Identification of a Novel Protein Interaction Motif in the Regulatory Subunit of Casein Kinase 2

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Casein kinase 2 (CK2) regulates multiple cellular processes and can promote oncogenesis. Interactions with the CK2 β regulatory subunit of the enzyme target its catalytic subunit (CK2 α or CK2 α') to specific substrates; however, little is known about the mechanisms by which these interactions occur. We previously showed that by binding CK2 β , the Epstein-Barr virus (EBV) EBNA1 protein recruits CK2 to promyelocytic leukemia (PML) nuclear bodies, where increased CK2-mediated phosphorylation of PML proteins triggers their degradation. Here we have identified a KSSR motif near the dimerization interface of CK2 β as forming part of a protein interaction pocket that mediates interaction with EBNA1. We show that the EBNA1-CK2 β interaction is primed by phosphorylation of EBNA1 on S393 (within a polyserine region). This phosphoserine is critical for EBNA1-induced PML degradation but does not affect EBNA1 functions in EBV replication or segregation. Using comparative proteomics of wild-type (WT) and KSSR mutant CK2 β , we identified an uncharacterized cellular protein, C18orf25/ARKL1, that also binds CK2 β through the KSSR motif and show that this involves a polyserine sequence resembling the CK2 β binding sequence in EBNA1. Therefore, we have identified a new mechanism of CK2 interaction used by viral and cellular proteins.

Casein kinase 2 (CK2) is a highly conserved pleiotropic kinase whose functions and regulation are only partly understood (1, 2). The CK2 holoenzyme exists in a heterotetrameric complex comprised of two catalytic subunits (CK2 α and/or CK2 α') and a dimer of two regulatory (CK2 β) subunits. CK2 has over 300 known substrates and is implicated in an array of cellular processes, including cell proliferation, cell cycle progression, apoptosis, DNA damage responses, transcription, and circadian rhythm (3, 4). It is also clear that there is a strong link between CK2 and cancer, as CK2 activity and levels are commonly elevated in tumor cells (4, 5). In addition, overexpression of the catalytic CK2 α subunit has been shown to induce the development of mammary tumors and lymphomas in transgenic mice (6–8). These effects likely stem from the roles of CK2 in regulating the activities and/or stabilities of several tumor suppressor and proto-oncogenic proteins, including p53, promyelocytic leukemia protein (PML), PTEN, and NF- κ B (9–16). CK2 negatively regulates the levels of PML proteins by phosphorylation of Ser517, which triggers their subsequent polyubiquitylation and degradation by the proteasome (12). Since PML proteins form the basis of PML nuclear bodies (NBs), which mediate several processes, including apoptosis and DNA repair (reviewed in reference 17), CK2 can impact these processes partly through its effect on PML.

CK2 is also a common player in viral infections, as a number of viruses (e.g., human papillomavirus [HPV], human immunodeficiency virus [HIV], hepatitis B and C viruses, and several herpesviruses) use CK2 for various aspects of their life cycle (reviewed in reference 18). For example, infection by herpes simplex virus 1 (HSV-1) stimulates CK2 activity, and the immediate early viral protein ICP27 translocates the CK2 holoenzyme into the cytoplasm to phosphorylate hnRNP K to facilitate the viral lytic cycle (19). In addition, CK2 phosphorylates several viral proteins, including Rev from HIV-1, ICP27 and VP16 from HSV-1, NS2 from hepatitis C virus, and BZLF1 from Epstein-Barr virus (EBV) to facilitate their functions in viral infection (20–24).

Through proteomic experiments, we previously revealed a link between the EBV EBNA1 protein and CK2 (25, 26). EBNA1 is

critical for EBV latent infection due to its roles in replicating and segregating the viral genomes in proliferating host cells, as well as in transactivating the expression of other EBV latency genes (reviewed in reference 27). In addition, several recent studies have identified multiple roles for EBNA1 in altering cellular pathways in ways that promote cell proliferation and survival, which may contribute to the development of EBV-associated cancers (reviewed in reference 28). This includes the findings that EBNA1 induces the degradation of PML proteins in multiple carcinoma cell lines, leading to loss of PML NBs that would otherwise promote apoptosis and suppress viral lytic infection (29–31). In keeping with this finding, EBNA1 has been shown to have an antiapoptotic effect and to positively contribute to EBV lytic infection in PML-positive but not PML-negative epithelial cells (29, 31, 32). EBNA1-induced PML loss also appears to occur in EBV-associated carcinomas, as EBV-positive gastric tumors were found to have considerably less PML staining than EBV-negative gastric tumors (32). Studies on the mechanism of EBNA1-mediated PML loss showed that EBNA1 binding to CK2 was critical for this effect and that EBNA1 recruited the CK2 holoenzyme to PML NBs, which resulted in increased phosphorylation of S517 of PML by CK2 (33), a trigger for polyubiquitylation and degradation (12). In addition, the interaction of EBNA1 with CK2 was shown to involve direct EBNA1 binding to the CK2 β subunit through a serine-rich region in EBNA1 between amino acids 387 and 394.

Unlike the regulatory subunits of other hetero-oligomeric kinases (e.g., cyclin-dependent kinases [CDKs] and protein kinase A

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[PKA]), the CK2 β regulatory subunit is not required for the activity of the catalytic subunits (34). Rather, CK2 β is generally thought to recruit substrates and/or regulators of the CK2 complex (35, 36). For example, CK2 β targets the CK2 catalytic activity to p53 and topoisomerase II (37, 38). However, despite the importance of CK2 β for targeting CK2 activity, in most cases, little is known about how specific proteins interact with CK2 β .

In this study, we have identified a novel binding pocket in CK2 β (based on a KSSR motif) that mediates interaction with EBNA1. This interaction is primed by phosphorylation of EBNA1 on S393 in the CK2 binding region and affects the ability of EBNA1 to induce PML degradation. Using comparative proteomics of the wild type (WT) and a KSSR mutant of CK2 β , we find that the KSSR motif is required for a small subset of CK2 β cellular protein interactions. The most prominent of these interactions is with a functionally uncharacterized cellular protein, C18orf25/ARKL1, which binds the KSSR binding pocket of CK2 β by using a serine-rich sequence similar to that in EBNA1. Therefore, we have identified a previously unknown mechanism of CK2 interaction used by viral and cellular proteins.

MATERIALS AND METHODS

Cell lines. The EBV-negative nasopharyngeal carcinoma cell line CNE2Z (39) was propagated in alpha minimal essential media (α MEM; Gibco) supplemented with 10% fetal calf serum (Gibco). BJAB cells (EBV-negative B cells) (40) and 293T cells were grown in RPMI (Invitrogen) and Dulbecco's modified Eagle's medium (DMEM) (Gibco), respectively, with 10% fetal calf serum (Invitrogen).

Plasmids. Plasmids expressing C-terminally sequential purification affinity (SPA)-tagged EBNA1 or LacZ (pMZS3F-EBNA1 or pMZS3F-lacZ) were previously described (33). Untagged EBNA1 was expressed from the pc3oriP plasmid (referred to as pc3oriPE), which contains the EBV *oriP* element, as was an EBNA1 mutant lacking the central Gly-Arg repeat (pc3oriPE Δ 325–376). The construction of these plasmids was previously described (41, 42). All of the EBNA1 point mutants used in this study were generated by QuikChange site-directed mutagenesis (Stratagene) of pMZS3F-EBNA1 and pc3oriPE Δ 325–376 and were sequenced to verify the mutation. A plasmid expressing N-terminally FLAG-tagged CK2 β was generated by PCR amplification of the CK2 β cDNA from pGEX3X-CK2 β (43) (kindly provided by David Litchfield) and insertion between the BamHI and XhoI sites of pcDNA5-FLAG plasmid (Invitrogen) (kindly provided by Anne-Claude Gingras). CK2 β with the KSSR sequence mutated to AAAA was generated by gene synthesis of the CK2 β cDNA sequence (Life Technology), which was inserted between the BamHI and XhoI sites of the pcDNA5-FLAG plasmid. A glutathione S-transferase (GST)-tagged version of the KSSR mutant was subsequently generated by PCR amplification of CK2 β KSSR mutant cDNA from the pcDNA5-FLAG and insertion between the XmaI and NotI sites of pGEX3X. All of the CK2 β plasmids were verified by DNA sequencing. A plasmid expressing N-terminally Myc-tagged ARKL1 was generated by PCR amplification of the C18orf25 cDNA sequence in pOTB7 (IMAGE ID 4040087) from the Mammalian Genome Consortium Library (TCAG Genome Resource Facility, Hospital for Sick Children), and insertion between the HindIII and NotI sites in pCMV-Myc. pCMV-myc was generated by (i) by inserting an N-terminal myc tag between the NheI and BglII sites and (ii) replacing the NotI-BamHI fragment of pEYFP-N1 (Clontech), containing the C-terminal yellow fluorescent protein (YFP) tag, with a NotI-BamHI fragment containing a triple FLAG tag. pCMV-Myc-ARKL1 contains a stop codon before the FLAG tag so that ARKL1 is expressed with only an N-terminal Myc tag. Myc-tagged ARKL1 lacking amino acids 202 to 225 (Δ S) was generated by gene synthesis (Life Technology) of the cDNA encoding the N-terminal portion of ARKL1 up to the endogenous BamHI site. This sequence was then used to replace the HindIII-BamHI fragment in pCMV-Myc-ARKL1. The ARKL1 sequence in the generated plasmids was verified by DNA sequencing.

Western blotting and antibodies. Protein samples were subjected to SDS-PAGE and transferred to nitrocellulose. Membranes were blocked in 5% nonfat dry milk in Tris-buffered saline (TBS) and then incubated with antibodies against CK2 β (sc-20710, 1:500 dilution [Santa Cruz Biotechnology]), CK2 α (ab10466 – 50; 1:5,000 dilution [Abcam]), EBNA1 (R4 rabbit serum against full-length EBNA1; 1:2,000 dilution) (25), USP7 (rabbit serum against full-length USP7; 1:1,000 dilution) (44), PML (A301-167A; 1:2,000 dilution [Bethyl]), phosphoserine 517 of PML (1:500 dilution) (12) (kindly supplied by Pier Pandolfi), actin (sc-1616; 1:1,000 dilution [Santa Cruz Biotechnology]), and myc (sc-789; 1:2,000 dilution [Santa Cruz Biotechnology]). After washing, blots were probed with goat anti-rabbit peroxidase (1:5,000 dilution [Santa Cruz Biotechnology]) and developed using chemiluminescence reagents (ECL enhanced chemiluminescence; PerkinElmer).

