Supporting Information (Pabla et al.)

Materials and Methods

Antibodies and Immunoblot Analysis. The following antibodies were used for initial immunoblot analysis of Chk1: anti-N-terminus Chk1 (Epitomics-E250), Chk1 (Santa Cruz-G4), anti-C-terminus Chk1 (Abcam- 71691), Chk1 (R&D- AF1630) and Chk1 (Proteintech-10362-1-AP). The anti-Chk1 antibody from Proteintech was used for subsequent immunoblot analysis in this study. The antibodies for other proteins were from the following sources: anti-CDK1, anti-phospho-CDK1, anti-CDC25A, anti-CDC25B, anti-CDC25C, anti-Myc, anti-phospho-histone-H3 and phospho-Chk1 (S317 and S345) from Cell Signaling, anti-Nek2 antibody from Santa Cruz Biotech and anti-β-actin and anti-FLAG antibodies from Sigma. Immunoblot analysis was performed by standard protocols using PVDF membranes and enhanced chemiluminescence method was used for signal detection.

Plasmid Construction. The cDNA of Chk1-S was cloned into pcDNA3.1-Myc (Invitrogen), pCMV2-FLAG (Sigma), pEGFP-C3 (Clontech), pEYFP-C3 (Clontech), and pcDNA3.1hygro (Invitrogen) to generate Myc-, FLAG-, GFP-, YFP-tagged, CFP-tagged or untagged Chk1-S plasmids by standard methods. pcDNA3-Chk1-Myc and pcDNA3-Chk1-KD-Myc plasmids were kindly provided by Dr. Piwnica-Worms at Washington University School of Medicine (St. Louis, MO). The pcDNA3-Chk1-Myc plasmid was used as a template to subclone Chk1 into pEGFP-C3 and pECFP vectors and also for site-directed mutagenesis (Stratagene) to generate the Chk1-S317A/S345A plasmid. To generate Tet-on inducible vectors, Myc-tagged Chk1, Chk1-KD, and Chk1-S were subcloned from above mentioned vectors into the pTRE-Tight

vector (Clontech). The YFP-γ-Tubulin plasmid used to express centrosomal marker was from the AFCS set of subcellular localization markers purchased from ATCC (Manassas, VA).

Cell culture, Synchronization and Stable cell generation. HEK293, HeLa, U2OS, MDA-MB-231, HCT116, and MCF-7 cells from ATCC were cultured in DMEM medium supplemented with antibiotics and 10% FBS. The rat kidney proximal tubular cell line (RPTC) was originally obtained from Dr. Hopfer at Case Western Reserve University (Cleveland, OH) and the mouse proximal tubular cell line (BUMPT) from Dr. Lieberthal and Dr. Shwartz from Boston University (Boston, MA). Cell cycle synchronization was achieved by protocols modified from others (44). Briefly, U2OS and HEK293 cells were either incubated in DMEM medium without serum for 48-72 hours to induce G0/G1 arrest or treated with 100 ng/ml Nocodazole (Sigma) and incubated for 16-18 hours to induce M phase arrest. For G1/S arrest, cells were synchronized by double thymidine block. Briefly, asynchronously growing cells were incubated with 2.5 mM thymidine (Sigma) for 17 hrs, followed by washing and releasing cells into fresh medium for 14 hrs and then incubating with 2.5 mM thymidine for another 17 hrs. In some experiments, the cells were then released into normal medium or medium with 100ng/ml nocodazole for 7-9 hrs to trap the cells in G2/M phase. The efficiency of synchronization was tested by Propidium Iodide-based cell cycle analysis kit (Genscript) using flow cytometry. To generate stable cell lines, U2OS and MDA-MB-231 cells were first transfected with pTet-on Advanced Vector (Clontech) and stable cells were selected with G418 (700 μg/ml). The cells were then transfected with pTRE-Tight constructs (empty vector, Chk1-Myc, Chk1-KD-Myc and Chk1-S-Myc) and linear Puromycin Vector (Clontech) and selected with Puromycin (1 μg/ml). The stable cells were then

treated with doxycycline (Clontech) 100 ng/ml for 48 hours and the expression of inducible protein was confirmed by immunoblot analysis.

Gene expression analysis. Total RNA was isolated from different human cell lines using RNA isolation kit from Epicentre Biotechnologies, followed by first-strand cDNA synthesis (Invitrogen) and gene specific PCR analysis. Both conventional RT-PCR and real time RT-PCR were conducted to determine Chk1, Chk1-S, and GAPDH mRNA expression. Conventional RT-PCR used following primers: forward primers (F1 - gactgggacttggtgcaaac and F2 – ctgaagaagcagtcgcagtg) and reverse primers (R1 - gcaggaagccaaatcttctg and R2 – tgggagactctgacacacca). As depicted in Fig. 1c, the primers were used in three sets: P1 (F2 & R1), P2 (F1 & R1), and P3 (F1 & R2). TaqMan-based real-time PCR was conducted using the following primers and probes: Chk1 (forward primer – GGTGCCTATGGAGAAGTTCAA, reverse primer - TCTACGGCACGCTTCATATC, and probe - CAATCTTCACTGCGACTGCTTCTTCAG) and Chk1-S (forward - GTGCAAACCCTGGGAGAA, reverse – CTCTGAGCATCTGGTTCAGG, and probe – TGCCTATGTCTGGCTTCTCCATAGGC). These custom-designed oligonucleotides were synthesized by Applied Biosystems. The relative change in gene expression was calculated using the ΔCT values.

To determine the expression of Chk1 and Chk1-S in normal and fetal human tissues, the cDNA panels were purchased from Clontech. To analyze mRNA expression in normal and cancer tissues, a widely used cDNA panel from Origene (cancer tissue panel 384-I) was used. To determine Chk1 and Chk1-S protein expression in normal human fetal tissues, tissue lysates

were purchased from Proteintech (Chicago, IL). Adult normal and testicular cancer human tissue lysates were purchased from Protein Biotechnologies (Ramona, CA).

Cell fractionation. HEK293 cells were fractionated into nuclear and cytoplasmic fractions using the NE-PER kit (Pierce) according to manufacturer's instruction. Centrosomal fractions were obtained using the method described previously (ref. 35). Briefly, synchronized HEK293 cells (5×10^7) were incubated with 1μM nocodazole and 1μg/ml cytochalasin D for 1 hour at 37°C. Cells were then lysed with 0.5% NP-40 and centrifuged at low speed to remove cell debris and nuclei. The supernatant was treated with DNAse, followed by sedimentation on a sucrose cushion by centrifugation at 10,000*g* for 0.5 hour at 4°C. The sample was further layered onto a discontinuous sucrose gradient and centrifuged to collect the purified centrosomal fraction at the bottom.

