and log10 transformed.

**Immunoblot analysis.** Whole cells were harvested and subsequently lysed by sonication in 50 mM Tris-Cl (pH 6.8), 2% SDS, 10% glycerol, 1 mM DTT, 2 µg/mL aprotinin, 10 µg/mL leupeptin, 1 µM pepstatin, 0.1 mM Pefabloc. The lysates were clarified by centrifugation and protein concentration was measured using the Bio-Rad DC protein assay. Lysates were adjusted to equal protein concentration, supplemented with 100 mM dithiothreitol and bromophenol blue, and boiled at 95°C for 3 min. For subcellular fractionation into nuclei and cytoplasmic extracts, cells were lysed by Dounce homogenization in hypotonic buffer, consisting of 10 mM Hepes (pH 8.0), 10 mM KCl, 1.5 mM MgCl₂, 0.34 M sucrose, 10% glycerol, 0.1 mM DTT and protease inhibitors. Nuclei were isolated by hand by low-speed centrifugation (4 min, 1300 g, 4°C). The supernatant was clarified by high-speed centrifugation (15 min, 20,000 g, 4°C). The nuclei were washed once in the hypotonic extraction buffer and lysed then as described above for the preparation of whole cell lysates. Proteins were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) and blotted to polyvinylidene fluoride membranes. To prove identity of membranes, binding, blots were treated with Triton-X-100 before probing with an antibody specific to the target protein. Protein bands were visualized using a chemiluminescence detection system (Thermo Fisher). All antibodies were diluted to 1 µg/mL in TBS, 5% skim milk. Uncropped scans of the immunoblots are provided in the Source Data file.

**Immunoprecipitation.** Cells were lysed by freezing-thawing in IP buffer (IPB): 50 mM Tris-Cl pH 7.4, 150 mM NaCl, 10 mM MgCl₂, 10 mM NaF, 0.5 mM Na3VO4, 0.5% Nonidet P-40, 10% glycerol, 1 mM DTT, 2 µg/mL aprotinin, 1 mM leupeptin, 1 mM Pefabloc. Cell extracts were clarified by centrifugation at 20,000 g. Cyclin A IPs were performed by incubating the IP extracts with Cyclin A, H432, conjugated to agarose beads (Santa Cruz) for 1 h at 4°C. For HA IPs, a γ-32P-AHis-tag isolation kit (Miltenyi Biotec) was employed according to the manufacturer’s instructions, except that IPB was used as both lysis and washing buffer.

**Kinase assay.** First, HA–M97 was immunoprecipitated from infected cells. To this end, IP extracts were prepared and incubated with HA antibody clone 3F10 (1 µg per mL) and Protein G-conjugated agarose beads. The precipitates were washed several times with IPB containing 0.1% Triton X-100 and 0.1 M MgCl₂, 1 mM DTT. Then, they were incubated under constant agitination for 60 min at 30°C in kinase reaction buffer: 20 mM Tris-HCl (pH 7.4), 10 mM MgCl₂, 1 mM DTT, 10 mM ATP, 100 mCi [γ-32P]ATP, 0.025% Nonidet P-40, 10% glycerol, 1 mM DTT, 2 µg/mL aprotinin, 1 mM leupeptin, 1 mM Pefabloc. Cell extracts were clarified by centrifugation at 20,000 g. Cyclin A IPs were performed by incubating the IP extracts with Cyclin A, H432, conjugated to agarose beads (Santa Cruz) for 1 h at 4°C. For HA IPs, a γ-32P-AHis-tag isolation kit (Miltenyi Biotec) was employed according to the manufacturer’s instructions, except that IPB was used as both lysis and washing buffer.

**Kinase assay.** First, HA–M97 was immunoprecipitated from infected cells. To this end, IP extracts were prepared and incubated with HA antibody clone 3F10 (1 µg per mL) and Protein G-conjugated agarose beads. The precipitates were washed several times with IPB containing 0.1% Triton X-100 and 0.1 M MgCl₂, 1 mM DTT. Then, they were incubated under constant agitination for 60 min at 30°C in kinase reaction buffer: 20 mM Tris-HCl (pH 7.4), 10 mM MgCl₂, 1 mM DTT,
10 mM β-glycerophosphate, 50 mM ATP, 5 μCi [γ-32P]ATP. Kinase reactions were analyzed by 8% SDS-PAGE followed by autoradiography.