IP of EBNA1 and EBNA1 mutants. For immunoprecipitation (IP), 293T cells (7×10^6 cells) in a 10-cm-diameter dish were transfected with 4 μ g of pMZS3F-EBNA1 plasmids (with the indicated EBNA1 mutations) or pMZS3F-lacZ (negative control) using Lipofectamine 2000 (Invitrogen), as per the manufacturer's directions (41). Cells were moved to a 15-cm-diameter dish after 24 h and harvested 72 h posttransfection. After being washed in phosphate-buffered saline (PBS), the cells were lysed on ice for 20 min in a $5 \times$ volume of radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl [pH 8], 150 mM NaCl, 0.1% sodium deoxycholate, 0.5% NP-40, 2 mM EDTA) with Complete protease inhibitors (P8340 [Sigma]), followed by sonication and centrifugation. One milligram of each clarified lysate was incubated with 30 μ l of M2 anti-FLAG resin (Sigma) for 2 h at 4°C with mixing. The resin was harvested by centrifugation, washed in RIPA buffer, and then boiled in SDS loading buffer. Immunoprecipitated proteins were separated by SDS-PAGE and analyzed by Western blotting, as described above.

Analysis of effect of S393 mutations on the EBNA1 phosphoshift. A total of 1.5×10^6 293T or CNE2Z cells in a 6-cm-diameter dish were transfected with 2 μ g of pc3oriPE Δ 325–376 (expressing a version of EBNA1 that migrates true to its size in SDS-PAGE) or with the same construct containing S393A, S393D, or S393T point mutations, using Lipofectamine 2000. Twenty-four hours later, cells were washed in PBS and then lysed in a mixture containing 150 mM NaCl, 20 mM Tris (pH 7.5), 30 mM MgCl₂, 0.5% NP-40, 0.5 mM, 1 mM dithiothreitol (DTT), and Complete protease inhibitors (P8340 [Sigma]). Lysates were clarified by centrifugation. Five micrograms of each clarified lysate was then incubated for 15 min at 37°C either with 10 U calf intestinal alkaline phosphatase (CIP; NEB) or with the same amount of CIP that had been heat inactivated by boiling for 2 min at 100°C. Samples were then analyzed by 13% SDS-PAGE, followed by Western blotting with anti-EBNA1 antibody.

IP of endogenous CK2 β . 293T cells were transfected with pc3oriPE Δ 325–376 expressing EBNA1 Δ 325–376 as described above for EBNA1 IPs. Seventy-two hours posttransfection, cells were lysed in hypotonic buffer (20 mM Tris [pH 8.0], 2 mM MgCl₂, 50 mM sodium bisulfate, 8.6% sucrose, 0.1% Triton, and Complete protease inhibitors) with Dounce homogenization. The nuclei were harvested by centrifugation at $300 \times g$ for 20 min and then extracted in a $2 \times$ volume of RIPA buffer for 20 min on ice. After centrifugation, 1.5 mg of the supernatant was added to 25 μ l protein A/G-agarose beads (Santa Cruz Biotechnology) pre-coupled to 1.0 μ g of rabbit CK2 β antibody (A301-984A [Bethyl]) or to 1.0 μ g rabbit total IgG (negative control [Santa Cruz Biotechnology]) and mixed for 4 h at 4°C. Beads were collected by centrifugation, washed in RIPA buffer, and where indicated, incubated with 30 U of CIP phosphatase (NEB) for 15 or 30 min at 37°C. Proteins were eluted from the beads by being boiled in SDS loading buffer and were analyzed by SDS-PAGE and Western blotting for CK2 β and EBNA1.

IP and Western blotting of PML proteins. For Western blots examining the effect of EBNA1 on PML proteins, CNE2Z cells were transfected with pc3oriP, pc3oriPE, or pc3oriPE-S393A, and 48 h posttransfection, cells were lysed in a mixture containing 9 M urea and 5 mM Tris-HCl (pH

6.8), followed by sonication. Fifty micrograms of each clarified lysate was analyzed by SDS-PAGE and Western blotting. For PML IP experiments, CNE2Z cells were transfected with pc3oriP, pc3oriPE, or pc3oriPE-S393A, and 24 h posttransfection, cells were lysed in IP buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM MgCl₂, 10% glycerol, 1% Triton X-100) on ice for 30 min. After centrifugation, 2 mg of the supernatant was added to 40 μ l rabbit ExactaCruz beads (Santa Cruz Biotechnology) precoupled to 0.5 μ g of rabbit PML antibody (Bethyl) and mixed for 4 h at 4°C. Beads were collected by centrifugation and washed in IP buffer, and proteins were eluted by boiling the beads in SDS loading buffer. Eluted proteins were analyzed by SDS-PAGE and Western blotting for PML, phospho-PML (on pS517), CK2 β , and USP7, as described above.

IP of FLAG-CK2 β . For CK2 β co-IPs with EBNA1, 7×10^6 293T cells in a 10-cm dish were cotransfected with 4 μ g of pcDNA5 or pcDNA5-FLAG-CK2 β (WT or KSSR mutant) and 4 μ g of pc3oriPE by using PolyJet (FroggaBio). Cells were propagated and lysed 72 h posttransfection, as for the EBNA1 IPs described above. Three hundred micrograms of clarified lysate was either incubated with 10 U of FastAP alkaline phosphatase (Thermo Scientific) for 30 min at 37°C (reaction stopped by addition of 20 mM NaF) or left untreated. In both cases, lysates were incubated with 20 μ l of M2 anti-FLAG resin for 2 h at 4°C with mixing. Resin was harvested by centrifugation, washed in RIPA buffer, and then boiled in SDS loading buffer. Recovered proteins were separated by SDS-PAGE and analyzed by Western blotting, as described above. For CK2 β co-IPs with ARKL1, 7×10^6 293T cells in a 10-cm dish were cotransfected with 4 μ g of pcDNA5 or pcDNA5-FLAG-CK2 β (WT or KSSR mutant) and 4 μ g of pCMV-myc-ARKL1 (WT or Δ S mutant, as indicated), using PolyJet. Cell lysates were either treated with FastAP alkaline phosphatase, as described above (see Fig. 7E), or left untreated, and then IPs were performed with anti-FLAG resin, as described above.

Immunofluorescence microscopy. For EBNA1 localization in epithelial cells, 6×10^5 CNE2Z cells grown on a polylysine-treated coverslip in 1 well of a 6-well dish were transfected with 2 μ g of pMZS3F-EBNA1 with or without the S393A mutation, using PolyJet transfection reagent, and fixed 24 h after transfection. For EBNA1 localization in B cells, 1×10^6 BJAB cells grown on a polylysine-treated coverslip in 1 well of a 6-well dish were infected with an adenovirus expressing EBNA1 (described in reference 26) or the S393A mutant of EBNA1. Cells were fixed 5 days postinfection. For PML experiments, 6×10^5 CNE2Z cells grown on a coverslip as described above were transfected with 2 μ g of pc3oriPE using PolyJet or with the same plasmids with the S393A or S393T mutation. Cells were fixed 24 h after transfection with 3.7% formaldehyde in PBS for 20 min, rinsed twice in PBS, and permeabilized with 1% Triton X-100 in PBS for 5 min. Samples were blocked with 4% bovine serum albumin (BSA) in PBS followed by incubation with primary antibodies against either EBNA1 (R4 rabbit serum at 1:300 dilution) (25), PML (PG-M3 at 1:50 dilution [Santa Cruz Biotechnology]), or Nm23 (Santa Cruz sc-343 at 1:50 dilution). The cells were then incubated with the secondary antibodies goat anti-rabbit antibody–Alexa Fluor 555 (Molecular Probes) and goat anti-mouse antibody–Alexa Fluor 488 (Molecular Probes) in 4% BSA. For ARKL1 localization, 6×10^5 CNE2Z cells grown on a coverslip were transfected with 2 μ g pCMVmycARKL1 or pCMVmycARKL1 Δ S and then fixed as for PML experiments and stained using primary antibodies against myc (sc70463 at 1:500 [Santa Cruz Biotechnology]) and PML (A301-167A at 1:500 [Bethyl]), as well as goat anti-rabbit antibody–Alexa Fluor 488 (Molecular Probes) and goat anti-mouse antibody–Alexa Fluor 647 (Molecular Probes) secondary antibodies. For all samples, coverslips were mounted onto slides using ProLong Gold antifade medium containing DAPI (4',6-diamidino-2-phenylindole) (Invitrogen). Images were obtained using the 40 $\times$ oil objective on a Leica inverted fluorescence microscope and processed using OpenLAB (version 4.0) software. PML NBs were quantified by counting all visible PML foci in 100 cells.

EBNA1 replication and plasmid maintenance assays. CNE2Z cells were plated at 1×10^6 cells per plate in three 6-cm plates. Twenty-four hours later, cells were transfected with 2 μ g pc3oriP (negative control),

pc3oriPE, or pc3oriPE with the S393A mutation, using Lipofectamine 2000. Transfected cells were grown for 3 days prior to harvesting of 5×10^6 cells for the replication assay. The remaining cells were propagated (without selection) for a total of 2 weeks prior to harvesting for plasmid maintenance assays. Plasmids were isolated from equal cell numbers (5×10^6 cells for replication assays and 1×10^7 cells for plasmid maintenance assays), linearized with XhoI, DpnI digested (with 10% kept back as input control without DpnI digestion), and Southern blotted, as previously described (41).

GST pulldown assays. GST-tagged CK2 β or the CK2 β KSSR mutant was expressed in *Escherichia coli* BL21 (pLysS) cells and purified, as previously described for GST-CK2 β (33, 43). EBNA1 was produced in insect cells and purified, as previously described (25). Where indicated, EBNA1 was incubated with active or heat-inactivated (5 min at 75°C) FastAP alkaline phosphatase (Thermo Scientific) at a ratio of 1 U to 50 μ g of EBNA1 for 15 min at 37°C, prior to being used in GST pulldown assays. Fifty-five micrograms of EBNA1 was incubated with equimolar quantities of GST alone, GST-CK2 β , or the GST-CK2 β KSSR mutant and 25 μ l glutathione-Sepharose (Pierce) at 37°C for 1 h with mixing in a total volume of 300 μ l binding buffer (50 mM Tris-HCl [pH 7.5], 250 mM NaCl [see Fig. 2C], or 150 mM NaCl [see Fig. 6B], 0.5 mM EDTA, 1 mM DTT, 5% glycerol). After the resin was washed in binding buffer, proteins were eluted with 20 mM reduced glutathione and detected by SDS-PAGE and Coomassie staining.