Cell transfection, Immunofluorescence, and Analysis of aberrant mitotic entry. Cells were transfected using Lipofectamine 2000 (Invitrogen). Immunofluorescence was carried out using phospho-histone H3 antibody (Cell Signaling) as previously (32, 33). For morphological analysis of aberrant mitotic entry, cells were transfected with GFP-Chk1 or GFP-Chk1-S followed by fixation with 4% Para formaldehyde and nuclear staining with Hoechst33342 (Sigma). The cells with features of aberrant mitotic nuclei (multiple or lobed nuclei, micronuclei and aberrant chromatin condensation) were counted to determine the percentage. Flow cytometric analysis of premature mitotic entry was analyzed by releasing U2OS cells after double thymidine block into nocodazole-containing medium and then staining the cells with anti-phospho-histone-H3 antibody and PI staining. The cells with phospho-histone-H3 positive staining were analyzed for

their DNA content. The cells with 4n or less than 4n DNA content was calculated to determine the percentage of cells entering mitosis before completion of DNA replication.

***In Vitro* translation, Immunoprecipitation and *In vitro* Kinase assay.** Various constructs were used for in vitro expression of Chk1 and Chk1-S using the TNT T7 Quick Coupled Transcription/Translation System (Promega). To determine the interaction between in vitro translated Chk1 and Chk1-S, 25 µl translation products of both Chk1 and Chk1-S were incubated for 1 hour at 30^{o}C followed co-immunoprecipitation (IP) analysis. IP was carried out from *in vitro* translated proteins or cellular lysate using the IP buffer of 1% Triton, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerol phosphate, 1 mM Na_3VO_4, protease inhibitors, 0.2% dodecyl-beta-D-maltoside, and 20 mM Tris as described previously (32). Before analysis, the immunoprecipitates were washed with the IP buffer or a more stringent RIPA buffer (1% NP40, 0.1% SDS, Na.Deoxycholate 0.5 %, 150 mM NaCl, 50 mM Tris). Agarose A/G beads (Santa Cruz Biotech) or anti-Myc conjugated beads (Cell Signaling) were used for different IP experiments. Chk1 kinase activity was measured using the Chktide (Enzo Lifesciences) as the substrate. Briefly, purified Chk1 or immunoprecipitated Chk1 was incubated for 30 minutes at 30°C in the kinase reaction containing 100 µM ATP, 2.5 µCi [γ-^{32}P]ATP (MP Biochemicals), 100 µM Checktide, 50 mM Hepes, 10 mM $MgCl_2$, 0.8 mM EDTA, 0.8 mM DTT, and 10 mM β-glycerophosphate. The reaction mixture was then spotted on filter paper, washed with phosphoric acid and acetone, and ^{32}P incorporation was measured to calculate the relative kinase activity. Chk1-S-Myc was purified from Chk1-S-Myc-transfected HEK293 cells using protein purification kit from Pierce and used for co-incubation experiments.

Tumor Xenograft Model. 10 million MDA-MB-231 cells stably transfected with Tet-On based empty vector, Chk1, Chk1-KD, or Chk1-S were injected into both flanks of 7-week old female nude mice (Harlan). Tumor volume was measured with a Vernier caliper and once the tumors reached ~100 mm^3 (in ~2 weeks), the mice were maintained on drinking water with or without 500μg/ml doxycycline. Tumor volume was then measured weekly. Four weeks later, the tumors were excised for further biochemical analysis.

Supplementary Figures

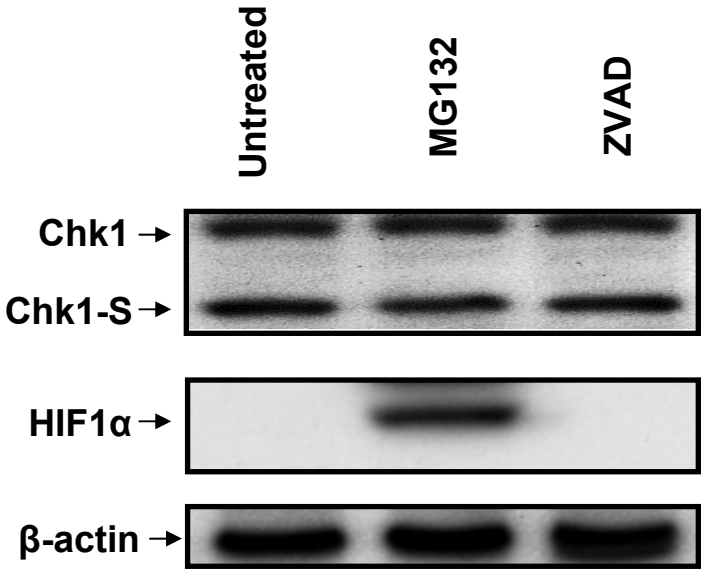

Fig. S1. Effects of proteosome and protease inhibitors on the 43 kD protein in Chk1 immunoblots. HEK293 cells were incubated for three hours with or without 10 μM MG132 (proteosome inhibitor) or 100 μM ZVAD (caspase inhibitor) to collect whole cell lysate for immunoblot analysis of Chk1, HIF1a, and β-actin. The results show that the expression of the 43 kD protein was not affected by proteasome or caspase inhibitors, whereas as a positive control proteosomal inhbition by MG132 induced the accumulation of HIF1a.

a

Chk1 cDNA

atggcagtgccctttgtggaagactgggacttggtgcaaaccctgggagaaggtgcctat
ggagaagttcaacttgctgtgaatagagtaactgaagaagcagtcgcagtgaagattgta
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Chk1-S cDNA