Immunofluorescence microscopy. 3T3 fibroblasts were grown and infected on glass coverslips. Where indicated, cells were incubated with 10 μM 5-ethyl-2'-deoxyuridine (EdU) for 30 min. To analyze for M79 localization and sites of EdU incorporation, coverslips were washed with PBS and incubated for 10 min in PBS-4% paraformaldehyde fixation solution, followed by additional washing and incubation in PBS-T permeabilization solution (PBS, 0.1% Triton X-100, 0.05% Tween 20) and 2% bovine serum albumin (BSA) fraction V/ PBS-T blocking solution. Afterwards, incorporated EdU was conjugated to Alexa Fluor 488 using the Click-IT EdU labeling kit (Thermo Fisher). Then samples were incubated overnight at 4 °C with the following antibodies: anti-HA clone 3F10 or anti-M57 clone M57.02 (Center for Proteomics, Rijeka), both diluted to 1 μg mL−1 in PBS-T containing 2% BSA. After washing in PBS, cells were incubated for 1 h at 25 °C with Alexa Fluor 488 or 647-coupled anti-lgG antibodies (Thermo Fisher). Coverslips were mounted on glass slides in 4',6-diamidino-2-phenylindole (DAPI) containing Fluoromount-G medium (Thermo Fisher). Images were acquired by an Eclipse Ti series scanning microscope, using NIS-Elements software (Nikon Instruments). Equal microscope settings and exposure times were used to allow direct comparison between samples. For quantification, the microscope slides were randomly scanned and all cells in the randomly acquired frames were analyzed by ImageJ.

Flow cytometry. Cells were fixed and permeabilized in ice-cold PBS-80% ethanol for at least 16 h. After washing, cells were incubated on ice for at least 16 h with one of the following primary antibodies: anti-IE1 (clone Croma 101) or anti M57 (clone M57.02), both diluted to 1 μg mL−1 in PBS-1% BSA. After washing, cells were incubated in Alexa Fluor 647 conjugated anti-mouse IgG for 1 h at 25 °C. Cells were washed again and incubated then for 15 min at 25 °C in PBS supplemented with 0.1 mg mL−1 RNase A and 25 μg mL−1 propidium iodide. All washing steps and antibody dilution were performed using PBS-1% BSA. Flow cytometry was performed using a FACSCanTI instrument equipped with FACSData and CellQuest Pro software (Becton Dickinson).

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
MS raw data and MaxQuant output tables have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD016334. URL. The source data underlying Figs. 1e, 2b, 3b–d, 4b, 5a, e, f and Supplementary Figs. 1a, 3c–f and 10b are provided as a Source Data file. The source data underlying Figs. 1b–d, 3a, 4c and Supplementary Figs. 1a, c, 3b, 6b–e, 8b, c are provided in Supplementary Data files 1–4. Fasta files for proteomic searches were downloaded from Uniprot (https://www.uniprot.org/). All other relevant data are available from the corresponding author upon reasonable request. Source data are provided with this paper.

Received: 17 December 2019; Accepted: 24 August 2020; Published online: 24 September 2020.