Proteomics comparison of CK2 β WT- and KSSR mutant-interacting proteins. Five 10-cm plates of 293T cells were transfected with 5 μ g of pcDNA5-FLAG-CK2 β , with or without the KSSR mutation, or with the pcDNA5-FLAG empty plasmid using polyethylenimine (PEI) cellulose (Polysciences), as per the manufacturer's instructions. The cells were moved to 15-cm plates 24 h later and harvested 72 h posttransfection. After PBS washes, the cell pellets were lysed on ice for 30 min in a 5 $\times$ volume of modified RIPA buffer (50 mM Tris-HCl [pH 8], 300 mM NaCl, 0.1% sodium deoxycholate, 0.5% NP-40, 2 mM EDTA) containing protease inhibitor cocktail (P8340 [Invitrogen]), 10 mM sodium fluoride, 0.25 mM sodium orthovanadate, and 5 mM calyculin A. After sonication and centrifugation at $3,000 \times g$ for 30 min, 2 mg of each clarified cell lysate (at a protein concentration of 8 mg/ml) was incubated with 30 μ l of M2 anti-FLAG resin (Sigma) for 2 h at 4°C with mixing. Resin was harvested by centrifugation, washed in modified RIPA buffer, and then washed in 50 mM ammonium bicarbonate–75 mM KCl. Immunoprecipitated proteins were eluted with fresh 5% ammonium hydroxide elution buffer at pH 12 and lyophilized as described by Chen and Gingras (45). Dried protein was resuspended in 50 mM ammonium bicarbonate (pH 8.5) containing 10 μ g/ml sequencing-grade modified trypsin (Promega Corp.) and incubated for 1 h at 37°C followed by a further 18-h incubation at room temperature. Samples were dried using rotary evaporation and then washed 3 times with high-performance liquid chromatography (HPLC)-grade water. The resulting tryptic peptides were detected by liquid chromatography-mass spectrometry (LC-MS) using a LTQ Orbitrap system (Thermo Finnigan) and identified using Mascot software (Matrix Science, United Kingdom).

RESULTS

EBNA1 S393 is critical for CK2 interaction and EBNA1 phosphorylation. We previously showed that EBNA1 interacts with the CK2 holoenzyme by binding directly to the CK2 β subunit and that deletion of EBNA1 amino acids 387 to 394 abrogated this interaction (33). To further define the EBNA1 residues critical for CK2 interaction, we performed alanine-scanning mutagenesis of this EBNA1 region and tested the ability of the FLAG-tagged EBNA1 mutants to interact with CK2 in 293T cells (Fig. 1A). As expected, FLAG immunoprecipitation (IP) of wild-type (WT) EBNA1, but not FLAG-tagged LacZ (negative control) recovered CK2 α , CK2 β , and USP7, another binding partner of EBNA1. However, recovery of CK2 α and CK2 β was greatly reduced by all

FIG 1 S393 is critical for EBNA1 binding to CK2 and the EBNA1 phosphoshift. (A) The EBNA1 sequence shown on the top was subjected to alanine-scanning mutations. 293T cells were transfected with plasmids expressing FLAG-tagged EBNA1 with the indicated alanine mutations or FLAG-tagged LacZ (negative control). The tagged proteins were immunoprecipitated with anti-FLAG resin and analyzed by Western blotting using antibodies against USP7, CK2α, CK2β, and FLAG. The positions of these FLAG-tagged proteins in the IP samples are indicated by “L” for LacZ and “E” for EBNA1. Western blots of starting lysates (10% of the amount used for IP) are also shown (Input). (B) 293T cells were transfected with plasmids expressing FLAG-tagged EBNA1 with WT sequence (EBNA1) or with S393A, S393D, or S393T point mutations, followed by IP and Western blotting as in panel A. (C) 293T cells (top panel) and CNE2Z cells (bottom panel) were transfected with plasmids expressing untagged EBNA1Δ325–376 (a version of EBNA1 lacking the Gly-Arg repeat region that causes anomalous migration) or with EBNA1Δ325–376 containing S393A, S393D, or S393T point mutations. Cell lysates were treated with either active (+) or heat-inactivated (–) alkaline phosphatase (Phos) and analyzed by Western blotting using an EBNA1 antibody. The migration of the phosphoshifted form of EBNA1 (p-E) and unshifted EBNA1 (E) is indicated.

of the EBNA1 serine mutations (S386A, S388A, S389A, S390A, S391A, and S393A), as well as by the P394A mutation, but was unaffected by the Q387A and G392A mutations. Importantly, none of the mutations affected binding to USP7 (known to bind to EBNA1 residues 442 to 228) (46), indicating that the alanine substitutions did not disrupt protein folding. The data indicate that all of the serine residues and P394 in the CK2-binding region of EBNA1 are important for the CK2 interaction.

S393 is the only residue in the CK2-binding region that is a known phosphosite (47, 48), and while mutation of the other serines had partial effects on CK2 binding, mutation of Ser393 consistently reduced CK2 binding to undetectable levels. This is best seen by following the recovery of CK2α, since the CK2α antibody is of higher affinity than the CK2β antibody (Fig. 1A, left panel). S393 is followed by P394, which generates a cyclin-dependent kinase (CDK) consensus site, the kinase previously reported to phosphorylate EBNA1 on S393 (48). The P394A mutation also disrupted the EBNA1-CK2 interaction, suggesting that phosphorylation of S393 may be important for the CK2 interaction. We further investigated this possibility by generating an EBNA1

FIG 2 Phosphorylated EBNA1 binds CK2β. (A) 293T cells were transfected with plasmids expressing untagged EBNA1Δ325–376, and then IPs were performed on nuclear lysates using CK2β antibody or nonspecific IgG (negative control). Where indicated (+), phosphatase (Phos) was added to proteins recovered from IP and incubated for 15 or 30 min (lanes 3 and 4, respectively) before analysis by Western blotting using antibodies against EBNA1 and CK2β. Results for 10% of the starting lysate (Input) are also shown. (B) 293T cells were cotransfected with plasmids expressing FLAG-tagged CK2β and untagged EBNA1. Lysates were incubated with phosphatase (+) or heat-inactivated phosphatase (–) prior to CK2β IP using anti-FLAG resin. The proteins recovered were analyzed by Western blotting using antibodies against EBNA1, CK2α, and CK2β. Results from 2% of the starting lysate with and without phosphatase treatment are also shown (Input). (C) Baculovirus-produced EBNA1 was treated with either active (lanes 5 to 7) or heat-inactivated (lanes 2 to 4) phosphatase prior to incubation with GST-tagged CK2β and glutathione-Sepharose. After being washed, proteins were eluted with glutathione and analyzed by SDS-PAGE and colloidal Coomassie staining. A negative-control experiment involving baculovirus-produced EBNA1 with GST alone is shown in the right panel. The samples shown are the input protein mixture (In [3% of the total]), the flowthrough not retained on the resin (FT [3% of the total]), and protein that was retained by the resin and eluted (EI [50% of the total]). The positions of EBNA1, GST-CK2β, and GST and molecular mass markers (M) are indicated.

S393D mutant (potential phosphomimetic) and an S393T mutant that would restore the CDK phosphosite. IP of these FLAG-tagged proteins from 293T cells showed that S393D did not rescue CK2 binding, whereas S393T partially restored the CK2 interaction (Fig. 1B). The degree to which the EBNA1 S393 mutations affected phosphorylation of EBNA1 was then examined by determining their effect on EBNA1 migration in SDS-PAGE with and without phosphatase treatment (Fig. 1C). Experiments performed in both 293T and CNE2Z cells (nasopharyngeal carcinoma cells in which EBNA1 regulates PML NBs) (29) identified a shifted form of EBNA1 that is sensitive to phosphatase treatment. However, this phosphoshift was not seen with the S393A mutant, indicating that S393A is a major phosphosite for EBNA1. The S393T mutation partially restored the phosphoshift, consistent with restoration of the CDK site. The correlation between phosphorylation of EBNA1 at residue 393 and CK2 binding suggests that phosphorylation of this site is an important determinant in binding to CK2 and that it cannot be mimicked by replacing the phosphate with a negative charge (as in the S393D mutant).

Phosphorylated EBNA1 binds CK2β. To further investigate the possibility that the phosphorylated form of EBNA1 binds CK2, we examined the migration of EBNA1 that immunoprecipitated with endogenous CK2β from 293T cells, before and after treatment of the recovered EBNA1 with alkaline phosphatase (Fig. 2A).

FIG 3 S393A mutation does not affect EBNA1 nuclear localization or replication and plasmid maintenance functions. (A) CNE2 epithelial cells were transfected with plasmids expressing EBNA1 or the EBNA1 S393A mutant and then stained for EBNA1. BJAB B cells were infected with adenovirus expressing EBNA1 or the EBNA1 S393A mutant and then stained for EBNA1 and Nm23 (a predominantly cytoplasmic protein). All cells were counterstained with DAPI to visualize the nucleus. (B and C) CNE2Z cells were transfected with *oriP* plasmids expressing EBNA1, the EBNA1 S393A mutant, or nothing (*oriP* negative control) and then propagated for 3 days (for replication assays in panel B) or for 14 days (for plasmid maintenance assays in panel C). Plasmids isolated from equal cell numbers were linearized and incubated with DpnI to digest any unreplicated plasmids (bottom gel in panel B). The DNA was then analyzed by agarose gel electrophoresis and Southern blotting using 32 P-labeled pc3oriP as a probe. Input plasmid levels were also assessed by analyzing 1/10 of each sample prior to DpnI digestion (top gel in panel B). Experiments were performed in triplicate (lanes 1, 2, and 3 in panel B). Plasmid bands were

A slower-migrating form of EBNA1 was recovered with CK2 β , which shifted to a faster-migrating form upon phosphatase treatment, indicating that the phosphorylated form of EBNA1 was bound to CK2 β . However, these results do not distinguish between the possibilities that CK2 β binds EBNA1 that is phosphorylated prior to the interaction or that EBNA1 is phosphorylated after being bound by CK2. To distinguish between these two possibilities, we pretreated the cell lysate with phosphatase prior to performing the CK2 β IP. A comparison of EBNA1 recovery with CK2 β with and without phosphatase treatment showed that the phosphatase treatment greatly decreased the interaction of EBNA1 with CK2 (Fig. 2B), suggesting that only the phosphorylated form of EBNA1 binds CK2 β .