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ggaga----------

----------agccagacata
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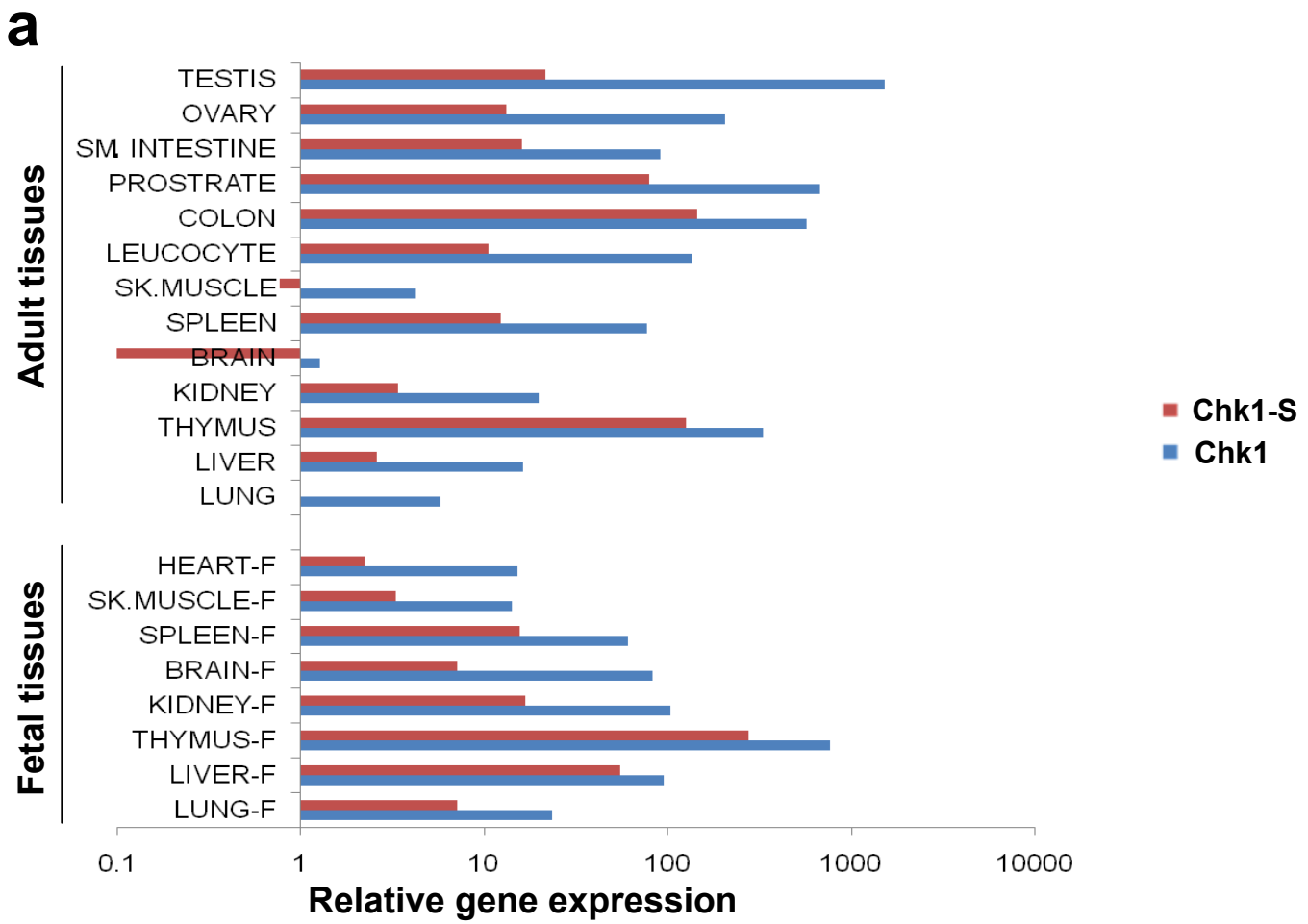
aagctgattgatattgtgagcagccagaagatttggcttcctgccacatga

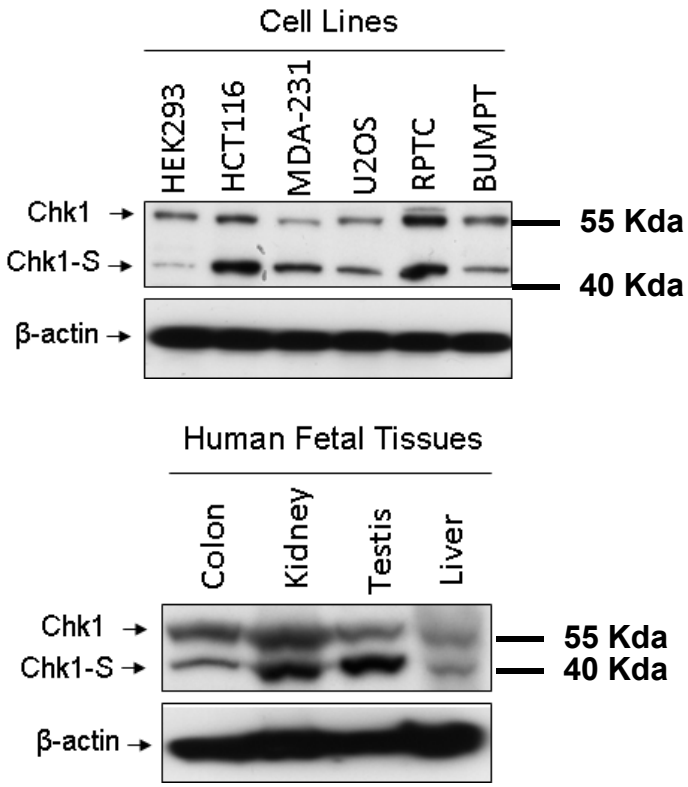
b

Chk1 protein	Chk1-S protein
MAVPFVEDWDLVQTLGEGAYGEVQLAVNR	----------
VTEEAVAVKIVDMKRAVDCPENIKKEICINK	----------
MLNHENVVKFYGHRREGNIQYLFLEYCSGG	----------
ELFDRIEPDIGMPEPDAQRFFHQLMAGVVY	-------MEKPDIGMPEPDAQRFFHQLMAGVVY
LHGIGITHRDIKPENLLLDERDNLKISDFGLAT	LHGIGITHRDIKPENLLLDERDNLKISDFGLAT
VFRYNNRERLLNKMCGTLPYVAPELLK	VFRYNNRERLLNKMCGTLPYVAPELLK
RREFHAEPVDVWSCGIVLTAMLAGELPWDQ	RREFHAEPVDVWSCGIVLTAMLAGELPWDQ
PSDSCQEYSDWKEKKTYLNPWKKIDSAPLA	PSDSCQEYSDWKEKKTYLNPWKKIDSAPLA
LLHKILVENPSARITIPDIKKDRWYNKPLKKGA	LLHKILVENPSARITIPDIKKDRWYNKPLKKGA
KRPRVTSGGVSESPSGFSKHIQSNLDF	KRPRVTSGGVSESPSGFSKHIQSNLDF
SPVNSASSEENVKYSSSQPEPRTGLSLWDT	SPVNSASSEENVKYSSSQPEPRTGLSLWDT
SPSYIDKLVQGISFSQPTCPDHMLLNSQLL	SPSYIDKLVQGISFSQPTCPDHMLLNSQLL
GTPGSSQNPWQRLVKRMTRFFTKLDADKSY	GTPGSSQNPWQRLVKRMTRFFTKLDADKSY
QCLKETCEKLGYQWKKSCMNQVTISTTDRR	QCLKETCEKLGYQWKKSCMNQVTISTTDRR
NNKLIFKVNLLEMDDKILVDFRLSKGDGLEFK	NNKLIFKVNLLEMDDKILVDFRLSKGDGLEFK
RHFLKIKGKLIDIVSSQKIWLPAT	RHFLKIKGKLIDIVSSQKIWLPAT

