# CKS Proteins Protect Mitochondrial Genome Integrity by Interacting with Mitochondrial Single-stranded DNA-binding Protein*

**Marko Radulovic‡, Eleanor Crane‡, Mark Crawford§, Jasminka Godovac-Zimmermann§, and Veronica P. C. C. Yu‡¶**

**Cyclin-dependent kinase subunit (CKS) proteins interact with cyclin-dependent kinases (CDKs) with high affinity. Mammalian CKS1 and CKS2 bind CDK1 and CDK2 and partake in the control of cell cycle progression. We identified CKS-interacting proteins by affinity purification followed by mass spectrometry in the human lymphocytic cell line Ramos. Apart from known interactors, such as CDKs, we identified a novel CDK-dependent interaction between CKS proteins and the mitochondrial single-stranded DNA-binding protein (mtSSB). mtSSB bound both CKS1 and CKS2 and underwent CDK-dependent phosphorylation. mtSSB is known to participate in replication of mitochondrial DNA. We demonstrated that mitochondrial morphology and DNA integrity were compromised in cells depleted of both CKS proteins or that had inhibited CDK activity. These features are consistent with the hypothesis of CKS-dependent regulation of mtSSB function and support a direct role of cell cycle proteins in controlling mitochondrial DNA replication.** ***Molecular & Cellular Proteomics 9:145–152, 2010.***

CKS[1] proteins are binding partners of cyclin-dependent kinases (CDKs) and their activating cyclins. CKS proteins covalently linked to Sepharose beads have been used historically as a reagent to purify active CDK complexes and to help identify CDK-dependent substrates (1).

CKS proteins are evolutionarily conserved. In higher eukaryotes, there are two orthologues, CKS1 and CKS2. Human *CKS1* and *CKS2* have been proposed as potential oncogenes and biomarkers for cancer prognosis (2–8). Murine knockdown of both CKS proteins results in lethality (9). Human CKS1 has been demonstrated to be responsible for degradation of the cell cycle inhibitor $p27^{kip}$ by binding to the E3 ligase complex $^{SCF}$SKP2 (10). This governs the entry of cells into S phase. Such function is unique to human CKS1 as human CKS2, which is 81% identical in amino acid sequence, cannot substitute for this function.

Cks1 in yeast has been shown to participate in the regulation of transcription of a subset of genes. This evolutionarily conserved function is also observed in mammalian cells (9, 11). This could potentially explain the lethal phenotype observed when both copies of *Cks* genes are deleted in mammalian cells.

In an effort to identify potential binding partners to CKS proteins apart from CDKs, we applied CKS-coupled Sepharose beads to mammalian total cell extracts and subjected the eluates to mass spectrometry for identification of proteins. Interestingly, we identified specific binding of both CKS1 and CKS2 to the mitochondrial single-stranded DNA-binding protein (mtSSB).

Single-stranded DNA-binding proteins are evolutionarily conserved, bind selectively to single-stranded DNA, and enable DNA replication (12). mtSSBs are small proteins (molecular mass range from 13 to 16 kDa) that adopt a homotetrameric structure (13). mtSSB has a stimulatory role on the rate of DNA unwinding by the mitochondrial TWINKLE helicase (14) and plays an important role in enhancing replication and repair of mitochondrial DNA.

Mitochondria control cellular energy metabolism and are also essential in vital biological pathways such as apoptosis. Mitochondrial DNA accumulates somatic mutations during aging and pathogenic processes such as cancer and diabetes (15). Mitochondrial DNA copy number is usually maintained during cell division, although it is not clear whether any unifying mechanism regulates this process. It is also uncertain how mtDNA replication is related to the cell cycle despite evidence of certain signaling factors such as RAS, which was reported to signal mitochondrial replication in $G_1$ (16).

This study provides a proteomics analysis of the CKS protein complex in Ramos cells. mtSSB was thereby identified as a novel interactor protein of CKS1 and CKS2. Given that CKS proteins and CDKs are key drivers of the cell cycle, we also aimed to examine whether CKS-CDK protein complexes have direct roles in regulating mtSSB function.

From the ‡Eukaryotic Chromatin Dynamics Group, Medical Research Council Clinical Sciences Centre, Imperial College Hammersmith Campus, Du Cane Road, London W12 0NN and §Centre for Molecular Medicine, Department of Medicine, The Rayne Institute, University College London, London WC1E 6JJ, United Kingdom

Received, February 11, 2009, and in revised form, June 24, 2009

Published, MCP Papers in Press, September 28, 2009, DOI 10.1074/mcp.M900078-MCP200

[1] The abbreviations used are: CKS, cyclin-dependent kinase subunit; CDK, cyclin-dependent kinase; MEF, murine embryonal fibroblast; mtSSB, mitochondrial single-stranded DNA-binding protein; shRNA, short hairpin RNA; SKP, S-phase kinase-associated protein; LTQ, linear trap quadrupole.

TABLE I
*Proteins identified by use of baits attached to Sepharose beads*

CKS1, CKS2, CKS1E63Q, and CKS2E63Q were attached to Sepharose beads and used as baits. BSA coupled to the beads was used as control. Interactor proteins were pulled down from lysates of Ramos cells and identified by use of LC-MS/MS. Proteins binding to control beads were subtracted from identified proteins binding to baits. Only proteins identified in three of four biological replicates are listed for CKS1 and CKS2. Proteins identified in two out of four experiments for CKS1E63Q and CKS2E63Q are not shown. LDH, lactate dehydrogenase.

| Bait proteins | NCBI accession number | Expectation value (protein) | SEQUEST score | Sequence coverage | Unique peptides |
|---|---|---|---|---|---|
| | | | | % | |
| CKS1 | | | | | |
| CDK1 | gi\|16306492 | $1.4e^{-14}$ | 230 | 70 | 16 |
| CDK2 | gi\|1942626 | $1.0e^{-10}$ | 146 | 53 | 13 |
| Cyclin A | gi\|4502611 | $6.2e^{-13}$ | 40 | 13 | 4 |
| Cyclin B1 | gi\|14327896 | $1.2e^{-12}$ | 50 | 16 | 4 |
| Cyclin B2 | gi\|4757930 | $1.8e^{-7}$ | 50 | 14 | 9 |
| mtSSB | gi\|4507231 | $4.8e^{-10}$ | 30 | 26 | 3 |
| HSP90 | gi\|20149594 | $1.4e^{-11}$ | 20 | 11 | 6 |
| CDC37 | gi\|5901922 | $9.2e^{-8}$ | 20 | 7 | 2 |
| SKP2 | gi\|16306595 | $6.8e^{-13}$ | 70 | 28 | 7 |
| SKP1A | gi\|25777713 | $1.9e^{-12}$ | 20 | 18 | 2 |
| Cullin 1 | gi\|32307161 | $9.2e^{-8}$ | 110 | 18 | 10 |
| LDH, H chain | gi\|13786847 | $5.2e^{-5}$ | 20 | 22 | 2 |
| CKS2 | | | | | |
| CDK1 | gi\|16306492 | $2.1e^{-14}$ | 200 | 75 | 15 |
| CDK2 | gi\|1942626 | $6.9e^{-12}$ | 106 | 47 | 10 |
| Cyclin A | gi\|4502611 | $9.0e^{-11}$ | 30 | 10 | 3 |
| Cyclin B1 | gi\|14327896 | $5.4e^{-11}$ | 80 | 30 | 8 |
| Cyclin B2 | gi\|4757930 | $1.2e^{-13}$ | 30 | 11 | 3 |
| mtSSB | gi\|4507231 | $5.7e^{-8}$ | 20 | 17 | 2 |
| HSP90 | gi\|20149594 | $1.4e^{-11}$ | 20 | 4 | 2 |
| CDC37 | gi\|5901922 | $8.3e^{-8}$ | 20 | 8 | 2 |
| LDH, H chain | gi\|13786847 | $2.0e^{-11}$ | 30 | 26 | 3 |