We further examined the requirement for EBNA1 phosphorylation for CK2 β binding by performing *in vitro* assays, in which the ability of baculovirus-produced EBNA1, with and without alkaline phosphatase treatment, was tested for binding to GST-CK2 β (Fig. 2C). When EBNA1 purified from insect cells was treated with heat-inactivated phosphatase and then incubated with GST-CK2 β , a proportion of the EBNA1 was retained on the glutathione resin by CK2 β and eluted with it upon addition of glutathione. This EBNA1 was phosphorylated, as evidenced by its slower migration relative to the same EBNA1 treated with active phosphatase (compare EBNA1 migration in lanes 2 to 4 to that in lanes 5 and 6). When the assay was repeated with dephosphorylated EBNA1 (active phosphatase treatment), no retention of EBNA1 by GST-CK2 β was detected. In addition, phosphorylated EBNA1 was not retained by glutathione resin containing GST alone. Therefore, the data indicate that CK2 β binds the phosphorylated form of EBNA1.

Effects of S393 mutation on EBNA1 functions. EBNA1 is known to have important roles in replicating and segregating EBV episomes during latent infection, through its interactions with the EBV latent origin of replication, *oriP*. A previous report suggested that mutation of S393 impairs its nuclear localization and therefore affects EBNA1 functions at *oriP* (48). However, we found that the EBNA1 S393A mutant was nuclear in all cells in which it was expressed (both epithelial and B-cell lines were examined) and that its localization was indistinguishable from that of WT EBNA1 (Fig. 3A). In addition, we found that the S393A mutation did not affect the ability of EBNA1 to support the replication of *oriP*-containing plasmids (Fig. 3B) or the long-term maintenance of *oriP*-containing plasmids (Fig. 3C). Since the long-term maintenance of *oriP* plasmids requires that the plasmids replicate and segregate stably in mitosis (both functions of EBNA1), the results indicate that the replication and segregation functions of EBNA1 are intact and that the effects of the S393 mutation are specific for the CK2 β interaction.

Currently, the only known functional role of the EBNA1-CK2 interaction is in the disruption of PML NBs by EBNA1, in which EBNA1 recruits CK2 to PML NBs resulting in increased CK2-mediated phosphorylation of PML proteins and their subsequent degradation (33). Therefore, to better understand the functional impor-

quantified by PhosphorImager analysis using ImageQuant software (Molecular Dynamics), and the intensities of the DpnI-resistant bands were quantified relative to the input band for the same sample. Average values for plasmid replication (B, right panel) and plasmid maintenance (C) assays relative to EBNA1 are shown along with standard deviations.

FIG 4 The ability of EBNA1 to disrupt PML nuclear bodies is abrogated by the S393A mutation and restored by the S393T mutation. (A) CNE2Z cells were transfected with plasmids expressing EBNA1 or the EBNA1 S393A or S393T mutants. Cells were then stained with antibodies against PML and EBNA1 and counterstained with DAPI. The number of PML nuclear bodies per cell was counted for 100 cells in two independent experiments. The average number of PML nuclear bodies per transfected cell is shown in the bar graph, along with standard deviations. (B to D) CNE2Z cells were transfected with plasmids expressing EBNA1 or the EBNA1 S393A mutant or empty plasmid (OriP). Cells were then lysed and analyzed directly for PML by Western blotting (B), or total PML in the lysate was immunoprecipitated, followed by Western blotting for PML, phosphorylated PML (pS517), CK2 β , or USP7, as indicated (C and D). In panel B, PML bands were quantified and normalized to actin, and this ratio is shown at the bottom of each lane relative to the OriP lane, which was set to 1.

tance of S393, we compared the abilities of the WT and S393A and S393T mutant versions of EBNA1 to disrupt PML NBs upon transient expression in CNE2Z cells (the cell line in which EBNA1-PML interactions have been previously defined [29]). The S393A mutation was found to abrogate the ability of EBNA1 to disrupt PML NBs, while the S393T mutation largely restored this ability (Fig. 4A). These results parallel the ability of these EBNA1 proteins to interact with CK2 and further emphasize the functional importance of the EBNA1-CK2 interaction for PML disruption.

It has been shown that CK2 phosphorylates PML proteins at Ser 517, triggering their polyubiquitylation and degradation (12). Our previous studies indicated that EBNA1 induces the loss of PML proteins, at least in part by increasing the association of CK2 with PML proteins, which increases PML phosphorylation (at S517) by CK2 (33). We further tested this model by determining how the S393A mutation in EBNA1 affected PML protein levels and CK2-mediated phosphorylation. Consistent with this model, EBNA1 but not the S393A mutant was found to decrease the levels of PML proteins, as detected by Western blotting, in which PML proteins are detected as a ladder of bands due to their multiple isoforms and modifications (Fig. 4B). Note that in these transient-transfection experiments, only ~50% of the cells express EBNA1; therefore, the degree to which EBNA1 induces loss of PML proteins would be about 2-fold more than that seen in Fig. 4B. In

addition, IP of total PML revealed that EBNA1 but not the S393A mutant increases the association of CK2 β with PML, as well as the amount of PML that is phosphorylated on S517 (as detected with an antibody specific to this modification) (Fig. 4C). Finally, we had previously shown that EBNA1 also increases the association of USP7 with PML proteins and NBs and suggested that this was independent of the EBNA1-CK2 interaction (33). We have further confirmed this independence by showing that both EBNA1 and the S393A mutant (which fails to bind CK2) increase the association of USP7 with PML proteins in a co-IP assay (Fig. 4D). Therefore, the effects of the S393A mutant are consistent with our model of the mechanism of EBNA1-mediated PML loss.

Identification of a KSSR motif in CK2 β that mediates the EBNA1 interaction. While several CK2-interacting proteins are known to interact directly with CK2 β , no clear binding pocket in CK2 β has been identified (36). Examination of the previously reported CK2 crystal structures revealed a region in CK2 β that coordinated phosphates (Protein Data Bank [PDB] no. 1JWH) or sulfates (PDB no. 3EED) from the crystallization buffer (Fig. 5) (49, 50). These negative-ion interactions were mediated by KSSR residues (amino acids 147 to 150) in the two CK2 β monomers in close proximity to the dimer interface, hereafter, referred to as the KSSR motif (Fig. 5). We hypothesized that the KSSR motif might be important for binding phosphorylated EBNA1 due to its pro-

FIG 5 The KSSR motif in CK2 β coordinates phosphates. Ribbon representation of the CK2 holoenzyme from the crystal structure (PDB no. 1JWH), where bound phosphates are shown by purple sticks (top panel). A closer look at the KSSR sequence from CK2 β is shown in the bottom panel, with bonds to phosphates shown by green dashed lines and phosphates shown by red and orange sticks.

pendency to bind phosphates. We tested this hypothesis by mutating the KSSR sequence to AAAA (referred to as the KSSR mutant), coexpressing FLAG-tagged WT CK2 β or the KSSR mutant in 293T cells with EBNA1 and performing co-IPs with anti-FLAG resin (Fig. 6A). EBNA1 was recovered with WT CK2 β but not with the KSSR mutant. Importantly, both CK2 β proteins were expressed at similar levels, and both recovered equal amounts of endogenous CK2 α , indicating that the mutations did not unfold CK2 β or interfere with its ability to form the CK2 holoenzyme. Therefore, the KSSR motif is important for specific interactions with EBNA1.

We also examined the importance of the KSSR motif for EBNA1 binding by using purified proteins in *in vitro* GST pull-down assays. GST-CK2 β proteins, with or without the KSSR mutation, were produced in *E. coli*, combined with baculovirus-produced EBNA1, and recovered on glutathione resin (Fig. 6B). Elution of the resin showed that EBNA1 bound to WT CK2 β but not to the KSSR mutant or to GST alone. These results support those of the *in vivo* experiments and confirm that the KSSR motif forms the binding interface for phosphorylated EBNA1.

The KSSR motif can also mediate cellular protein interactions. Viral proteins typically mimic cellular proteins in the mechanisms by which they mediate cellular protein interactions. Therefore, we hypothesized that the KSSR motif in CK2 β would also mediate some cellular protein interactions. To test this hypothesis, we performed proteomics experiments to compare cellular protein interactions with WT and KSSR mutant CK2 β . To this end,

FIG 6 KSSR mutation abrogates CK2 β binding to EBNA1 *in vivo* and *in vitro*. (A) 293T cells were cotransfected with plasmids expressing EBNA1 and FLAG-tagged CK2 β or the CK2 β KSSR mutant (labeled as KSSR) or empty FLAG plasmid (control [Ctrl]). The tagged proteins were immunoprecipitated with anti-FLAG resin and analyzed by Western blotting using antibodies against EBNA1 CK2 α and CK2 β . Ten percent of the starting lysate (Input) is also shown. The positions of FLAG-tagged CK2 β and endogenous CK2 β are indicated on the right. (B) Purified EBNA1 was incubated with purified GST-tagged CK2 β or the CK2 β KSSR mutant or with GST alone and then mixed with glutathione-Sepharose. After washing, proteins were eluted with glutathione and analyzed by SDS-PAGE and colloidal Coomassie staining. Samples shown are the input protein mixture (In [3% of the total]), the flowthrough that was not retained on the resin (FT [3% of the total]), and protein that was retained by the resin and eluted (El [50% of the total]). The positions of EBNA1, GST-CK2 β , GST, and molecular mass markers (M) are indicated.

the FLAG-tagged CK2 β proteins were transiently expressed in 293T cells, followed by recovery on anti-FLAG resin and identification of recovered proteins by LC-MS/MS. As shown in Table 1, WT and KSSR mutant versions of CK2 β were recovered equally, and as expected, they interacted equally well with the CK2 α subunit and the alternative CK2 α' isoform. The 50 most prevalent interactions detected for WT CK2 β are shown in Table 1, and most of these interactions are unchanged by the KSSR mutation, indicating that the KSSR mutation has minimal effects on the structure of CK2 β . Only three protein interactions with CK2 β were detected in which the KSSR mutation reduced the interaction to the background levels seen with the empty plasmid control, namely, C18orf25, Bcl2-associated transcription factor 1 (BCLAF1/Btf1), and DDX54 dead-box RNA helicase (shown in boldface in Table 1). Of these, the interaction with the uncharacterized C18orf25 protein was the most prominent and its dependence on the KSSR motif was the most striking, since no peptides from this protein were detected with the CK2 β KSSR mutant.