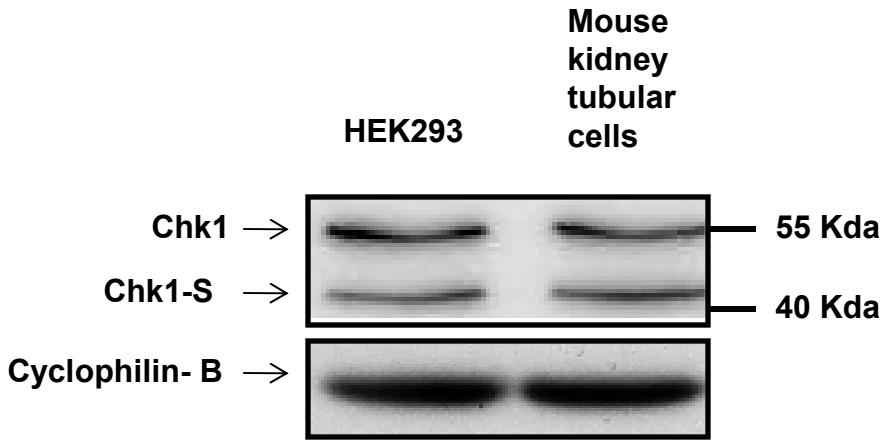
Fig. S2. Sequences of Chk1 and Chk1-S. **a**. cDNA sequences of Chk1 and Chk1-S aligned using ClustalW. The exon-3 coded region in the Chk1 cDNA is marked in blue and is missing in Chk1-S. The start and stop codons are marked in red. **b**. Amino acid sequences of Chk1 and Chk1-S aligned using ClustalW. The N-terminal region of Chk1 marked in blue is missing in Chk1-S. The three amino acids marked in red in Chk1-S are unique to this protein.


a
Adult tissues
Fetal tissues
TESTIS
OVARY
SM. INTESTINE
PROSTRATE
COLON
LEUCOCYTE
SK.MUSCLE
SPLEEN
BRAIN
KIDNEY
THYMUS
LIVER
LUNG
HEART-F
SK.MUSCLE-F
SPLEEN-F
BRAIN-F
KIDNEY-F
THYMUS-F
LIVER-F
LUNG-F
0.1
1
10
100
1000
10000
Relative gene expression
Chk1-S
Chk1

b


Cell Lines
HEK293
HCT116
MDA-231
U2OS
RPTC
BUMPT
Chk1
Chk1-S
β-actin
55 Kda
40 Kda
Human Fetal Tissues
Colon
Kidney
Testis
Liver
Chk1
Chk1-S
β-actin
55 Kda
40 Kda

c


HEK293
Mouse kidney tubular cells
Chk1
Chk1-S
Cyclophilin- B
55 Kda
40 Kda

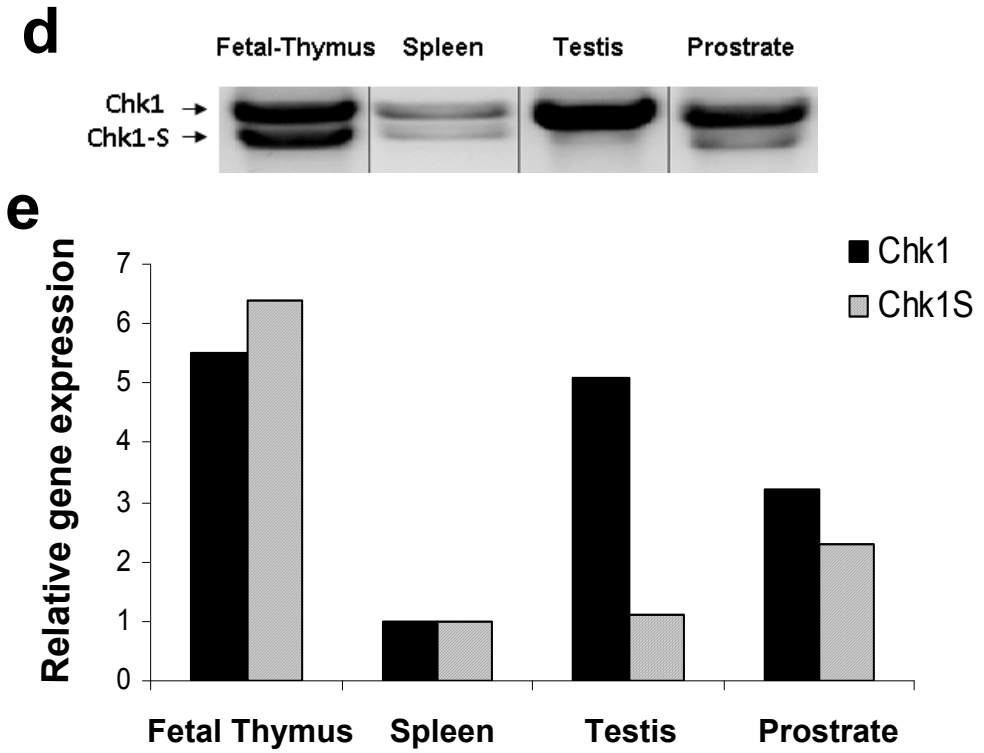
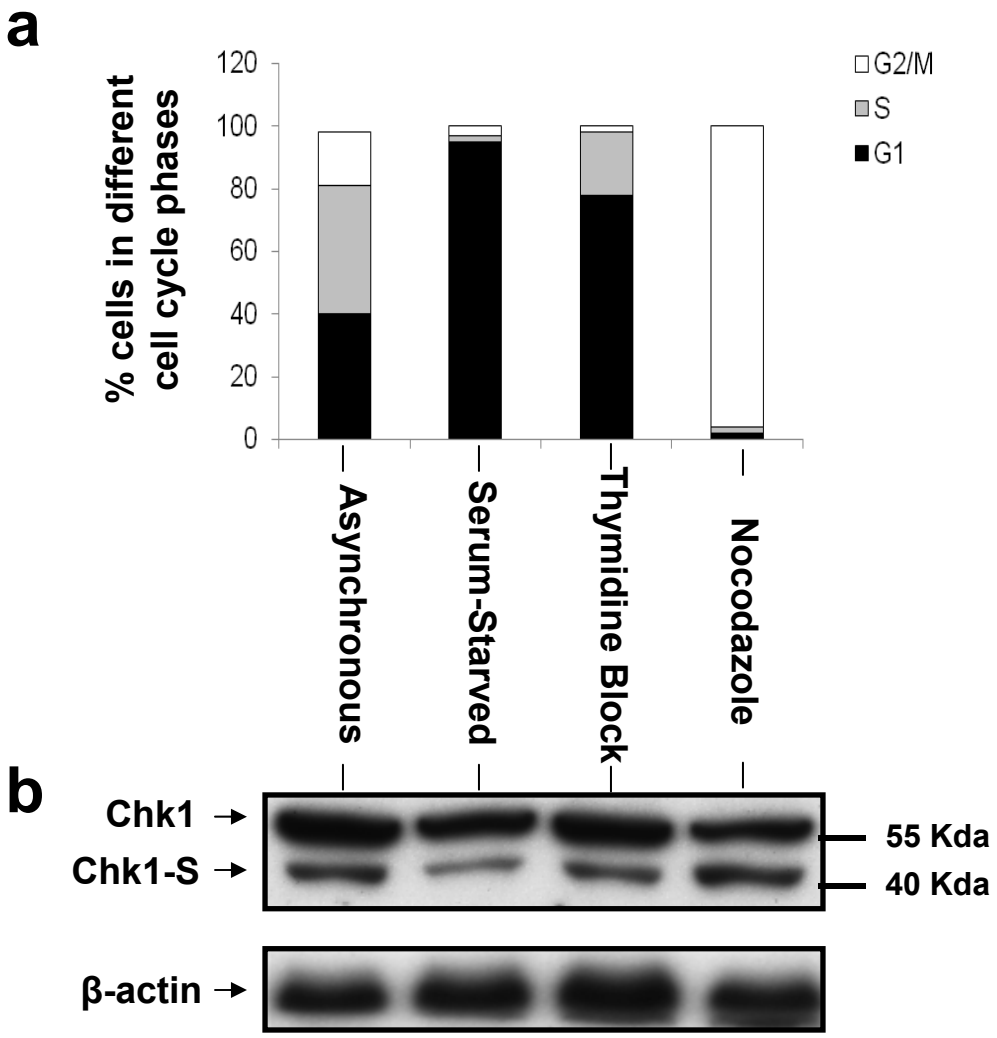