References
1. Adler, B., Sattler, C. & Adler, H. Herpesviruses and their host cells: a successful liaison. Trends Microbiol. 25, 229–241 (2017).
2. Mocarski, E. S. Jr. in Human Herpesviruses: Biology, Therapy, and Immunopathophysiology Ch. 4 (eds Arvin, A. et al.) (Cambridge Univ. Press, 2011).
3. Gershburg, E. & Pagano, J. S. Conserved herpesvirus protein kinases. Biochim. Biophys. Acta 1784, 203–212 (2008).
4. Li, R. & Hayward, S. D. Potential of protein kinase inhibitors for treating herpesvirus-associated disease. Trends Microbiol. 21, 286–295 (2013).
5. Topalis, D., Gillemot, S., Snoeck, R. & Andrei, G. Thymidine kinase and cyclin-dependent kinase-like function is shared by the beta- and gamma- subset of the conserved herpesvirus protein kinases. PLoS Pathog. 6, e1001092 (2010).
6. Hamirally, S. et al. Viral mimicry of Cdc2/cyclin-dependent kinase 1 mediates disruption of nuclear lamina during human cytomegalovirus nuclear egress. PLoS Pathog. 5, e1000275 (2009).
7. Rokman, D. et al. Analysis of the structure–activity relationship of four herpesviral UL97 subfamily protein kinases reveals partial but not full functional conservation. J. Med. Chem. 49, 7044–7053 (2006).
8. Li, C.-P. et al. Epstein–Barr virus BGLF4 kinase induces premature chromosome condensation through activation of condensin and topoisomerase II. J. Virol. 81, 5166–5180 (2007).
9. Hume, A. J. et al. Phosphorylation of retinoblastoma protein by viral protein with cyclin-dependent kinase function. Science 320, 797–799 (2008).
10. Steingruber, M. et al. Cyclins B1, T1 and H differ in their molecular mode of interaction with cytomegalovirus protein kinase UL97. J. Biol. Chem. 294, 6188–6203 (2019).
11. Preichle, N. M. et al. Human cytomegalovirus UL97 kinase activity is required for the hyperphosphorylation of retinoblastoma protein and inhibits the formation of nuclear aggresomes. J. Virol. 82, 5054–5067 (2008).
12. Zhang, K., Lv, D.-W. & Li, R. Conserved herpesvirus protein kinases target SAMHD1 to facilitate virus replication. Cell Rep. 28, 449–459.e5 (2019).
13. Busunger, R. et al. Human cytomegalovirus overcomes SAMHD1 restriction in macrophages via pUL97. Nat. Microbiol. 4, 2260–2272 (2019).
14. Milbradt, J. et al. The prolyl isomerase Pin1 promotes the herpesvirus-induced phosphorylation-dependent disassembly of the nuclear lamina required for nucleocytoplasmic egress. PLoS Pathog. 12, e1005825 (2016).
15. Deutschmann, J. et al. A viral kinase counterattacks in vivo restriction of murine cytomegalovirus by SAMHD1. Nat. Microbiol. 4, 2272–2284 (2019).
16. Iwahori, S., Hakki, M., Chou, S. & Kalejta, R. F. Molecular determinants for the inactivation of the retinoblastoma tumor suppressor by the viral cyclin-dependent kinase UL97. J. Virol. 80, 19666–19680 (2015).
17. Li, R. et al. SUMO binding by the Epstein-Barr virus protein kinase BGLF4 is crucial for BGLF4 function. J. Virol. 86, 5412–5421 (2012).
18. Avey, D. et al. Discovery of a corollary interaction between Kaposi’s sarcoma-associated herpesvirus ORF45 and the viral protein kinase ORF36. J. Virol. 90, 5959–5964 (2016).
19. Uetz, P. et al. Herpesviral protein networks and their interaction with the human proteome. Science 319, 239–242 (2008).
20. Calderwood, M. A. et al. Epstein–Barr virus and virus human protein interaction maps. Proc. Natl Acad. Sci. USA 104, 7606–7611 (2007).
21. Li, R. et al. Phosphoproteomic profiling reveals Epstein–Barr virus protein kinase gene integration of DNA damage response and mitotic signaling. PLoS Pathog. 11, e1005346 (2015).
22. Umaña, A. C., Iwahori, S. & Kalejta, R. F. Direct substrate identification with an analog sensitive (AS) Viral Cyclin-dependent Kinase (v-Cdk). ACS Chem. Biol. 11, 189–199 (2016).
23. Iwahori, S., Umaña, A. C., VanDeusen, H. R. & Kalejta, R. F. Human cytomegalovirus-encoded viral cyclin-dependent kinase (v-CDK) UL97 phosphorylates and inactivates the retinoblastoma protein-related p107 and p130 proteins. J. Biol. Chem. 292, 6583–6599 (2017).
24. Taipale, M. et al. Quantitative analysis of HSP90-client interactions reveals principles of substrate recognition. Cell 150, 987–1001 (2012).
25. Sun, X. et al. Hsp90 inhibitor 17-DMAG decreases expression of conserved herpesvirus protein kinases and reduces virus production in Epstein-Barr virus-infected cells. J. Virol. 87, 10126–10138 (2013).
26. Schulman, B. A., Lindstrom, D. L. & Harlow, E. Substrate recruitment to cyclin-dependent kinase 2 by a multipurpose docking site on cyclin A. Proc. Natl Acad. Sci. USA 95, 10453–10458 (1998).
27. Isogawa, Y. et al. Characterization of the human herpesvirus 6 U69 gene product and identification of its nuclear localization signal. J. Virol. 82, 710–718 (2008).
28. Deng, G. & Stammers, K. Mapping of nuclear localization signals by simultaneous fusion to green fluorescent protein and to beta-galactosidase. Biotechniques 26, 858–862 (1999).
37. Muranyi, W., Haas, J., Wagner, M., Krohne, G. & Koszinowski, U. H. Cytomegalovirus recruitment of cellular kinases to dissolve the nuclear lamina. Science 297, 854–857 (2002).