C18orf25 is a protein of unknown function that is homologous to the N terminus of Arkadia/RNF111, a SUMO-targeted ubiquitin E3 ligase (Fig. 7A), and therefore was named “Arkadia-like 1” (ARKL1) (51, 52). To validate the ARKL1-CK2 β interaction and confirm that it is mediated by the KSSR motif, we cotransfected 293T cells with constructs expressing myc-tagged ARKL1 and FLAG-tagged CK2 β or the FLAG-tagged CK2 β KSSR mutant and then performed IPs with anti-FLAG (Fig. 7B). Western blots for the myc tag confirmed that myc-ARKL1 bound CK2 β but not the KSSR mutant.

TABLE 1 Top 50 proteins recovered with FLAG-CK2β compared to recovery with the FLAG-CK2β KSSR mutant^a

Protein designation	Description	Total no. of peptides with empty plasmid	CK2β WT			CK2β KSSR		
			No. of peptides		% coverage	No. of peptides		% coverage
			Total	Unique		Total	Unique	
LY6G5B	Casein kinase II subunit β	0	323	13	62	312	13	62
CSNK2A1	Casein kinase II subunit α	0	150	30	66	157	27	63
PRKDC	DNA-dependent protein kinase catalytic subunit	7	82	64	18	82	60	16
RPS6KA3	Ribosomal protein S6 kinase α-3	0	80	38	63	86	37	62
CSNK2A2	Casein kinase II subunit α'	0	68	24	64	65	22	63
C18orf25	Uncharacterized protein C18orf25	0	60	9	32	0	0	0
DYNC1H1	Cytoplasmic dynein 1 heavy chain 1	1	51	45	13	42	38	11
MYBBP1A	Myb-binding protein 1A	1	47	25	24	34	21	23
BCLAF1	Bcl-2-associated transcription factor 1	10	43	20	25	9	8	11
CHD4	Chromodomain helicase DNA-binding protein 4	0	41	29	20	22	17	13
CDC42BPA	Serine/threonine-protein kinase MRCK α	0	36	32	25	54	41	32
RPL28	60S ribosomal protein L28	9	36	5	28	44	6	29
ASCC3L1	U5 small nuclear ribonucleoprotein 200-kDa helicase	4	35	32	22	25	24	16
DDX5	Probable ATP-dependent RNA helicase DDX5	6	29	15	22	16	14	23
NUMA1	Nuclear mitotic apparatus protein 1	1	27	24	16	27	25	15
CAD	CAD protein (includes glutamine-dependent carbamoyl-phosphate synthase)	2	26	24	15	30	24	14
CLTC	Clathrin heavy chain 1 (CLH-17)	5	26	21	17	26	20	16
ATP5A1	ATP synthase subunit α, mitochondrial precursor	5	25	15	36	22	17	39
PRPF8	Pre-mRNA-processing-splicing factor 8	5	25	23	14	22	21	11
RPS16	40S ribosomal protein S16	7	24	9	53	23	9	51
ATAD3A	ATPase family AAA domain-containing protein 3A	4	24	18	38	23	15	29
SLC25A5	ADP/ATP translocase 2	6	22	13	39	19	10	29
ATP1A1	Sodium/potassium-transporting ATPase subunit α-1 precursor	3	21	16	25	22	17	26
RPS14	40S ribosomal protein S14	4	22	9	43	18	7	41
COPA	Coatomer subunit α	1	21	14	14	11	11	11
DDX54	ATP-dependent RNA helicase DDX54	1	21	14	23	2	2	3.5
EFTUD2	116-kDa U5 small nuclear ribonucleoprotein component	6	20	17	28	15	11	15
SF3B2	Splicing factor 3B subunit 2	0	20	11	16	10	8	12
MKI67	Antigen KI-67	0	20	17	7.5	8	7	3.6
RPS6KA1	Ribosomal protein S6 kinase α-1	0	19	16	44	17	13	38
TUFM	Elongation factor Tu	6	18	15	42	18	15	38
TRIM28	Transcription intermediary factor 1-β (TIF1-β)	2	17	13	19	14	11	17
RPS23	40S ribosomal protein S23	2	17	7	25	10	5	18
AC080112.15	TOP2A_HUMAN isoform 2 of P11388	0	17	15	12	9	8	5.7
DNAJA1	DnaJ homolog subfamily A member 1 (heat shock 40-kDa protein 4)	2	16	13	37	14	9	33
GCN1L1	Translational activator GCN1 (GCN1-like protein 1)	0	16	16	8.8	10	10	6
SMC2	Structural maintenance of chromosomes protein 2	0	16	15	14	9	9	8.3
MTA2	Metastasis-associated protein MTA2 (metastasis-associated 1-like 1)	0	16	4	8.2	5	5	9.7
EIF3S9	Eukaryotic translation initiation factor 3 subunit B	6	15	10	15	33	7	9.9
MCM3	DNA replication licensing factor MCM3	7	15	14	24	9	9	15
RPL23	60S ribosomal protein L23 (ribosomal protein L17)	2	15	9	28	14	9	41
RPS6KA4	Ribosomal protein S6 kinase α-4	0	15	7	25	10	7	18
RPL38	60S ribosomal protein L38	5	15	9	53	9	4	50
RBM14	RNA-binding protein 14 (RNA-binding motif protein 14)	2	15	12	24	11	10	13
ATP5C1	ATP synthase subunit γ, mitochondrial precursor	3	15	10	37	9	5	25
TP53	Cellular tumor antigen p53 (tumor suppressor p53)	0	14	9	18	12	8	20
PCBP2	Poly(rC)-binding protein 2 (α-CP2) (hnRNP-E2)	2	14	10	38	13	7	29
NSUN2	tRNA (cytosine-5-)-methyltransferase (NSUN2)	2	14	11	14	12	10	17
SMC1A	Structural maintenance of chromosomes protein 1A (SMC1α protein)	1	14	13	12	13	13	12
KPNA2	Importin subunit α-2	0	14	11	29	13	10	27

^a Results for proteins that were decreased to background levels by the KSSR mutation are shown in boldface.

Sequence alignments between Arkadia and ARKL1 from several organisms show multiple conserved sequences, including two SUMO-interacting motifs (SIMs) (Fig. 7A). In addition, a highly conserved polyserine stretch (amino acids 202 to 225 in human ARKL1) is evident that resembles the CK2 binding region in EBNA1. To determine if this polyserine sequence mediates the interaction of ARKL1 with CK2β, we deleted it in ARKL1 (ΔS) and repeated the myc-ARKL1/FLAG-CK2β co-IP assays comparing WT and ΔS versions of ARKL1 (Fig. 7C). Unlike WT ARKL1, the ΔS ARKL1 mutant failed to detectably bind to CK2β. However, both WT and ΔS versions of ARKL1 had similar cellular localizations, including the ability to associate with PML NBs, as

seen by their ability to form foci that correspond to PML NBs (Fig. 7D). This localization was expected due to the presence of the SIMs, which typically mediate interactions with the highly SUMOylated PML proteins and have been previously reported to target Arkadia to PML NBs (53). The localization of the ΔS ARKL1 mutant to PML indicates that this mutation did not disrupt interactions with sumoylated proteins, and the data as a whole indicate that the polyserine stretch in ARKL1 mediates an interaction with the CK2β KSSR motif. Finally, we also asked whether ARKL1 is phosphorylated and whether phosphorylation affects CK2β binding. Phosphatase treatment of lysates expressing myc-ARKL1 resulted in a faster-migrating form of the protein, suggesting that, like EBNA1,

A **B** **C** **D** **E**

most of the ARKL1 is phosphorylated (Fig. 7E). However, recovery of myc-ARKL1 with FLAG-CK2 β in FLAG IP experiments showed that phosphatase treatment only slightly decreased recovery of myc-ARKL1 (Fig. 7E), so the degree to which phosphorylation of ARKL1 contributes to CK2 β binding is unclear.

DISCUSSION

Viral proteins have long been recognized as valuable tools for identifying cellular proteins central to important pathways, as well as the mechanisms by which these proteins are regulated. Our study of the EBV EBNA1 protein interaction with the cellular kinase CK2 has led to the identification of a new protein binding site in the CK2 regulatory subunit (CK2 β) that is used by both viral and cellular proteins to interact with the active kinase. This binding pocket includes a KSSR motif from each CK2 β monomer that binds negatively charged ions that may give this interface specificity for phosphorylated sequences. Although the complete sequence requirements for interactions with the KSSR pocket are not yet known, polyserine regions appear to be a preferred target, since both EBNA1 and ARKL1 interact with the CK2 β KSSR motif through similar polyserine tracts.

Our results indicate that the EBNA1-CK2 β interaction is primed by phosphorylation of EBNA1 on S393. S393 is the only residue in the CK2-binding region (amino acids 387 to 394) that is known to be phosphorylated (47, 48). It is part of a CDK consensus site, and this kinase was previously reported to phosphorylate S393 (48). We have shown that phosphorylation of S393 is critical for the shifted migration of EBNA1 in SDS-PAGE, but it is not clear whether this shift is due solely to phosphorylation of this single site or whether phosphorylation of S393 might prime EBNA1 for additional phosphorylation events. Either way, the phosphorylated form of EBNA1 appears to be a prevalent form of EBNA1 (as shown in Fig. 1C).

It was previously reported that phosphorylation of S393 was important for nuclear entry of EBNA1 and therefore for EBNA1 functions at the EBV *oriP* sequence (48). However, we found no difference in the cellular localization or ability of the EBNA1 S393A mutant to support the replication and stable maintenance of *oriP*-based plasmids relative to those of WT EBNA1. The reason for this discrepancy is not clear but could be the result of secondary mutations in the EBNA1 construct used by Kang et al. (48). Our data indicate that EBNA1 S393 plays a specific role in mediating an interaction with CK2, which we previously reported is important for disruption of PML NBs by EBNA1 (33). The mutational analysis presented here further strengthens the connection between CK2 binding and PML disruption by EBNA1, as the S393A mutation abrogates both CK2 binding and PML disruption, while the S393T mutation partially restores both. The S393T

mutation also partially restores EBNA1 phosphorylation, suggesting that phosphorylation of S393 controls both CK2 binding and PML disruption by EBNA1. The importance of phosphorylation of S393 for CK2 binding was further supported by experiments showing that IP of EBNA1 with endogenous CK2 β recovered a phosphophorylated form of EBNA1 and was abrogated by pretreatment of the lysate with phosphatase prior to IP (Fig. 2A and B, respectively). In addition, EBNA1 purified from insect cells bound purified CK2 β before but not after phosphatase treatment (Fig. 2C).