Fig. S3. Chk1 and Chk1-S expression in a variety of cell types and human tissues. a. Real time PCR analysis was performed on cDNA panels of adult and fetal human tissues. Chk1-S was detected in all tissues except adult lung, brain, and skeletal muscle. Chk1-S expression was generally higher in fetal tissues. **b.** Lysates of human, rat (RPTC) and mouse (BUMPT) cell lines and human fetal tissues were used for immunoblot analysis, confirming Chk1-S expression in multiple species and tissue types. **C**. Kidney tubular cells were isolated from C57/Bl6 mice for culture to collect whole cell lysate for immunoblot analysis. HEK293 cell lysate was run in parallel for comparison. **D.** RT-PCR was performed to analyze Chk1 and Chk1-S expression in human tissues using primers amplifying both cDNAs. Two amplicons were obtained. Sequencing confirmed that the longer and shorter amplicons were Chk1 and Chk1-S, respectively. **e**. Taqman-based real-time PCR was performed to analyze the expression of Chk1 and Chk1-S in human tissues using specific primers and probes. The real-time PCR showed consistent results as the RT-PCR, verifying the specificity of the real-time PCR primers and probes.



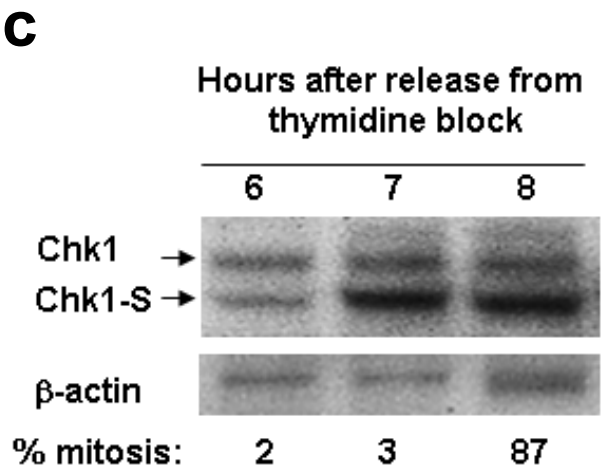


Fig. S4. Cell cycle-dependent expression of Chk1-S. HEK293 cells were asynchronized or synchronized by serum-starvation, double thymidine block, or nocodazole treatment. **a.** Cell cycle profile analyzed by PI staining and FACS analysis. **b**. Immunoblot analysis of Chk1 and Chk1-S. Chk1-S was low in serum-starved G1/G0 cells, increased in thymidine block arrested G1/S phase cells, and reached the highest level in nocodazole arrested G2/M cells. **c**. HEK293 cells were synchronized by double thymidine block and then released for 6-8 hours for immunblot analysis or phospho-histone H3 staining to estimate % mitotic cells.

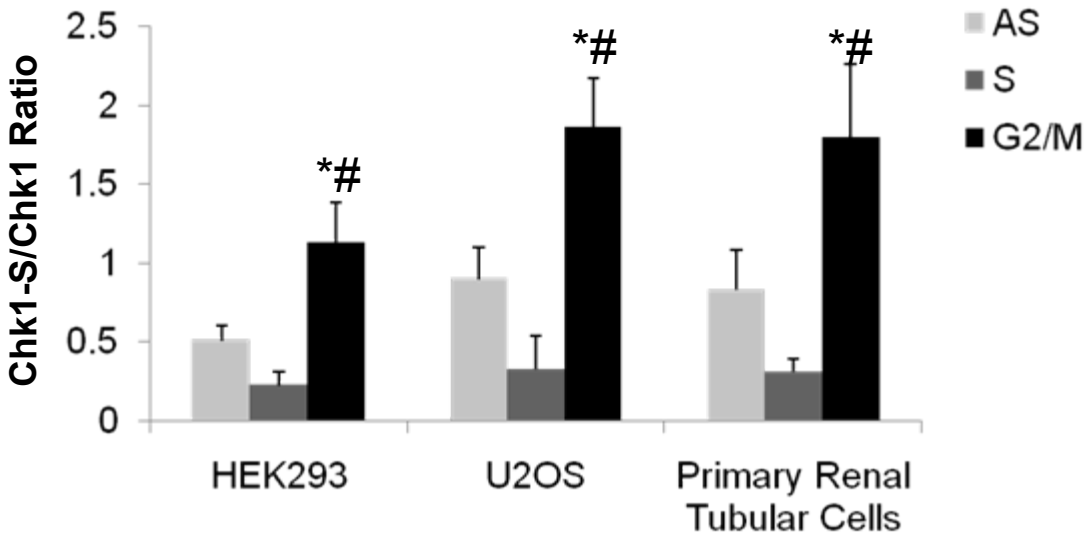


Fig. S5. Relative Chk1 and Chk1-S expression in different cells. HEK293, U2OS and mouse primary kidney tubular cells were synchronized by double thymidine block. Whole cell lysates were collected at different time points after release to determine the expression of Chk1 and Chk1-S proteins at different cell cycle phases (AS-asynchronous). Immunoblot results from three independent experiments were used for densitometric analysis to determine the Chk1-S/Chk1 ratio. The results show that although the Chk1-S/Chk1 ratio varies in different cells; the ratio is >1 in all cells synchronized at G2/M phase. * significant difference vs. asynchronous cells, # significant difference vs. S phase cells.