## EXPERIMENTAL PROCEDURES

*Recombinant Protein Production and Purification*—Full-length *CKS1*, *CKS2*, *CKS1E63Q*, *CKS2E63Q*, and *CDK1* were cloned into the pET-22HT vector (Novagen). Recombinant protein was purified from bacterial lysates by nickel-chelating affinity chromatography (nickel-nitrilotriacetic acid, Qiagen, Crawley, UK) followed by size exclusion chromatography with the AKTAexplorer system on a HiLoad 16/60 Superdex 75 prep grade column (GE Healthcare). Bovine serum albumin (>96%) was purchased from Sigma and additionally purified by use of the HiLoad 16/60 Superdex 75 prep grade column. Purified recombinant proteins were covalently immobilized on beads by the use of *N*-hydroxysuccinimidyl-Sepharose 4 Fast Flow (*N*-hydroxysuccinimidyl-Sepharose, Sigma-Aldrich). Unreacted sites were blocked with 1 M ethanolamine, pH 8.0. Measuring of coupling efficiency was done as described by the GE Biosciences *N*-hydroxysuccinimidyl-Sepharose manual. Briefly, coupling solution was acidified to pH 2.5 by use of 2 M glycine-HCl, pH 2.0, and the absorbance was measured at 280 nm.

*Binding Experiments*—CKS1, CKS2, CKS1E63Q, CKS2E63Q, and CDK1 were attached to Sepharose beads as explained above and incubated at room temperature for 1 h in a solution of recombinant human mtSSB at a concentration of 50 $\mu$g/ml in PBS supplemented with 1% BSA. After three washes with 10 ml of PBS with or without increasing salt stringency washes (to final NaCl concentrations of 150, 300, and 500 mM), proteins were eluted with SDS sample buffer and applied to SDS-PAGE and Western blotting using an anti-mtSSB antibody (Abcam, Cambridge, MA; catalog number Ab26205-50).

*Pulldown Experiments*—Purified recombinant CKS proteins were covalently immobilized on beads by use of *N*-hydroxysuccinimidyl-Sepharose 4 Fast Flow (Sigma-Aldrich) as described above. Ramos cells ($1.5 \times 10^9$ per sample) were lysed by slow rotation for 15 min in 10 ml of hypotonic buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 2 mM EDTA, 0.3 mM sodium vanadate, 5 mM sodium pyrophosphate, 5 mM sodium fluoride, 5 mM $MgCl_2$, 1 mM DTT, complete protease inhibitors (Roche Applied Science), and ATP regeneration system (2.4 mM ATP, 100 mg/ml creatine phosphokinase, and 10 mM creatine phosphate) per sample. Nuclei were pelleted at 300 × *g* for 10 min. Nuclear proteins were extracted with 4 volumes of high salt buffer B (20 mM HEPES, pH 7.9, 0.65 M NaCl, 1 mM EDTA, and 10% glycerol together with all other additives as in buffer A) by rotation for 2 h at 4 °C. Cytoplasmic and nuclear fractions were combined in a 4:1 ratio, resulting in final salt concentration of 150 mM. The lysate was then spun three times at 16,000 × *g*. 100 $\mu$l of protein-coated Sepharose per sample was mixed with the cellular lysate and incubated overnight at 4 °C under slow rotation. Sepharose beads were washed three times with 12 ml of buffer identical in composition to the final lysis buffer.

Bound proteins were eluted with 7 M urea, 2 M thiourea, and 4% CHAPS solution. Eluate was extracted by methanol and chloroform and precipitated by methanol. Briefly, 600 ml of methanol, 150 ml of chloroform, and 450 ml of water were added to 200 ml of eluate and spun at 16,000 × *g* for 1 min. The upper aqueous layer was discarded, and the protein partitioned at the interphase was precipitated by the addition of 450 $\mu$l of methanol. After a 2-min spin at 16,000 × *g*, pellets were dissolved in 6 M urea, reduced, alkylated, and digested by sequencing grade modified trypsin (Promega, Southampton, UK) overnight at 37 °C. Tryptic peptides were desalted by use of Sep-Pak C18 cartridges (Waters) and analyzed by LC-MS/MS. A fraction of the protein pellets was analyzed by two-dimensional electrophoresis as described previously in detail (39). Briefly, proteins from all fractions