CK2 β is remarkably conserved in evolution, as the protein sequence is identical between birds and mammals (54). CK2 β forms a stable dimer that constitutes the core of the CK2 holoenzyme (35, 50, 55). In fact, formation of the CK2 tetrameric complex requires the dimerization of the CK2 β subunits prior to association with the catalytic subunits (55). We have identified the KSSR motif as forming part of a docking site on CK2 β . KSSR is adjacent to the zinc finger dimerization motif, with the side chains exposed to solvent and therefore free to accommodate protein interactions. The propensity of the KSSR motif to coordinate negatively charged ions (e.g., phosphates and sulfates) in two independent crystal structures suggested that it may coordinate interactions with phosphorylated amino acids such as Ser393 in EBNA1. In support of this hypothesis, mutation of KSSR to AAAA abrogated the EBNA1 interaction without affecting CK2 holoenzyme formation. However, alanine-scanning mutagenesis indicated that the 5 additional serines in the region from amino acids 386 to 391 (none of which have been found to be phosphorylated) (47) also contribute to the CK2 β interaction, suggesting that they make additional contacts with CK2 β . Despite the importance of the EBNA1 region from amino acids 386 to 391 for CK2 β binding, in our hands, synthetic peptides spanning this sequence fail to bind CK2 β , even when S393 is phosphorylated, suggesting either that this sequence requires a folded structure that does not occur in the synthesized peptides or that additional contacts outside this peptide also contribute to CK2 binding.

CK2 has been reported to interact with numerous proteins, and many of these interact with the CK2 β subunit (36, 56, 57). Some of these proteins (e.g., A-Raf, c-Mos, and Chk1 kinases) bind the CK2 β C-terminal region in a similar manner to CK2 α and can replace CK2 α (58–61). Therefore, these kinases may use CK2 β to regulate or direct their kinase activity. However, many CK2 β interactors bind to CK2 β in the context of the CK2 holoenzyme. These include Nopp140, ribosomal protein L41, CD5, topoisomerase II, p53, and p21^{WAF1}, which interact with various regions of CK2 β (37, 38, 57, 62–65). Therefore, it appears that there are multiple ways in which proteins can bind to CK2 β , and

FIG 7 A polyserine region of ARKL1 mediates an interaction with the KSSR motif of CK2 β . (A) Protein sequence alignment between the N terminus of Arkadia and ARKL1 from multiple organisms: *Homo sapiens* (Hs), *Gallus gallus* (Gg), *Xenopus tropicalis* (Xr), *Takifugu rubripes* (Ts), *Danio rerio* (Dr), and *Anolis carolinensis* (Ac). The two conserved SUMO-interacting motifs (SIM) and a polyserine stretch that resembles that in EBNA1 (underlined) are indicated. (B) 293T cells were cotransfected with plasmids expressing myc-tagged ARKL1 and FLAG-tagged CK2 β , the CK2 β KSSR mutant (KSSR), or empty FLAG plasmid (Ctrl). CK2 β proteins were coimmunoprecipitated from cell lysate using anti-FLAG resin, and recovered proteins were analyzed by Western blotting using antibodies against myc, CK2 α and CK2 β . Ten percent of the starting lysate (Input) is also shown. (C) 293T cells were cotransfected with plasmids expressing FLAG-tagged CK2 β and myc-tagged ARKL1 with WT sequence or the Δ S mutation (underlined region in panel A). CK2 β IP and Western blotting were performed as in panel B. (D) CNE2Z cells transfected with plasmids expressing myc-tagged ARKL1 with WT sequence or Δ S mutation were fixed and stained with antibodies against myc and PML and counterstained with DAPI. (E) 293T cells were cotransfected with plasmids expressing FLAG-tagged CK2 β and myc-tagged ARKL1. Cell lysates were incubated with phosphatase (+) or heat-inactivated phosphatase (–), and then CK2 β was immunoprecipitated with anti-FLAG resin and Western blots were performed as in panel A. The result for 2% of the starting lysate with and without phosphatase treatment is also shown (Input).

the role of the KSSR motif in protein interactions has not been previously investigated.

Since viral proteins often mimic cellular proteins in their mechanism of protein interactions, we designed a proteomic experiment to identify cellular proteins that bind CK2 β through the KSSR motif, like EBNA1. The most prevalent interaction we detected with WT CK2 β that was abrogated by the KSSR mutation was with a previously uncharacterized protein, C18orf25/ARKL1. The other two most prevalent interactions (which were not disrupted by KSSR mutation) were with the catalytic subunit of DNA-dependent protein kinase and with ribosomal protein S6 kinase. Both of these proteins have been previously shown to physically and functionally interact with CK2, supporting the validity of our data; although only the S6 kinase was known to interact with CK2 through the β subunit (66–68). In addition, we detected the previously characterized interactions of CK2 β with topoisomerase II and p53, neither of which was disrupted by the KSSR mutation (37, 38). P53 interacts with CK2 β through sequences very close to the KSSR motif (38), so the fact that the KSSR mutation did not disrupt p53 binding suggests that this mutation did not cause structural changes in adjacent regions. Only three of the 50 most prevalent protein interactions that we identified with CK2 β were disrupted by the KSSR mutation, indicating that only a small percentage of CK2-binding proteins interact through this site. The KSSR-interacting proteins that we did identify likely have a variety of cellular functions, suggesting that the KSSR motif contributes to multiple cellular processes.

The most striking KSSR-mediated CK2 β interaction that we detected was with C18orf25/ARKL1. The gene for this protein is located in a region of chromosome 18 where pericentric inversions have been seen in two families with schizophrenia and bipolar disorders, making it one of five candidate genes for these psychiatric disorders (51). C18orf25 is also homologous to the N terminus of the SUMO-targeted ubiquitin E3 ligase Arkadia/RNF111 and therefore was coined “ARKL1” (Arkadia-like 1) (51, 52, 69). ARKL1 contains two of the three SUMO-interacting motifs (SIMs) found in Arkadia (Fig. 7A) but lacks the RING domain needed for the ubiquitin ligase activity (70). Our protein sequence alignments between ARKL1 and Arkadia in multiple organisms have shown that they also share a highly conserved serine-rich region resembling that in EBNA1, and like that in EBNA1, the polyserine region of ARKL1 is critical for the CK2 β interaction. In EBNA1, phosphorylation of at least one serine in this polyserine stretch is required for CK2 β binding. While ARKL1 also appears to be phosphorylated, it is not known if this phosphorylation occurs within the polyserine region that mediates the CK2 β interaction, although this region does contain consensus sites for several kinases, including GSK3, CK1, and CK2. Phosphatase treatment of ARKL1 subtly decreased the CK2 β interaction but also decreased the total levels of ARKL1, complicating interpretation of whether or not phosphorylation contributes to the ARKL1-CK2 β interaction.

The fact that Arkadia and ARKL1 both contain the serine-rich region that mediates the ARKL1 interaction with the CK2 β KSSR motif suggests that Arkadia could also bind CK2 β through the same binding pocket as ARKL1 and EBNA1. While we did not recover Arkadia with WT CK2 β in our proteomics experiment, this could be due to incompatibility of Arkadia peptides for mass spectrometry (due to unusual sizes or composition) or due to low levels of Arkadia in the cell. In support of the latter possibility,

FIG 8 Model of EBNA1 and ARKL1 interactions with CK2. (A and B) EBNA1 and ARKL1 interact similarly with the KSSR motif in CK2 β . This interaction can facilitate CK2 phosphorylation of specific target proteins by bridging an interaction between the target protein and CK2. (C) EBNA1 may interfere with functions of ARKL1 (or other CK2 β binders) by outcompeting it for the KSSR motif on CK2 β .

Arkadia is known to be a very unstable protein, which is likely due to its autoubiquitylation (53, 71). Arkadia has been reported to have multiple functions, including transforming growth factor β (TGF- β) signaling and DNA damage responses, and can also induce PML degradation in response to arsenic trioxide, which causes hyper-SUMOylation of PML (53, 71–73). Interestingly, in a recent proteomic experiment for Arkadia interactors, Poulsen et al. (73) identified an interaction with CK2 (shown in their supplemental data). It will be interesting to determine whether Arkadia interacts with CK2 through CK2 β , as predicted by its serine-rich region, and how this interaction contributes to any of the functions of Arkadia.

In conclusion, we have identified a previously unknown protein interaction motif in CK2 β (KSSR) that can mediate protein docking with the active CK2 holoenzyme, enabling CK2 to be recruited to specific substrates (Fig. 8 A and B). This mechanism of CK2 interaction is used by EBNA1 and a subset of cellular CK2-interacting proteins, including ARKL1. The ability of EBNA1 to efficiently bind to this docking site on CK2 β suggests that EBNA1 could affect the functions of cellular proteins that also bind to this site, by interfering with their ability to bind and recruit CK2 (Fig. 8C). While our present knowledge on the significance of the EBNA1-CK2 interaction is limited to its role in inducing PML protein degradation, we predict that identification of the cellular proteins that dock at the CK2 β KSSR site will lead to additional cellular processes modulated by EBNA1 and therefore will be important for a more complete understanding of EBV infection and EBV-induced cancers. In addition, elucidation of CK2 β interactions at the KSSR motif will increase our understanding of CK2 functions and the complexes that it forms.