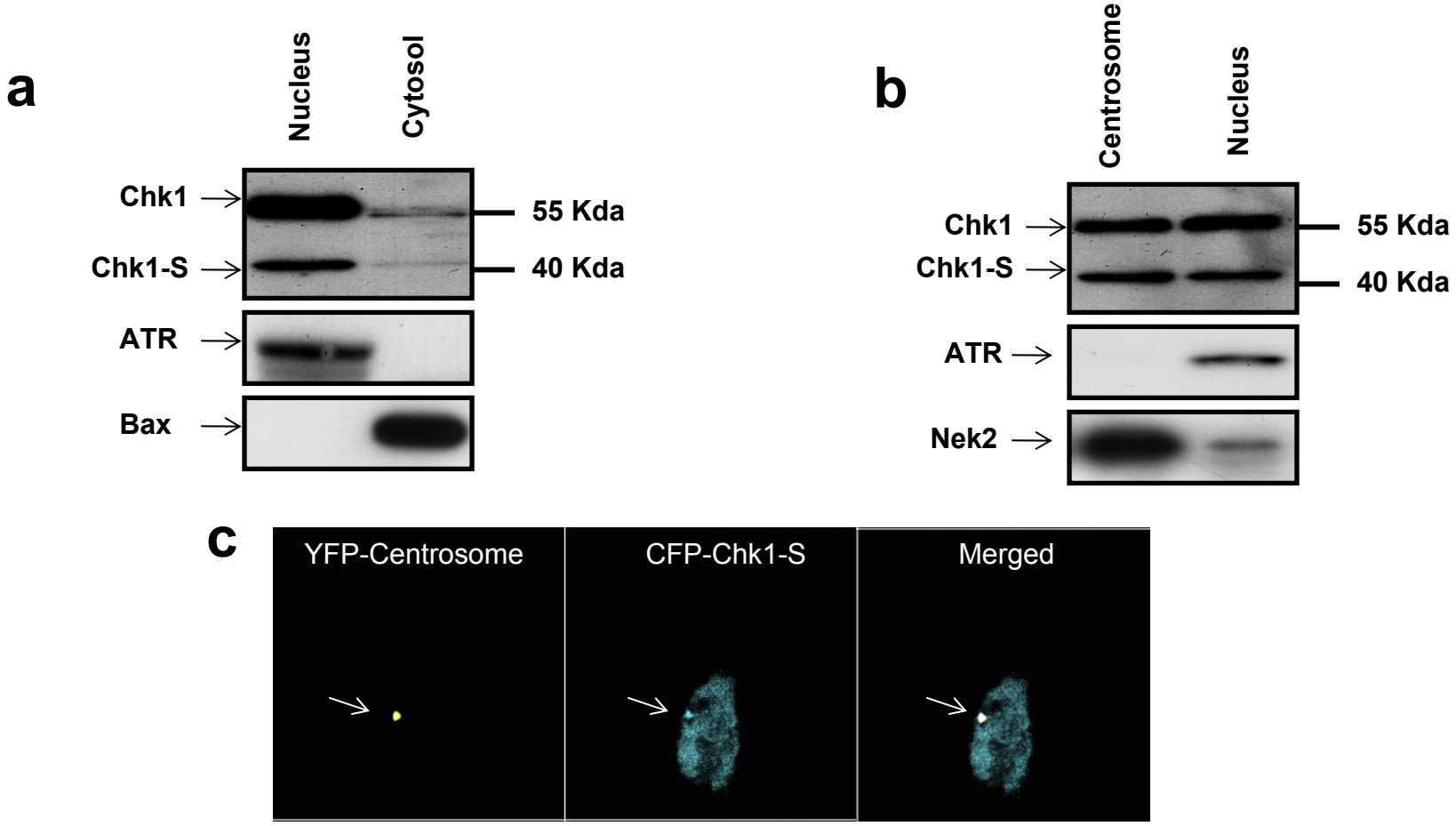


Fig. S6. Sub-cellular localization of Chk1-S. a. Asynchronously growing HEK293 cells were fractionated to collect nuclear and cytosolic fractions for immunoblot analysis of Chk1/Chk1-S. The nuclear protein ATR and cytosolic protein Bax were probed to verify the fractionation. The results suggest that Chk1 and Chk1-S are localized both in the nucleus and cytosol in asynchronized cells. **b.** HEK293 cells were synchronized by double thymidine block and released in fresh medium for 7 hours to obtain cells synchronized in G2 phase. Centrosome and nuclear fractions were isolated for analysis of Chk1 and Chk1-S. ATR and NeK2 were also probed to verify the fractionation. The results show Chk1 and Chk1-S localizations in centrosome and nucleus in G2 phase cells. Note: the centrosome sample was from about 10 times more cells than the nuclear sample analyzed on this blot. **c.** HEK293 cells were transfected with YFP-γ-Tubulin (centrosomal marker) and CFP-Chk1-S and examined 14-16 hours later by confocal microscopy. These results show Chk1-S localization in centrosomes, in addition to the nucleus.