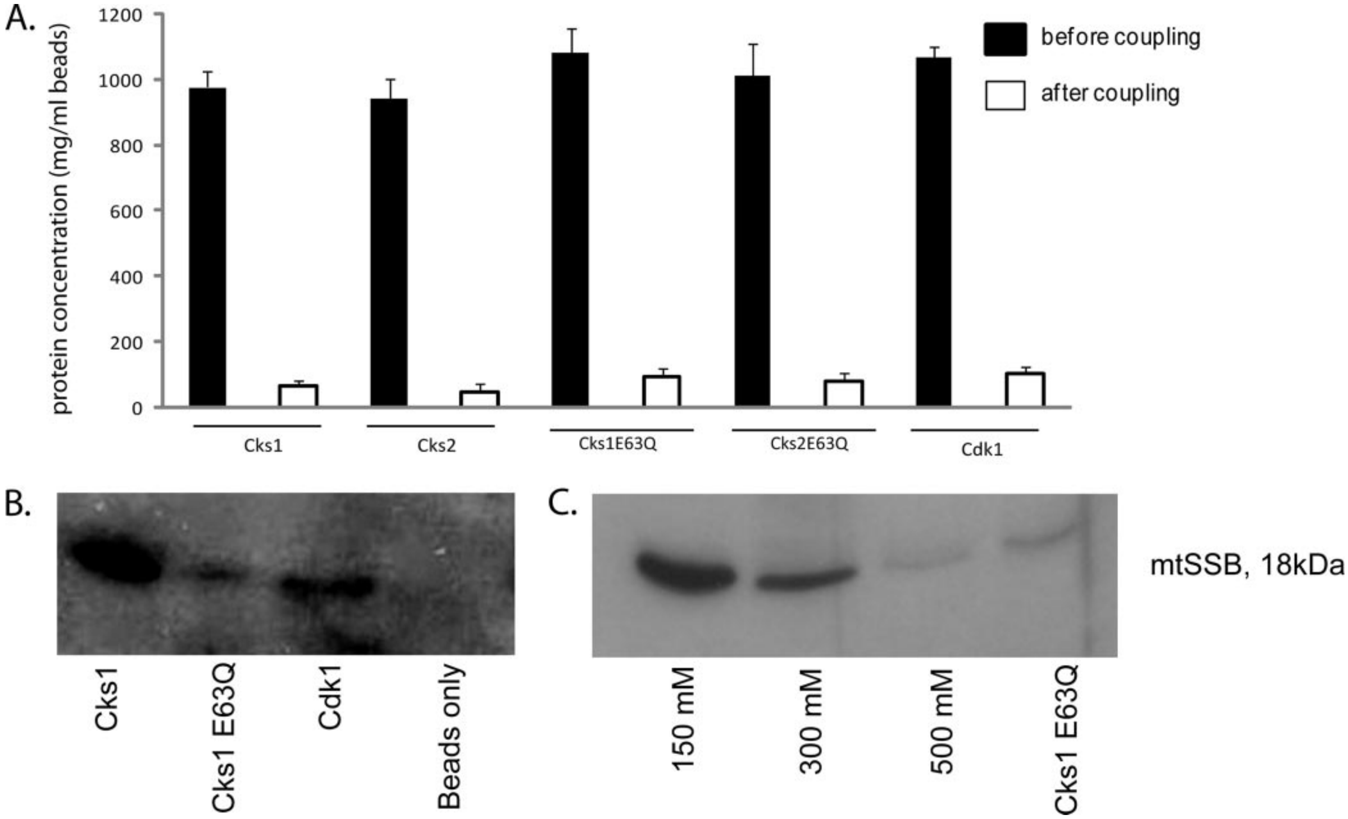


FIG. 1. **mtSSB binds CKS1 directly.** *A*, binding of proteins to the Sepharose beads. The coupling efficiency was determined spectrophotometrically at 280 nm after the pH of protein solutions was adjusted to 2.5. The amount of protein bound to beads was calculated by subtracting the total protein amount in solution before and after the coupling process, taking into account the wet volume of beads used for coupling. The average amount of bound protein was 938 ± 58 mg/ml of beads. Bar chart shows the mean ± standard error of 4 independent experiments. *B*, recombinant CKS1, CKS1E63Q, and CDK1 were bound to Sepharose beads and used as baits for mtSSB in solution (50 $\mu$g/ml). Relative amounts of mtSSB in eluates were assessed by use of the mtSSB antibody and Western blotting. CKS1 binds mtSSB efficiently but not CKS1E63Q or CDK1. *C*, the experiment in *B* was repeated with more stringent salt washes. CKS1 binding to mtSSB was stable up to a final salt concentration of 300 mM. CKS1E63Q beads washed with PBS only were added as a negative control (*right lane*).

were solubilized in IEF buffer (7 M urea, 2 M thiourea, 65 mM DTT, 2% ASB-14, and 4% CHAPS) and separated according to their isoelectric point using immobilized pH gradient strips (pH 3–10) 7 cm in length (GE Healthcare). The proteins were then additionally separated by SDS-PAGE and visualized by silver staining.

*LC-MS/MS*—LC-MS/MS analysis was performed on an LTQ-Orbitrap (Thermo Fisher Scientific) equipped with a SURVEYOR pump and Thermo autosampler. Peptides were resolved using a fused silica $C_{18}$ capillary column (Nikkyo Technos Co.) with an initial desalting step using a Michrom $C_{18}$ Captrap. Liquid chromatography was carried out at ambient temperature at a flow of 50 $\mu$l/min using a gradient of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). Full profile data were acquired on the LTQ-Orbitrap. The tune parameters were as follows: spray voltage, 1.40 kV; capillary temperature, 200 °C. A full scan was collected for eluted peptides in the range of 400–1600 amu with the Orbitrap portion of the instrument at a resolution of 60,000 followed by MS/MS using CID with a dynamic exclusion of 40 s and a maximum number in the dynamic exclusion list of 500 in the LTQ portion of the instrument with a minimum count threshold of 500. An activation q value of 0.25 and activation time of 30 ms were applied for MS2 acquisitions.

The RAW files were analyzed with Bioworks 3.3.1 (Thermo Electron Corp.) as the peak list-generating software using default parameters. The SEQUEST search engine was subsequently used to search against the NCBInr database using the following settings: *Homo sapiens* species restriction (justification: our cell line was of human origin), number of protein entries searched, 218,357; number of missed cleavages permitted, 2; precursor ion mass tolerance, 10 ppm; fragment ion mass tolerance, 0.8 Da; fixed modification, carbamidomethyl (residue specificity, cysteine); variable modification, oxidation (residue specificity, methionine); enzyme specificity, trypsin. The search results were subsequently filtered using a $\Delta$CN of 0.1; XCorr *versus* charge state of 1.5, 2.0, 2.5, and 3.0; cutoff expectation values: default peptide probability, 0.001; default protein probability, 0.001; and number of distinct peptides, 2. Other parameters were at default settings. The result was subsequently run through X! Tandem software embedded within SCAFFOLD 2.1.03 software, and results were validated.

*Two-dimensional Electrophoresis*—Two-dimensional electrophoresis was performed as described previously in detail (39). Briefly, proteins from all fractions were solubilized in IEF buffer (7 M urea, 2 M thiourea, 65 mM DTT, and 4% CHAPS) and separated according to their isoelectric point using immobilized pH gradient strips (pH 3–10) 7–13 cm in length (GE Healthcare). The proteins were then additionally separated by SDS-PAGE and visualized by silver staining.

*MALDI-TOF MS*—Protein spots were excised from the gel and processed automatically by Progest (Genomic Solutions, Huntingdon, UK). Briefly, gel pieces were soaked in 50 mM $NH_4HCO_3$, dehy-