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REFERENCES

- Pinna LA. 2002. Protein kinase CK2: a challenge to canons. *J. Cell Sci.* 115:3873–3878. <http://dx.doi.org/10.1242/jcs.00074>.
- Pinna LA, Allende JE. 2009. Protein kinase CK2 in health and disease: protein kinase CK2: an ugly duckling in the kinome pond. *Cell. Mol. Life Sci.* 66:1795–1799. <http://dx.doi.org/10.1007/s00018-009-9148-9>.
- Meggio F, Pinna LA. 2003. One-thousand-and-one substrates of protein

- kinase CK2? *FASEB J.* 17:349–368. <http://dx.doi.org/10.1096/fj.02-0473rev>.
4. Ruzzene M, Pinna LA. 2010. Addiction to protein kinase CK2: a common denominator of diverse cancer cells? *Biochim. Biophys. Acta* 1804:499–504. <http://dx.doi.org/10.1016/j.bbapap.2009.07.018>.
5. Duncan JS, Litchfield DW. 2008. Too much of a good thing: the role of protein kinase CK2 in tumorigenesis and prospects for therapeutic inhibition of CK2. *Biochim. Biophys. Acta* 1784:33–47. <http://dx.doi.org/10.1016/j.bbapap.2007.08.017>.
6. Seldin DC, Leder P. 1995. Casein kinase II alpha transgene-induced murine lymphoma: relation to theileriosis in cattle. *Science* 267:894–897. <http://dx.doi.org/10.1126/science.7846532>.
7. Kelliher MA, Seldin DC, Leder P. 1996. Tal-1 induces T cell acute lymphoblastic leukemia accelerated by casein kinase IIalpha. *EMBO J.* 15:5160–5166.
8. Landesman-Bollag E, Romieu-Mourez R, Song DH, Sonenshein GE, Cardiff RD, Seldin DC. 2001. Protein kinase CK2 in mammary gland tumorigenesis. *Oncogene* 20:3247–3257. <http://dx.doi.org/10.1038/sj.onc.1204411>.
9. Keller DM, Lu H. 2002. p53 serine 392 phosphorylation increases after UV through induction of the assembly of the CK2.hSPT16.SSRP1 complex. *J. Biol. Chem.* 277:50206–50213. <http://dx.doi.org/10.1074/jbc.M209820200>.
10. Cox ML, Meek DW. 2009. Phosphorylation of serine 392 in p53 is a common and integral event during p53 induction by diverse stimuli. *Cell. Signal.* 22:564–571.
11. Kapoor M, Lozano G. 1998. Functional activation of p53 via phosphorylation following DNA damage by UV but not gamma radiation. *Proc. Natl. Acad. Sci. U. S. A.* 95:2834–2837. <http://dx.doi.org/10.1073/pnas.95.6.2834>.
12. Scaglioni PP, Yung TM, Cai LF, Erdjument-Bromage H, Kaufman AJ, Singh B, Teruya-Feldstein J, Tempst P, Pandolfi PP. 2006. A CK2-dependent mechanism for degradation of the PML tumor suppressor. *Cell* 126:269–283. <http://dx.doi.org/10.1016/j.cell.2006.05.041>.
13. Al-Khoury AM, Ma Y, Togo SH, Williams S, Mustelin T. 2005. Cooperative phosphorylation of the tumor suppressor phosphatase and tensin homologue (PTEN) by casein kinases and glycogen synthase kinase 3beta. *J. Biol. Chem.* 280:35195–35202. <http://dx.doi.org/10.1074/jbc.M503045200>.
14. Eddy SF, Guo S, Demicco EG, Romieu-Mourez R, Landesman-Bollag E, Seldin DC, Sonenshein GE. 2005. Inducible IkappaB kinase/IkappaB kinase epsilon expression is induced by CK2 and promotes aberrant nuclear factor-kappaB activation in breast cancer cells. *Cancer Res.* 65:11375–11383. <http://dx.doi.org/10.1158/0008-5472.CAN-05-1602>.
15. Romieu-Mourez R, Landesman-Bollag E, Seldin DC, Sonenshein GE. 2002. Protein kinase CK2 promotes aberrant activation of nuclear factor-kappaB, transformed phenotype, and survival of breast cancer cells. *Cancer Res.* 62:6770–6778.
16. Torres J, Pulido R. 2001. The tumor suppressor PTEN is phosphorylated by the protein kinase CK2 at its C terminus. Implications for PTEN stability to proteasome-mediated degradation. *J. Biol. Chem.* 276:993–998.
17. Lallemand-Breitenbach V, de The H. 2010. PML nuclear bodies. *Cold Spring Harb. Perspect. Biol.* 2:a000661. <http://dx.doi.org/10.1101/cshperspect.a000661>.
18. St-Denis NA, Litchfield DW. 2009. Protein kinase CK2 in health and disease: from birth to death: the role of protein kinase CK2 in the regulation of cell proliferation and survival. *Cell. Mol. Life Sci.* 66:1817–1829. <http://dx.doi.org/10.1007/s00018-009-9150-2>.
19. Koffa MD, Kean J, Zachos G, Rice SA, Clements JB. 2003. CK2 protein kinase is stimulated and redistributed by functional herpes simplex virus ICP27 protein. *J. Virol.* 77:4315–4325. <http://dx.doi.org/10.1128/JVI.77.7.4315-4325.2003>.
20. Franck N, Le Seyec J, Guguen-Guillouzo C, Erdtmann L. 2005. Hepatitis C virus NS2 protein is phosphorylated by the protein kinase CK2 and targeted for degradation to the proteasome. *J. Virol.* 79:2700–2708. <http://dx.doi.org/10.1128/JVI.79.5.2700-2708.2005>.
21. El-Guindy AS, Miller G. 2004. Phosphorylation of Epstein-Barr virus ZEBRA protein at its casein kinase 2 sites mediates its ability to repress activation of a viral lytic cycle late gene by Rta. *J. Virol.* 78:7634–7644. <http://dx.doi.org/10.1128/JVI.78.14.7634-7644.2004>.
22. Meggio F, D'Agostino DM, Ciminale V, Chieco-Bianchi L, Pinna LA. 1996. Phosphorylation of HIV-1 Rev protein: implication of protein kinase CK2 and pro-directed kinases. *Biochem. Biophys. Res. Commun.* 226:547–554. <http://dx.doi.org/10.1006/bbrc.1996.1392>.
23. O'Reilly D, Hanscombe O, O'Hare P. 1997. A single serine residue at position 375 of VP16 is critical for complex assembly with Oct-1 and HCF and is a target of phosphorylation by casein kinase II. *EMBO J.* 16:2420–2430. <http://dx.doi.org/10.1093/emboj/16.9.2420>.
24. Zhi Y, Sandri-Goldin RM. 1999. Analysis of the phosphorylation sites of herpes simplex virus type 1 regulatory protein ICP27. *J. Virol.* 73:3246–3257.
25. Holowaty MN, Zeghouf M, Wu H, Tellam J, Athanasopoulos V, Greenblatt J, Frappier L. 2003. Protein profiling with Epstein-Barr nuclear antigen-1 reveals an interaction with the herpesvirus-associated ubiquitin-specific protease HAUSP/USP7. *J. Biol. Chem.* 278:29987–29994. <http://dx.doi.org/10.1074/jbc.M303977200>.
26. Malik-Soni N, Frappier L. 2012. Proteomic profiling of EBNA1-host protein interactions in latent and lytic Epstein-Barr virus infections. *J. Virol.* 86:6999–7002. <http://dx.doi.org/10.1128/JVI.00194-12>.
27. Frappier L. 2012. EBNA1 and host factors in Epstein-Barr virus latent DNA replication. *Curr. Opin. Virol.* 2:733–739. <http://dx.doi.org/10.1016/j.coviro.2012.09.005>.
28. Frappier L. 2011. Role of EBNA1 in NPC tumorigenesis. *Semin. Cancer Biol.* 22:154–161. <http://dx.doi.org/10.1016/j.semcancer.2011.12.002>.
29. Sivachandran N, Sarkari F, Frappier L. 2008. Epstein-Barr nuclear antigen 1 contributes to nasopharyngeal carcinoma through disruption of PML nuclear bodies. *PLoS Pathog.* 4:e1000170. <http://dx.doi.org/10.1371/journal.ppat.1000170>.
30. Everett RD, Chelbi-Alix MK. 2007. PML and PML nuclear bodies: implications in antiviral defence. *Biochimie* 89:819–830. <http://dx.doi.org/10.1016/j.biochi.2007.01.004>.
31. Sivachandran N, Wang X, Frappier L. 2012. Functions of the Epstein-Barr virus EBNA1 protein in viral reactivation and lytic infection. *J. Virol.* 86:6146–6158. <http://dx.doi.org/10.1128/JVI.00013-12>.
32. Sivachandran N, Dawson CW, Young LS, Liu FF, Middeldorp J, Frappier L. 2011. Contributions of the Epstein-Barr virus EBNA1 protein to gastric carcinoma. *J. Virol.* 86:60–68. <http://dx.doi.org/10.1128/JVI.06776-11>.
33. Sivachandran N, Cao JY, Frappier L. 2010. Epstein-Barr virus nuclear antigen 1 hijacks the host kinase CK2 to disrupt PML nuclear bodies. *J. Virol.* 84:11113–11123. <http://dx.doi.org/10.1128/JVI.01183-10>.
34. Sarno S, Marin O, Boschetti M, Pagano MA, Meggio F, Pinna LA. 2000. Cooperative modulation of protein kinase CK2 by separate domains of its regulatory beta-subunit. *Biochemistry* 39:12324–12329. <http://dx.doi.org/10.1021/bi0011431>.
35. Canton DA, Zhang C, Litchfield DW. 2001. Assembly of protein kinase CK2: investigation of complex formation between catalytic and regulatory subunits using a zinc-finger-deficient mutant of CK2beta. *Biochem. J.* 358:87–94. <http://dx.doi.org/10.1042/0264-6021:3580087>.
36. Bibby AC, Litchfield DW. 2005. The multiple personalities of the regulatory subunit of protein kinase CK2: CK2 dependent and CK2 independent roles reveal a secret identity for CK2beta. *Int. J. Biol. Sci.* 1:67–79. <http://dx.doi.org/10.7150/ijbs.1.67>.
37. Bojanowski K, Filhol O, Cochet C, Chambaz EM, Larsen AK. 1993. DNA topoisomerase II and casein kinase II associate in a molecular complex that is catalytically active. *J. Biol. Chem.* 268:22920–22926.
38. Appel K, Wagner P, Boldyreff B, Issinger OG, Montenarh M. 1995. Mapping of the interaction sites of the growth suppressor protein p53 with the regulatory beta-subunit of protein kinase CK2. *Oncogene* 11:1971–1978.
39. Sun Y, Hegamyer G, Cheng YJ, Hildesheim A, Chen JY, Chen IH, Cao Y, Yao KT, Colburn NH. 1992. An infrequent point mutation of the p53 gene in human nasopharyngeal carcinoma. *Proc. Natl. Acad. Sci. U. S. A.* 89:6516–6520. <http://dx.doi.org/10.1073/pnas.89.14.6516>.
40. Menezes J, Leibold W, Klein G, Clements G. 1975. Establishment and characterization of an Epstein-Barr virus (EBV)-negative lymphoblastoid B cell line (BJA-B) from an exceptional, EBV-genome-negative African Burkitt's lymphoma. *Biomedicine* 22:276–284.
41. Shire K, Ceccarelli DF, Avolio-Hunter TM, Frappier L. 1999. EBP2, a human protein that interacts with sequences of the Epstein-Barr virus nuclear antigen 1 important for plasmid maintenance. *J. Virol.* 73:2587–2595.
42. Shire K, Kapoor P, Jiang K, Hing MN, Sivachandran N, Nguyen T, Frappier L. 2006. Regulation of the EBNA1 Epstein-Barr virus protein by serine phosphorylation and arginine methylation. *J. Virol.* 80:5261–5272. <http://dx.doi.org/10.1128/JVI.02682-05>.
43. Bosc DG, Slominski E, Sichler C, Litchfield DW. 1995. Phosphorylation