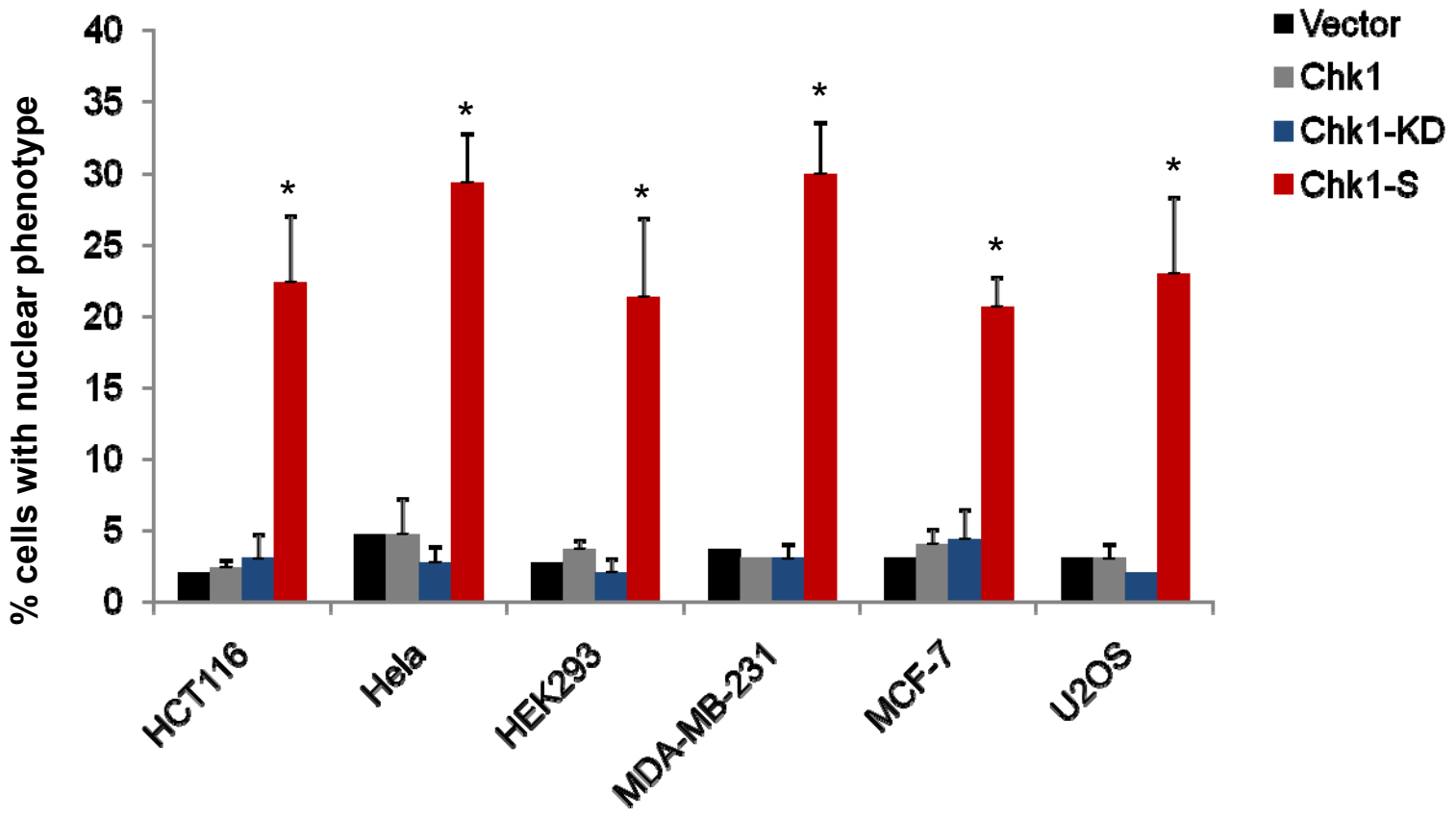


Fig. S7. Chk1-S overexpression induces mitotic catastrophe in different cell lines. Indicated cell lines were transfected with empty vector, Chk1, Chk1-KD, or Chk1-S. The cells were examined 36 hours later for the nuclear phenotype of mitotic catastrophe as described in methods. Overexpression of Chk1-S, but not Chk1 or Chk1-KD, induced mitotic catastrophe in all cell lines tested.

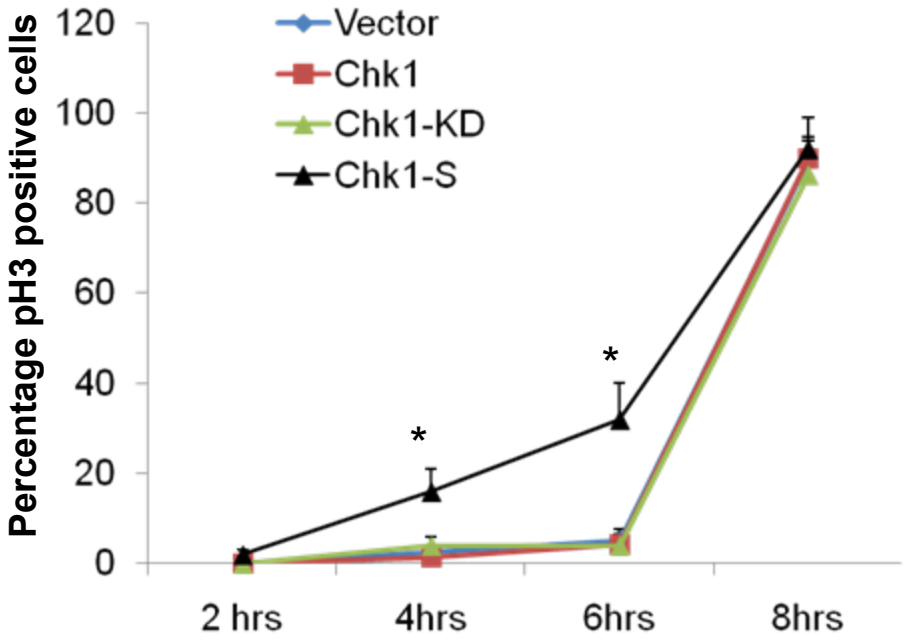
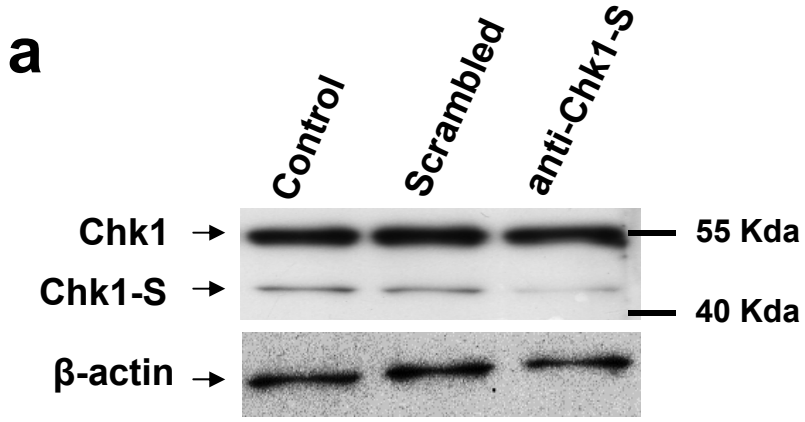
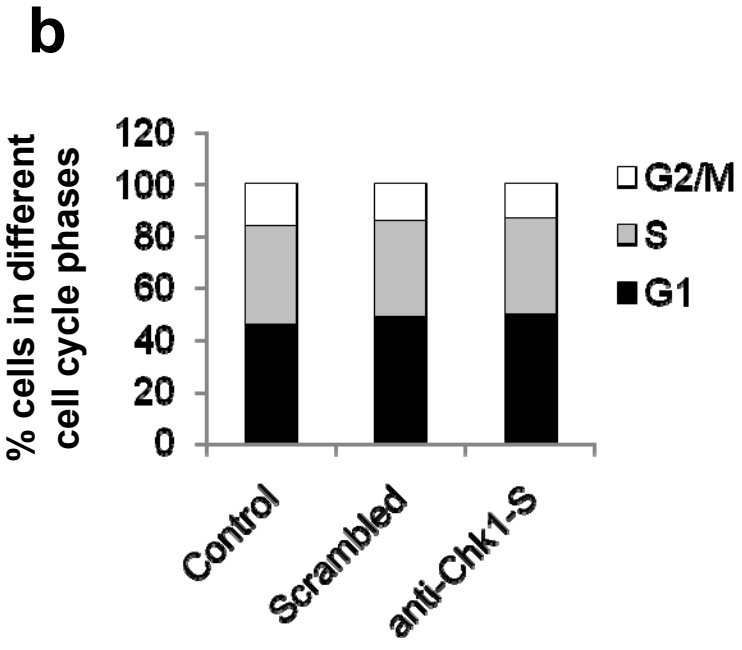