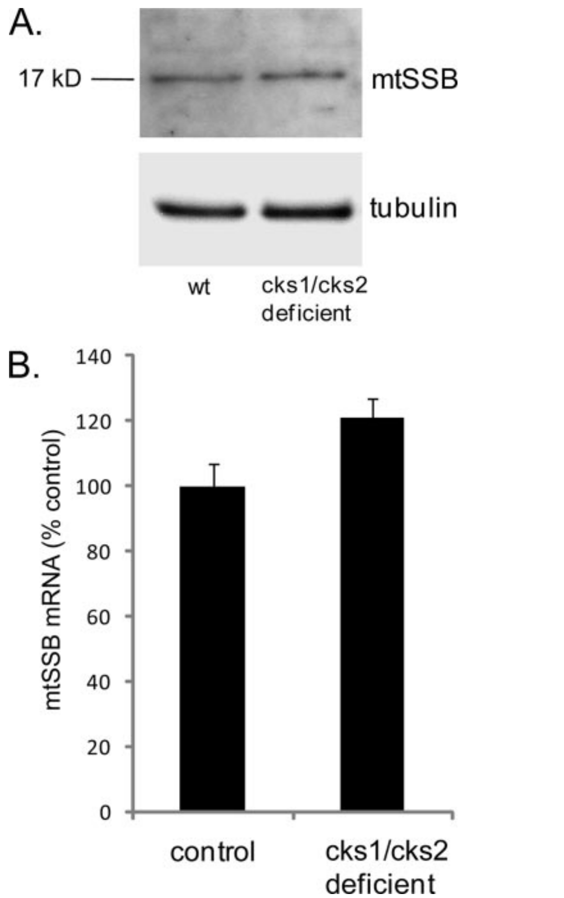


FIG. 2. **mtSSB protein and mRNA levels in *Cks1/Cks2*-silenced cells.** *A*, protein levels of mtSSB as determined by immunoblotting. Tubulin staining was used as a loading control. *B*, mtSSB mRNA levels by quantitative RT-PCR. The experiments shown are representative of the three performed. The results show the mean ± S.E. of four independent experiments.

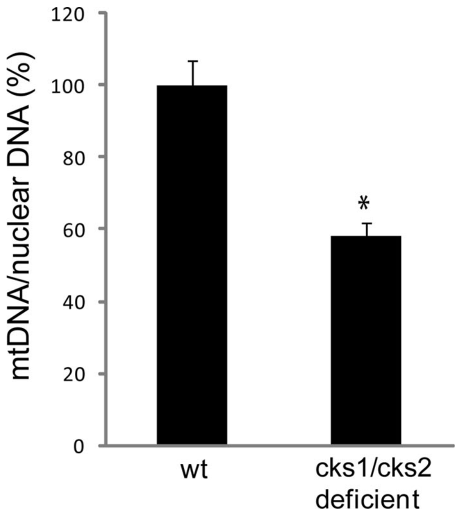


FIG. 3. **Mitochondrial DNA replication is dependent on functional CKS-CDK complex.** The relative content of mitochondrial *versus* nuclear DNA was determined by quantitative RT-PCR. Relative mtDNA content in wild type (*wt*) MEFs in comparison with *Cks* knockdown MEFs. Results are expressed as mean values ± S.E. of the representative experiment from the three performed. Statistically significant differences were determined by analysis of variance: *, $p < 0.05$.

drated with acetonitrile, then reduced by 10 mM DTT, and alkylated with 100 mM iodoacetamide. Dried gel pieces were rehydrated with 3 μl of a 30 ng/μl solution of sequencing grade porcine trypsin (Promega) in 50 mM $NH_4HCO_3$ buffer for 15 min. An additional 9 μl of 50 mM $NH_4HCO_3$ was added thereafter, and digestion was carried out at 30 °C overnight. Tryptic digests (0.7 μl) were applied to the target plate and allowed to air dry, and then 0.7 μl of a saturated solution of α-cyano-4-hydroxycinnamic acid in 33% acetonitrile and 0.1% TFA (v/v) was overlaid.

Mass spectra of the tryptic digests were obtained on an Autoflex MALDI-TOF mass spectrometer (Bruker Daltonics, Billerica, MA). External calibration was done with a mixture of seven standard peptides by use of the FLEXControl Version 1.1.62.0 peak list-generating software with default parameters. Spectra were analyzed by use of the peak picking Bruker DataAnalysis for TOF 1.6g software. Internal calibration was carried out by use of trypsin autolysis peaks. Monoisotopic peptide masses of clearly defined peaks over the intensity threshold of at least three times the base line were assigned manually, trypsin autolysis and keratin peaks were excluded, and the mass lists were used for protein identification in the NCBInr 20090123 nonredundant protein database by Mascot 2.0 search engine (Matrix Science Inc., London, UK) using the following settings: fixed modification, carbamidomethyl; variable modification, oxidation (Met); taxonomy, restricted to *H. sapiens*, number of protein entries searched, 218,357; enzyme specificity, trypsin. The mass accuracy of our analysis was set at 70 ppm, and up to one missed cleavage was allowed. The following acceptance criteria were used: a Mascot score above the cutoff score of 76 (considered significant; over 95% probability that the result is not false positive) and at least four matching masses.

*Cks1 and Cks2 Knockdown in Immortalized Murine Embryonal Fibroblasts (MEFs)*—Two shRNAs against *Cks1* (17) were cloned into pRETRO SUPER, and the constructs were verified by sequencing. Recombinant retroviruses encoding shRNA against *Cks1* or a control sequence were produced in 293T cells by transient transfection. The viruses were used to infect wild-type or *Cks2* knock-out immortalized MEFs, three rounds of infections were carried out over 24 h, and after another day, the cells were selected with puromycin at 1 μg/ml for 3 days after which the knockdown was verified by quantitative RT-PCR.

*Analysis of Mitochondrial DNA Content*—Quantification of mitochondrial DNA content by quantitative PCR and analysis of mitochondrial DNA morphology were carried out according to published work (18, 19).

*Phosphoprotein Enrichment and Immunoblotting*—The mitochondrial fraction (20) of Ramos cells or MEFs was enriched for phosphoproteins by use of the Talon PMAC (phosphate metal affinity chromatography) phosphoprotein enrichment kit (Clontech) as instructed by the manufacturer. Proteins in the eluted phosphoprotein and flow-through fractions were concentrated by use of methanol precipitation as described above, and 40 μg of protein was then loaded into each lane of an SDS-PAGE gel. Chicken polyclonal antibody to mtSSB came from Abcam, rabbit polyclonal antibody to HSP90 was from New England Biolabs (Hitchin, UK), and rabbit anti-tubulin antibody was obtained from Sigma-Aldrich. Immunoblotting was carried out according to standard protocols.