- of casein kinase II by p34cdc2. Identification of phosphorylation sites using phosphorylation site mutants in vitro. *J. Biol. Chem.* 270:25872–25878.
44. Holowaty MN, Sheng Y, Nguyen T, Arrowsmith C, Frappier L. 2003. Protein interaction domains of the ubiquitin-specific protease, USP7/HAUSP. *J. Biol. Chem.* 278:47753–47761. <http://dx.doi.org/10.1074/jbc.M307200200>.
 45. Chen GI, Gingras AC. 2007. Affinity-purification mass spectrometry (AP-MS) of serine/threonine phosphatases. *Methods* 42:298–305. <http://dx.doi.org/10.1016/j.ymeth.2007.02.018>.
 46. Saridakis V, Sheng Y, Sarkari F, Holowaty MN, Shire K, Nguyen T, Zhang RG, Liao J, Lee W, Edwards AM, Arrowsmith CH, Frappier L. 2005. Structure of the p53 binding domain of HAUSP/USP7 bound to Epstein-Barr nuclear antigen 1: implications for EBV-mediated immortalization. *Mol. Cell* 18:25–36. <http://dx.doi.org/10.1016/j.molcel.2005.02.029>.
 47. Duellman SJ, Thompson KL, Coon JJ, Burgess RR. 2009. Phosphorylation sites of Epstein-Barr virus EBNA1 regulate its function. *J. Gen. Virol.* 90:2251–2259. <http://dx.doi.org/10.1099/vir.0.012260-0>.
 48. Kang MS, Lee EK, Soni V, Lewis TA, Koehler AN, Srinivasan V, Kieff E. 2011. Roscovitine inhibits EBNA1 serine 393 phosphorylation, nuclear localization, transcription, and episome maintenance. *J. Virol.* 85:2859–2868. <http://dx.doi.org/10.1128/JVI.01628-10>.
 49. Niefind K, Guerra B, Ermakowa I, Issinger OG. 2001. Crystal structure of human protein kinase CK2: insights into basic properties of the CK2 holoenzyme. *EMBO J.* 20:5320–5331. <http://dx.doi.org/10.1093/emboj/20.19.5320>.
 50. Raaf J, Brunstein E, Issinger OG, Niefind K. 2008. The interaction of CK2alpha and CK2beta, the subunits of protein kinase CK2, requires CK2beta in a preformed conformation and is enthalpically driven. *Protein Sci.* 17:2180–2186. <http://dx.doi.org/10.1110/ps.037770.108>.
 51. Pickard BS, Malloy MP, Clark L, Lehellard S, Ewald HL, Mors O, Porteous DJ, Blackwood DH, Muir WJ. 2005. Candidate psychiatric illness genes identified in patients with pericentric inversions of chromosome 18. *Psychiatr. Genet.* 15:37–44. <http://dx.doi.org/10.1097/00041444-200503000-00007>.
 52. Sun H, Hunter T. 2012. Poly-small ubiquitin-like modifier (Poly-SUMO)-binding proteins identified through a string search. *J. Biol. Chem.* 287:42071–42083. <http://dx.doi.org/10.1074/jbc.M112.410985>.
 53. Erker Y, Neyret-Kahn H, Seeler JS, Dejean A, Atfi A, Levy L. 2013. Arkadia, a novel SUMO-targeted ubiquitin ligase involved in PML degradation. *Mol. Cell. Biol.* 33:2163–2177. <http://dx.doi.org/10.1128/MCB.01019-12>.
 54. Maridor G, Park W, Krek W, Nigg EA. 1991. Casein kinase II. cDNA sequences, developmental expression, and tissue distribution of mRNAs for alpha, alpha', and beta subunits of the chicken enzyme. *J. Biol. Chem.* 266:2362–2368.
 55. Graham KC, Litchfield DW. 2000. The regulatory beta subunit of protein kinase CK2 mediates formation of tetrameric CK2 complexes. *J. Biol. Chem.* 275:5003–5010. <http://dx.doi.org/10.1074/jbc.275.7.5003>.
 56. Filhol O, Martiel JL, Cochet C. 2004. Protein kinase CK2: a new view of an old molecular complex. *EMBO Rep.* 5:351–355. <http://dx.doi.org/10.1038/sj.embor.7400115>.
 57. Bolanos-Garcia VM, Fernandez-Recio J, Allende JE, Blundell TL. 2006. Identifying interaction motifs in CK2beta—a ubiquitous kinase regulatory subunit. *Trends Biochem. Sci.* 31:654–661. <http://dx.doi.org/10.1016/j.tibs.2006.10.005>.
 58. Chen M, Li D, Krebs EG, Cooper JA. 1997. The casein kinase II beta subunit binds to Mos and inhibits Mos activity. *Mol. Cell. Biol.* 17:1904–1912.
 59. Hagemann C, Kalmes A, Wixler V, Wixler L, Schuster T, Rapp UR. 1997. The regulatory subunit of protein kinase CK2 is a specific A-Raf activator. *FEBS Lett.* 403:200–202. [http://dx.doi.org/10.1016/S0014-5793\(97\)00011-2](http://dx.doi.org/10.1016/S0014-5793(97)00011-2).
 60. Lieberman SL, Ruderman JV. 2004. CK2 beta, which inhibits Mos function, binds to a discrete domain in the N-terminus of Mos. *Dev. Biol.* 268:271–279. <http://dx.doi.org/10.1016/j.ydbio.2003.12.009>.
 61. Guerra B, Issinger OG, Wang JY. 2003. Modulation of human checkpoint kinase Chk1 by the regulatory beta-subunit of protein kinase CK2. *Oncogene* 22:4933–4942. <http://dx.doi.org/10.1038/sj.onc.1206721>.
 62. Li D, Meier UT, Dobrowolska G, Krebs EG. 1997. Specific interaction between casein kinase 2 and the nucleolar protein Nopp140. *J. Biol. Chem.* 272:3773–3779. <http://dx.doi.org/10.1074/jbc.272.6.3773>.
 63. Ahn BH, Lee JH, Bae YS. 2003. Identification of mutations in protein kinase CKIIbeta subunit that affect its binding to ribosomal protein L41 and homodimerization. *J. Biochem. Mol. Biol.* 36:344–348. <http://dx.doi.org/10.5483/BMBRep.2003.36.4.344>.
 64. Raman C, Kuo A, Deshane J, Litchfield DW, Kimberly RP. 1998. Regulation of casein kinase 2 by direct interaction with cell surface receptor CD5. *J. Biol. Chem.* 273:19183–19189. <http://dx.doi.org/10.1074/jbc.273.30.19183>.
 65. Romero-Oliva F, Allende JE. 2001. Protein p21(WAF1/CIP1) is phosphorylated by protein kinase CK2 in vitro and interacts with the amino terminal end of the CK2 beta subunit. *J. Cell. Biochem.* 81:445–452. [http://dx.doi.org/10.1002/1097-4644\(20010601\)81:3<445::AID-JCB1058>3.0.CO;2-2](http://dx.doi.org/10.1002/1097-4644(20010601)81:3<445::AID-JCB1058>3.0.CO;2-2).
 66. Akten B, Tangredi MM, Jauch E, Roberts MA, Ng F, Raabe T, Jackson FR. 2009. Ribosomal s6 kinase cooperates with casein kinase 2 to modulate the Drosophila circadian molecular oscillator. *J. Neurosci.* 29:466–475. <http://dx.doi.org/10.1523/JNEUROSCI.4034-08.2009>.
 67. Olsen BB, Wang SY, Svenstrup TH, Chen BP, Guerra B. 2012. Protein kinase CK2 localizes to sites of DNA double-strand break regulating the cellular response to DNA damage. *BMC Mol. Biol.* 13:7. <http://dx.doi.org/10.1186/1471-2199-13-7>.
 68. Olsen BB, Issinger OG, Guerra B. 2010. Regulation of DNA-dependent protein kinase by protein kinase CK2 in human glioblastoma cells. *Oncogene* 29:6016–6026. <http://dx.doi.org/10.1038/onc.2010.337>.
 69. Episkopou V, Arkell R, Timmons PM, Walsh JJ, Andrew RL, Swan D. 2001. Induction of the mammalian node requires Arkadia function in the extraembryonic lineages. *Nature* 410:825–830. <http://dx.doi.org/10.1038/35071095>.
 70. Hecker CM, Rabiller M, Haglund K, Bayer P, Dikic I. 2006. Specification of SUMO1- and SUMO2-interacting motifs. *J. Biol. Chem.* 281:16117–16127. <http://dx.doi.org/10.1074/jbc.M512757200>.
 71. Briones-Orta MA, Levy L, Madsen CD, Das D, Erker Y, Sahai E, Hill CS. 2013. Arkadia regulates tumor metastasis by modulation of the TGF-beta pathway. *Cancer Res.* 73:1800–1810. <http://dx.doi.org/10.1158/0008-5472.CAN-12-1916>.
 72. Ma T, Chen Y, Zhang F, Yang CY, Wang S, Yu X. 2013. RNF111-dependent neddylation activates DNA damage-induced ubiquitination. *Mol. Cell* 49:897–907. <http://dx.doi.org/10.1016/j.molcel.2013.01.006>.
 73. Poulsen SL, Hansen RK, Wagner SA, van Cuijk L, van Belle GJ, Streicher W, Wikstrom M, Choudhary C, Houtsmuller AB, Marteiijn JA, Bekker-Jensen S, Mailand N. 2013. RNF111/Arkadia is a SUMO-targeted ubiquitin ligase that facilitates the DNA damage response. *J. Cell Biol.* 201:797–807. <http://dx.doi.org/10.1083/jcb.201212075>.