38. Marceau, A. H. et al. Structural basis for LIN54 recognition of CHR elements. Nature 589, 279–282 (2020).

39. Iwahori, S. & Kaleja, R. F. Phosphorylation of transcriptional regulators in the retinoblastoma protein pathway by UL97, the viral cyclin-dependent kinase encoded by murine cytomegalovirus. Virology 325, 95–103 (2004).

40. Girard, F., Strausfeld, U., Fernandez, A. & Lamb, N. J. Cyclin A is required for the onset of DNA replication in mammalian fibroblasts. Cell 67, 1169–1179 (1991).

41. Cardoso, M. C., Leonhardt, H. & Nadal-Ginard, B. Reversal of terminal differentiation and control of DNA replication: cyclin A and CDK2 specifically localize at subnuclear sites of DNA replication. Cell 74, 979–992 (1993).

42. Maridor, G., Gallant, P., Golsteyn, R. & Nigg, E. A. Nuclear localization of vertebrate cyclin A correlates with its ability to form complexes with cdk catalytic subunits. J. Cell Sci. 106, 535–544 (1993).

43. Wiebusch, L., Neuwirth, A., Gräbhenrich, L., Voigt, S. & Hagemeier, C. Cell cycle-independent expression of immediate-early gene 3 results in G1 and G2 arrest in murine cytomegalovirus-infected cells. J. Virol. 82, 10188–10198 (2008).

44. Wagner, M. et al. Comparison between human cytomegalovirus pUL97 and murine cytomegalovirus (MCMV) pM97 expressed by MCMV and vaccinia virus: pM97 does not confer ganciclovir sensitivity. J. Virol. 74, 10729–10736 (2000).

45. Adams, P. D. et al. Identification of a cyclin-CDK2 recognition motif present in substrates and p21-like cyclin-dependent kinase inhibitors. Mol. Cell. Biol. 16, 6623–6632 (1996).

46. Eifler, M. et al. PUL21a-Cyclin A2 interaction is required to protect human cytomegalovirus-infected cells from the deleterious consequences of mitotic entry. PLoS Pathog. 10, e1004514 (2014).

47. Caffarelli, N., Fehr, A. R. & Yu, D. Cyclin A degradation by primate T. Trendsto Biochem. Sci. 16, 459–459 (1993).

48. Webel, R. et al. Nuclear import of isoforms of the cytomegalovirus kinase pUL97 is mediated by differential activity of NLS1 and NLS2 both acting through classical importin-α/binding. J. Gen. Virol. 93, 1756–1768 (2012).

49. Chemerda, L. B., de Prat-Gay, G. & Sánchez, I. E. Convergent evolution and mimicry of protein linear motifs in host-pathogen interactions. Curr. Opin. Struct. Biol. 32, 91–101 (2015).