## RESULTS

*Identification of CKS-interacting Proteins*—CKS proteins covalently coupled to Sepharose beads have been used as a tool to purify active CDKs from eukaryotes and plants (21). We

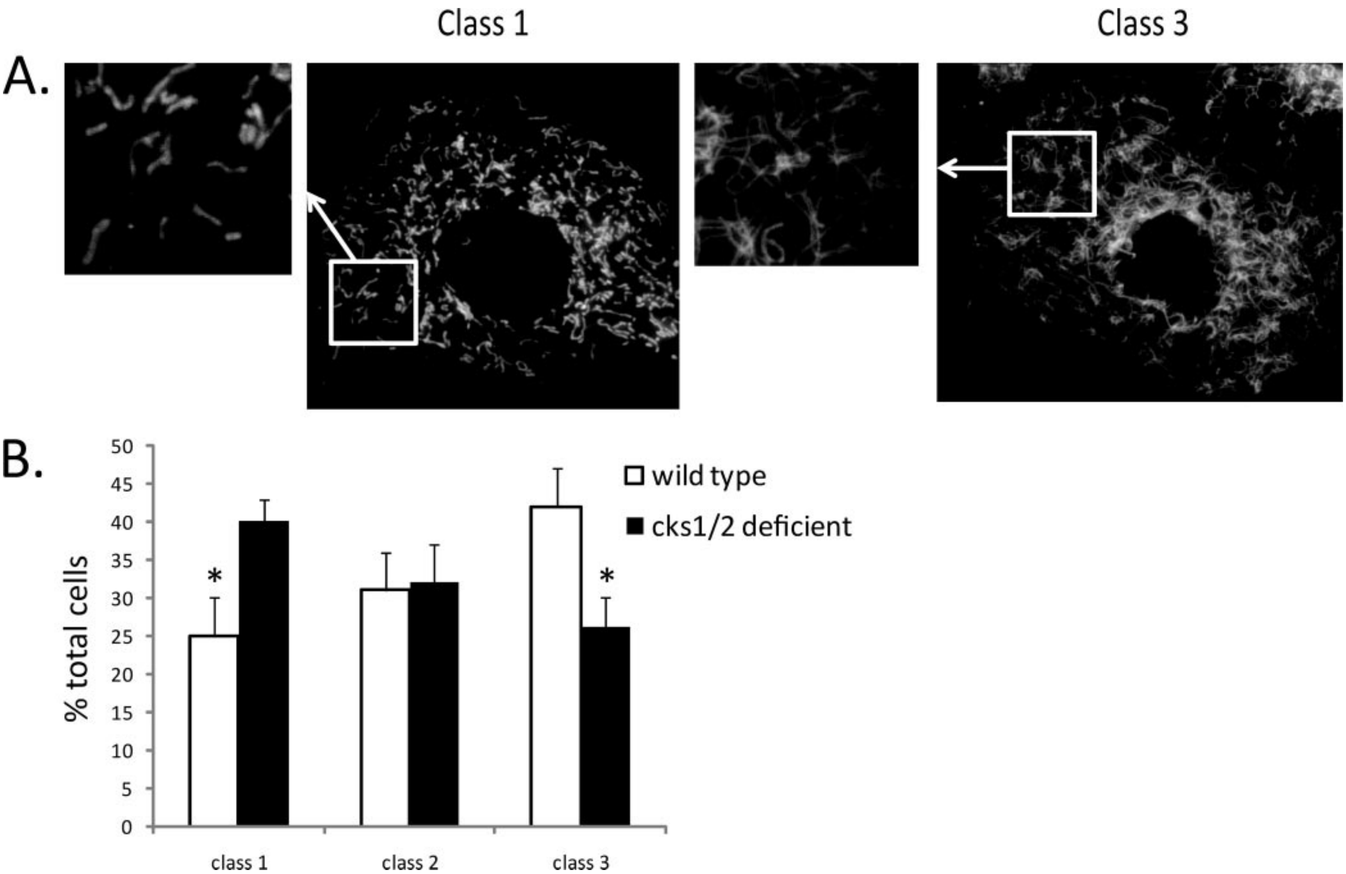


FIG. 4. **Knockdown of *Cks1/2* leads to increased fragmentation of mitochondria.** *A*, mitochondrial morphology was observed under a fluorescence microscope using a 60× objective. Mitochondria were classified according to their morphology as Class 1 (fragmented mitochondria), Class 2 (mixed), or Class 3 (tubular mitochondria). *B*, the number of cells with the indicated mitochondrial morphology is shown as a percentage of the total number counted (minimum, 200). The results show the mean ± S.E. of four independent experiments. *, $p < 0.05$.

coupled human CKS1 or CKS2 to Sepharose beads and used these as affinity matrix to pull down CKS-interacting proteins from Ramos cells. This cell line was chosen because it expressed high endogenous levels of CKS1 and CKS2 (data not shown).

Table I shows our results following affinity purification and analysis by LC-MS/MS. These results were also confirmed using two-dimensional electrophoresis (supplemental Fig. S1). In agreement with published work (22, 23), CKS1 and CKS2 co-purified with CDK1, CDK2, and their respective activating cyclins, cyclin B and cyclin A (Table I). Furthermore, the Skp2 complex (which comprises CUL-1, SKP1, and SKP2), which is known to interact only with CKS1, was identified here in the CKS1 sample but not in CKS2 (10). This validates the specificity and sensitivity of our method. Additional hits included the mtSSB, lactate dehydrogenase H chain and the HSP90-CDC37 chaperone complex (Table I). This study will only focus on the biological significance of the CKS-mtSSB interaction.

*CKS Proteins Interact Specifically with mtSSB*—We chose to focus our study on the mtSSB as it has not previously been reported to associate with CKS or CDK proteins. The interaction of mtSSB with the CKS-CDK protein complex was further confirmed by a binding assay using recombinant proteins expressed in bacteria. Fig. 1 shows that recombinant mtSSB bound directly to CKS1 (similar results were obtained for CKS2; data not shown). Monomeric CDK1 and mutant CKS1 that harbors a point mutation in its loop that interacts with CDK1, CKS1E63Q (24), had much reduced capacity to bind purified mtSSB. In agreement with this, when a pulldown from Ramos cell lysate was performed using CKS1E63Q and CKS2E63Q coupled to Sepharose beads, binding to mtSSB as well as CDKs was abrogated (data not shown). This suggests that *in vivo* mtSSB only binds to CKS proteins that are in complex with CDKs.

*CKS-deficient Cells Have Compromised Mitochondria*—Because both CKS1 and CKS2 bound mtSSB, and it is known that CKS1 and CKS2 play redundant roles in the maintenance of cell viability (9), we decided to test mitochondrial integrity in cells deficient in both CKS1 and CKS2 proteins. CKS proteins have been shown to regulate transcription of a subset of proteins (25). Expression of mtSSB is strictly regulated, and mtDNA content is directly proportional to the abundance of mtSSB mRNA (26). We examined both the protein levels and

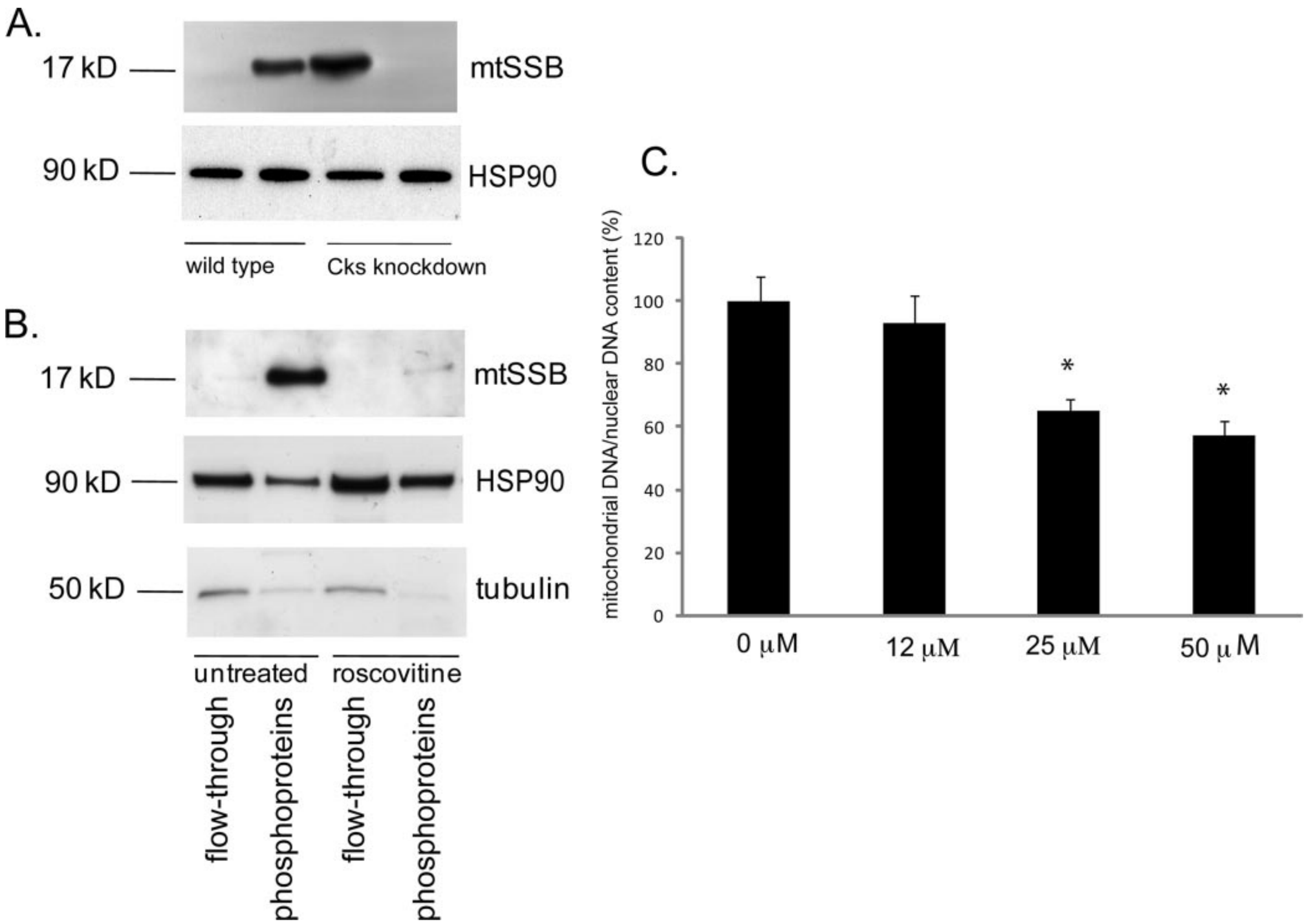


FIG. 5. **mtSSB is phosphorylated by CDK1.** Mitochondrial extracts were isolated from Ramos cells and applied to phosphoprotein enrichment columns. Western blotting was carried out using mtSSB, HSP90, and tubulin antibodies. 40 μg of protein was loaded in each lane. Molecular weight markers are indicated on the *left*. The experiment shown is representative of the three performed. *A*, in wild-type Ramos cells (*left*), all mtSSB was phosphorylated (found in the phosphorylated fraction). Knockdown of *Cks* genes by shRNA (*right*) abrogated the phosphorylation (all mtSSBs were detected in the flow-through unphosphorylated fraction). HSP90, a protein found abundantly in mitochondria that is known to be phosphorylated, acts as a "loading" control. *B*, Ramos cells were either treated or not treated with 20 μM roscovitine for 16 h prior to lysis. Roscovitine inhibited expression of mtSSB and its phosphorylation. *C*, wild type MEFs were treated with increasing concentrations of roscovitine for 16 h. The relative amount of mitochondrial *versus* nuclear DNA was determined by quantitative RT-PCR as detailed in Fig. 3. *, $p < 0.05$.

expression of mtSSB mRNA to determine whether *Cks* knockdown cells have reduced mtSSB (we refer to MEF cells with *Cks2* genetically knocked out and *Cks1* knocked down by shRNA as "*Cks* knockdown cells"). This was not the case (Fig. 2, *A* and *B*).

mtSSB ensures faithful replication of mitochondrial DNA (27). Therefore, we performed a standard assay to measure relative mitochondrial DNA content in control *versus Cks* knockdown cells by quantitative PCR (19). Concomitant with a reduced amount of mitochondrial DNA (Fig. 3), *Cks* knockdown cells showed abnormal mitochondrial morphology with increased fragmentation (Fig. 4). This is consistent with compromised mitochondrial DNA replication as reduced mitochondrial DNA has been linked to a more fragmented morphology of mitochondria (28).

*mtSSB Is Phosphorylated by CDK*—Given that mtSSB binding to CKS proteins is CDK-dependent, we tested whether mtSSB is a substrate of CDK. We isolated mitochondria from Ramos cells and applied a commercially available phosphate metal affinity chromatography kit to enrich for phosphorylated proteins. mtSSB was enriched in the phosphorylated fraction. Phosphorylation is dependent on CKS binding as MEFs deplete of CKS proteins did not phosphorylate mtSSB (Fig. 5*A*). Roscovitine is a specific CDK inhibitor that displays selectivity toward CDK1, CDK2, and CDK5 (29). Application of roscovitine abrogated mtSSB phosphorylation and decreased overall mtSSB expression (Fig. 5*B*). Incidentally, roscovitine was able to inhibit mitochondrial DNA replication in a fashion similar to that of the depletion of CKS proteins (Fig. 5*C*). These results suggest that mtSSB undergoes CKS-CDK-dependent phosphorylation *in vivo* and that inhibition of phosphorylation results in defective replication of mitochondrial DNA.

## DISCUSSION

Here we describe a direct interaction between CKS proteins and mtSSB and reveal a previously unknown pathway whereby cell cycle-regulating proteins modulate mitochondrial DNA replication. The lists of proteins pulled down by CKS1 and CKS2 were similar with the only exception being SKP2 and associated proteins Cullin and SKP1. These were found only in complexes containing CKS1 and are in accordance with published data (10, 30, 31). Apart from mtSSB, we also identified specific CKS-mediated binding to lactate dehydrogenase H chain and the HSP90 complex. These hits are currently subjects of further investigation. The absence of any reproducible hits in the pulldowns with CKS1E63Q and CKS2E63Q confirms that our assay is specific and that all the hits identified were mediated by effective CDK binding.

mtSSB is a key component of the mitochondrial DNA replication machinery and is an essential gene in some organisms (27, 32). To investigate whether the interaction between CKS and mtSSB is functionally relevant, we tested whether *Cks* knockdown cells exhibited a defect in mtDNA replication. Based on the reduced mitochondrial DNA content and abnormal mitochondrial morphology we observed in *Cks* knockdown fibroblasts and what is known about the role of mtSSB in mitochondrial DNA replication (33), we speculate that CKS proteins are required for the efficient mtDNA replication through the function of mtSSB. CDKs phosphorylate a number of proteins involved in nuclear DNA replication (34, 35). However, their involvement in replication of mitochondrial DNA has not yet been reported. The ratio of mitochondrial to nuclear DNA is maintained as cells divide, although how this takes place has remained unclear. A number of studies have indicated that mtDNA is mostly replicated in the pre-S phase and that mitochondrial morphology changes as the cell cycle progresses (36). There is only one report so far suggesting that cell cycle proteins can directly control mitochondrial processes (Taguchi *et al.* report a CDK1-Cyclin B-dependent phosphorylation of the mitochondrial protein Drp1 in mitosis).