50. Davey, N. E., Trévé, G. & Gibson, T. J. How viruses hijack cell regulation. Trends Biochem. Sci. 36, 159–169 (2011).

51. Lange, A. et al. Classical nuclear localization signals: definition, function, and interaction with importin alpha. J. Biol. Chem. 282, 5101–5105 (2007).

52. Christie, M. et al. Structural biology and regulation of protein import into the nucleus. J. Mol. Biol. 428, 2060–2090 (2016).

53. Zackshenaus, E., Bremmer, R., Phillips, R. A. & Gallie, B. L. A bipartite nuclear localization signal in the retinoblastoma gene product and its importance for biological activity. Mol. Cell. Biol. 13, 4588–4599 (1993).

54. Adams, P. D. et al. Retinoblastoma protein contains a C-terminal motif that targets it for phosphorylation by cyclin-CDK complexes. Mol. Cell. Biol. 19, 1068–1080 (1999).

55. Rodríguez-Vilarupla, A. et al. Identification of the nuclear localization signal of p21cip1 and consequences of its mutation on cell proliferation. FEBS Lett. 531, 319–323 (2002).

56. Krek, W. et al. Negative regulation of the growth-promoting transcription factor E2F-1 by a stably bound cyclin A-dependent protein kinase. Cell 78, 161–172 (1994).

57. Müller, H. et al. Induction of S-phase entry by E2F transcription factors depends on their nuclear localization. Mol. Cell. Biol. 17, 5508–5520 (1997).

58. Sugimoto, N. et al. Cdk1 phosphorylation by cyclin A-dependent kinases negatively regulates its function without affecting geminin binding. J. Biol. Chem. 279, 19691–19697 (2004).

59. Nishiyama, H., Lygerou, Z. & Nishimoto, T. Protein lysine methylation by recruitment of Ccr4 loss. Nat. Struct. Mol. Biol. 18, 582–589 (2011).

60. Jiao, W. et al. Alleviation of perturbation of SUMOylation on the retinoblastoma tumor suppressor protein in human cancer correlates with moderate/poor tumor differentiation. Oncogene 27, 3156–3164 (2008).

61. Coqueret, O. New roles for p21 and p27 cell-cycle inhibitors: a function for each cell compartment? Trends Cell Biol. 13, 65–70 (2003).

62. Petersen, B. O., Lukas, J., Sörensen, C. S., Bartek, J. & Helin, K. Phosphorylation of mammalian CDC6 by cyclin A/CDK2 regulates its subcellular localization. EMBO J. 18, 396–410 (1999).

63. Rauwel, B. et al. Release of human cytomegalovirus from latency by a KAP1/TRIM28 phosphorylation switch. eLife 4, e06086 (2015).

64. Svrčanská, A. et al. A noncanonical function of polycomb repressive complexes promotes human cytomegalovirus lytic DNA replication and serves as a novel cellular target for antiviral intervention. J. Virol. 93, e02143–18 (2019).

65. Rai, T. S. et al. Histone chaperone HIRA Deposits histone H3.3 onto viral DNA and contributes to anti-viral intrinsic immunity. Nucleic Acids Res. 45, 11673–11683 (2017).

66. Jordan, J. S. et al. Virus progeny of murine cytomegalovirus bacterial artificial chromosome pSM36 show reduced growth in salivary Glands due to a fixed mutation of MCK-2. J. Virol. 85, 10346–10353 (2011).

67. Tischer, B. K., Smith, G. A. & Osterrieder, N. En passant mutagenesis: a two step markerless red recombination method. Methods Mol. Biol. 634, 421–430 (2010).

68. Cox, J. & Mann, M. MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. Nat. Biotechnol. 26, 1367–1372 (2008).

69. Tyanova, S. et al. The Perseus computational platform for comprehensive analysis of (pro)teomics data. Nat. Methods 13, 731–740 (2016).

70. Zhou, Y. et al. Metascape provides a biologist-oriented resource for the analysis of systems-level datasets. Nat. Commun. 10, 1523 (2019).