In this study, we observed a significantly increased proportion of fragmented mitochondria in *Cks* knockdown cells. This may be due to the fact that mtDNA depletion leads to mitochondrial fragmentation (38). We have established that neither the reduced mtDNA content nor changed mitochondrial morphology in *Cks* knockdown cells was due to diminished mtSSB mRNA expression or mtDNA protein levels. Post-translational modifications of mtSSB have not been reported so far. We postulate that the observed phosphorylation of mtSSB is functionally significant because the addition of roscovitine (which specifically inhibits CDK activity) resulted in reduced levels of mitochondrial DNA, similar to the effect of silencing of *Cks* genes from cells. In fact, *Cks* knockdown in MEFs resulted in the abrogation of mtSSB phosphorylation. This is consistent with a model of CKS-CDK-driven phosphorylation of mtSSB.

We envisage a novel pathway whereby CKS-CDK-dependent binding and phosphorylation of mtSSB directly impact its ability to promote replication of mitochondrial DNA. Further investigations are underway to determine the nature of this phosphorylation, whether this fluctuates throughout the cell cycle, and how this controls the function of mtSSB in *in vitro* assays.

* This work was supported by the Medical Research Council and Cancer Research UK (to the Yu laboratory) and European Union Grant NEUPROCF (to J. G. Z.).

S The on-line version of this article (available at http://www.mcponline.org) contains supplemental Fig. S1 and Table S1.

¶ To whom correspondence should be addressed. Phone: 44-2083838261; Fax: 44-2083838306; E-mail: vyu@imperial.ac.uk.

## REFERENCES

1. Zhang, Y., Lin, Y., Bowles, C., and Wang, F. (2004) Direct cell cycle regulation by the fibroblast growth factor receptor (FGFR) kinase through phosphorylation-dependent release of Cks1 from FGFR substrate 2. *J. Biol. Chem.* **279,** 55348–55354
2. Masuda, T. A., Inoue, H., Nishida, K., Sonoda, H., Yoshikawa, Y., Kakeji, Y., Utsunomiya, T., and Mori, M. (2003) Cyclin-dependent kinase 1 gene expression is associated with poor prognosis in gastric carcinoma. *Clin. Cancer Res.* **9,** 5693–5698
3. Lan, Y., Zhang, Y., Wang, J., Lin, C., Ittmann, M. M., and Wang, F. (2008) Aberrant expression of Cks1 and Cks2 contributes to prostate tumorigenesis by promoting proliferation and inhibiting programmed cell death. *Int. J. Cancer* **123,** 543–551
4. Wang, X. C., Tian, J., Tian, L. L., Wu, H. L., Meng, A. M., Ma, T. H., Xiao, J., Xiao, X. L., and Li, C. H. (2009) Role of Cks1 amplification and overexpression in breast cancer. *Biochem. Biophys. Res. Commun.* **379,** 1107–1113
5. Wong, Y. F., Cheung, T. H., Tsao, G. S., Lo, K. W., Yim, S. F., Wang, V. W., Heung, M. M., Chan, S. C., Chan, L. K., Ho, T. W., Wong, K. W., Li, C., Guo, Y., Chung, T. K., and Smith, D. I. (2006) Genome-wide gene expression profiling of cervical cancer in Hong Kong women by oligonucleotide microarray. *Int. J. Cancer* **118,** 2461–2469
6. Li, M., Lin, Y. M., Hasegawa, S., Shimokawa, T., Murata, K., Kameyama, M., Ishikawa, O., Katagiri, T., Tsunoda, T., Nakamura, Y., and Furukawa, Y. (2004) Genes associated with liver metastasis of colon cancer, identified by genome-wide cDNA microarray. *Int. J. Oncol.* **24,** 305–312
7. Chang, H., Qi, X., Trieu, Y., Xu, W., Reader, J. C., Ning, Y., and Reece, D. (2006) Multiple myeloma patients with CKS1B gene amplification have a shorter progression-free survival post-autologous stem cell transplantation. *Br. J. Haematol.* **135,** 486–491
8. Shaughnessy, J. (2005) Amplification and overexpression of CKS1B at chromosome band 1q21 is associated with reduced levels of p27Kip1 and an aggressive clinical course in multiple myeloma. *Hematology* **10,** Suppl. 1, 117–126
9. Martinsson-Ahlzén, H. S., Liberal, V., Grünenfelder, B., Chaves, S. R., Spruck, C. H., and Reed, S. I. (2008) Cyclin-dependent kinase-associated proteins Cks1 and Cks2 are essential during early embryogenesis and for cell cycle progression in somatic cells. *Mol. Cell. Biol.* **28,** 5698–5709
10. Spruck, C., Strohmaier, H., Watson, M., Smith, A. P., Ryan, A., Krek, T. W., and Reed, S. I. (2001) A CDK-independent function of mammalian Cks1: targeting of SCF(Skp2) to the CDK inhibitor p27Kip1. *Mol. Cell* **7,** 639–650
11. Westbrook, L., Manuvakhova, M., Kern, F. G., Estes, N. R., 2nd, Ramanathan, H. N., and Thottassery, J. V. (2007) Cks1 regulates CDK1 expression: a novel role during mitotic entry in breast cancer cells. *Cancer Res.* **67,** 11393–11401
12. Chase, J. W., and Williams, K. R. (1986) Single-stranded DNA binding proteins required for DNA replication. *Annu. Rev. Biochem.* **55,** 103–136

13. Li, K., and Williams, R. S. (1997) Tetramerization and single-stranded DNA binding properties of native and mutated forms of murine mitochondrial single-stranded DNA-binding proteins. *J. Biol. Chem.* **272,** 8686–8694
14. Korhonen, J. A., Gaspari, M., and Falkenberg, M. (2003) TWINKLE has 5′-> 3′ DNA helicase activity and is specifically stimulated by mitochondrial single-stranded DNA-binding protein. *J. Biol. Chem.* **278,** 48627–48632
15. Shadel, G. S. (2008) Expression and maintenance of mitochondrial DNA: new insights into human disease pathology. *Am. J. Pathol.* **172,** 1445–1456
16. Trinei, M., Berniakovich, I., Pelicci, P. G., and Giorgio, M. (2006) Mitochondrial DNA copy number is regulated by cellular proliferation: a role for Ras and p66(Shc). *Biochim. Biophys. Acta* **1757,** 624–630
17. Zhan, F., Colla, S., Wu, X., Chen, B., Stewart, J. P., Kuehl, W. M., Barlogie, B., and Shaughnessy, J. D., Jr. (2007) CKS1B, over expressed in aggressive disease, regulates multiple myeloma growth and survival through SKP2- and p27Kip1-dependent and independent mechanisms. *Blood* **109,** 4995–5001
18. Arakaki, N., Nishihama, T., Kohda, A., Owaki, H., Kuramoto, Y., Abe, R., Kita, T., Suenaga, M., Himeda, T., Kuwajima, M., Shibata, H., and Higuti, T. (2006) Regulation of mitochondrial morphology and cell survival by Mitogenin I and mitochondrial single-stranded DNA binding protein. *Biochim. Biophys. Acta* **1760,** 1364–1372
19. Biesecker, G., Karimi, S., Desjardins, J., Meyer, D., Abbott, B., Bendele, R., and Richardson, F. (2003) Evaluation of mitochondrial DNA content and enzyme levels in tenofovir DF-treated rats, rhesus monkeys and woodchucks. *Antiviral Res.* **58,** 217–225
20. Jiang, X. S., Zhou, H., Zhang, L., Sheng, Q. H., Li, S. J., Li, L., Hao, P., Li, Y. X., Xia, Q. C., Wu, J. R., and Zeng, R. (2004) A high-throughput approach for subcellular proteome: identification of rat liver proteins using subcellular fractionation coupled with two-dimensional liquid chromatography tandem mass spectrometry and bioinformatic analysis. *Mol. Cell. Proteomics* **3,** 441–455
21. Labbé, J. C., Capony, J. P., Caput, D., Cavadore, J. C., Derancourt, J., Kaghad, M., Lelias, J. M., Picard, A., and Dorée, M. (1989) MPF from starfish oocytes at first meiotic metaphase is a heterodimer containing one molecule of cdc2 and one molecule of cyclin B. *EMBO J.* **8,** 3053–3058
22. Arvai, A. S., Bourne, Y., Hickey, M. J., and Tainer, J. A. (1995) Crystal structure of the human cell cycle protein CksHs1: single domain fold with similarity to kinase N-lobe domain. *J. Mol. Biol.* **249,** 835–842
23. Parge, H. E., Arvai, A. S., Murtari, D. J., Reed, S. I., and Tainer, J. A. (1993) Human CksHs2 atomic structure: a role for its hexameric assembly in cell cycle control. *Science* **262,** 387–395
24. Watson, M. H., Bourne, Y., Arvai, A. S., Hickey, M. J., Santiago, A., Bernstein, S. L., Tainer, J. A., and Reed, S. I. (1996) A mutation in the human cyclin-dependent kinase interacting protein, CksHs2, interferes with cyclin-dependent kinase binding and biological function, but preserves protein structure and assembly. *J. Mol. Biol.* **261,** 646–657
25. Yu, V. P., Baskerville, C., Grünenfelder, B., and Reed, S. I. (2005) A kinase-independent function of Cks1 and CDK1 in regulation of transcription. *Mol. Cell* **17,** 145–151
26. Tomáska, L., Nosek, J., and Kucejová, B. (2001) Mitochondrial single-stranded DNA-binding proteins: in search for new functions. *Biol. Chem.* **382,** 179–186
27. Falkenberg, M., Larsson, N. G., and Gustafsson, C. M. (2007) DNA replication and transcription in mammalian mitochondria. *Annu. Rev. Biochem.* **76,** 679–699
28. Gilkerson, R. W., Margineantu, D. H., Capaldi, R. A., and Selker, J. M. (2000) Mitochondrial DNA depletion causes morphological changes in the mitochondrial reticulum of cultured human cells. *FEBS Lett.* **474,** 1–4
29. Meijer, L., Borgne, A., Mulner, O., Chong, J. P., Blow, J. J., Inagaki, N., Inagaki, M., Delcros, J. G., and Moulinoux, J. P. (1997) Biochemical and cellular effects of roscovitine, a potent and selective inhibitor of the cyclin-dependent kinases cdc2, cdk2 and cdk5. *Eur. J. Biochem.* **243,** 527–536
30. Sitry, D., Seeliger, M. A., Ko, T. K., Ganoth, D., Breward, S. E., Itzhaki, L. S., Pagano, M., and Hershko, A. (2002) Three different binding sites of Cks1 are required for p27-ubiquitin ligation. *J. Biol. Chem.* **277,** 42233–42240
31. Ganoth, D., Bornstein, G., Ko, T. K., Larsen, B., Tyers, M., Pagano, M., and Hershko, A. (2001) The cell-cycle regulatory protein Cks1 is required for SCF(Skp2)-mediated ubiquitinylation of p27. *Nat. Cell Biol.* **3,** 321–324
32. Maier, D., Farr, C. L., Poeck, B., Alahari, A., Vogel, M., Fischer, S., Kaguni, L. S., and Schneuwly, S. (2001) Mitochondrial single-stranded DNA-binding protein is required for mitochondrial DNA replication and development in Drosophila melanogaster. *Mol. Biol. Cell* **12,** 821–830
33. Wanrooij, S., Fusté, J. M., Farge, G., Shi, Y., Gustafsson, C. M., and Falkenberg, M. (2008) Human mitochondrial RNA polymerase primes lagging-strand DNA synthesis in vitro. *Proc. Natl. Acad. Sci. U.S.A.* **105,** 11122–11127
34. Chi, Y., Welcker, M., Hizli, A. A., Posakony, J. J., Aebersold, R., and Clurman, B. E. (2008) Identification of CDK2 substrates in human cell lysates. *Genome Biol.* **9,** R149
35. Diffley, J. F. (2004) Regulation of early events in chromosome replication. *Curr. Biol.* **14,** R778–R786
36. McBride, H. M., Neuspiel, M., and Wasiak, S. (2006) Mitochondria: more than just a powerhouse. *Curr. Biol.* **16,** R551–R560
37. Taguchi, N., Ishihara, N., Jofuku, A., Oka, T., and Mihara, K. (2007) Mitotic phosphorylation of dynamin-related GTPase Drp1 participates in mitochondrial fission. *J. Biol. Chem.* **282,** 11521–11529
38. Karbowski, M., and Youle, R. J. (2003) Dynamics of mitochondrial morphology in healthy cells and during apoptosis. *Cell Death Differ.* **10,** 870–880
39. Stannard, C., Soskic, V., and Godovac-Zimmermann, J. (2003) Rapid changes in the phosphoproteome show diverse cellular responses following stimulation of human lung fibroblasts with endothelin-1. *Biochemistry* **42,** 13919–13928