Fig. S8. Chk1-S overexpression induces early entry of mitosis. U2OS cells expressing either an empty vector or Chk1, Chk1-KD and Chk1-S were synchronized by double thymidine block, followed by release into nocodazole containing medium. The cells were fixed at different time-points to determine the percentage of cells with positive pH3 staining. The results showed that the cells overexpressing Chk1-S enter G2/M phase (pH3 staining) earlier than others. * indicates significant difference as compared to the vector transfected group.





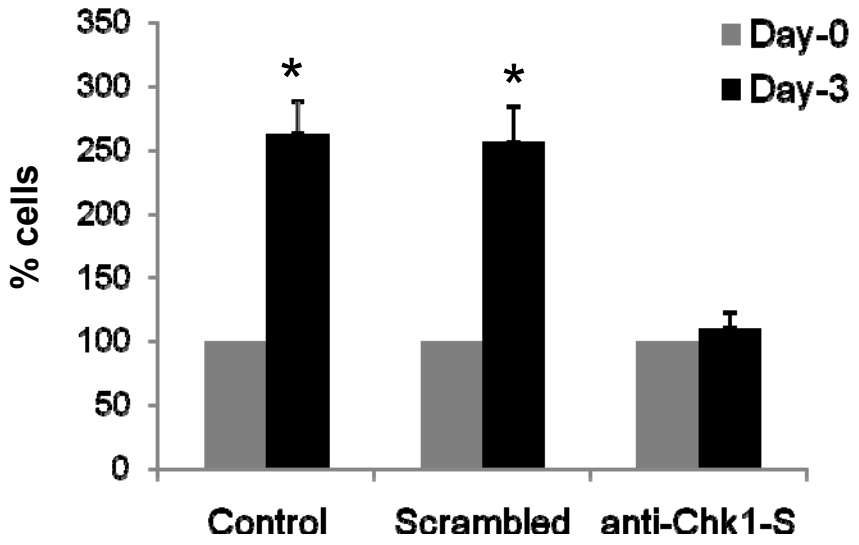


Fig. S9. Antisense-mediated knockdown of Chk1-S leads to decreased cell proliferation. U2OS cells were transfected with 50 nM of antisense oligonucleotides against Chk1-S (anti-Chk1-S) or scrambled sequence. **a**. Whole cell lysate was collected 48 hours later for immunoblot analysis to confirm the specific effect of anti-Chk1-S on Chk1-S expression. **b.** Cell cycle profile analyzed by PI staining and FACS analysis. **c**. Cell numbers were counted prior to (Day 0) and three days (Day 3) after the oligonucleotide transfection. Cellular prolioferation was assessed by % of the Day 3 cell number over Day 0 cell number which was arbitarily set as 100. The results show that blockade of Chk1-S reduced cell proliferation.

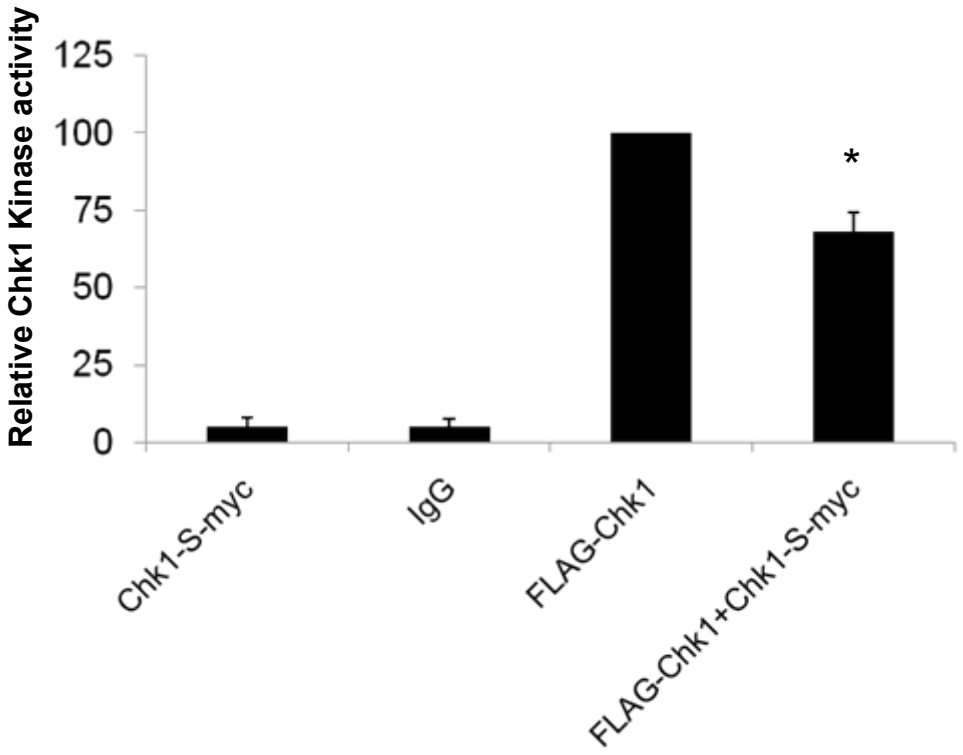


Fig. S10. Chk1-S inhibits FLAG-Chk1 kinase activity in vitro. Purified FLAG-Chk1 and Chk1-S-myc proteins were incubated separately or together and then added to the kinase activity assay using Chktide as substrate. The kinase activities of various incubations were normalized with the kinase activity of FLAG-Chk1 which was arbitrarily set as 100. The results show that Chk1-S can inhibit Chk1 kinase activity.

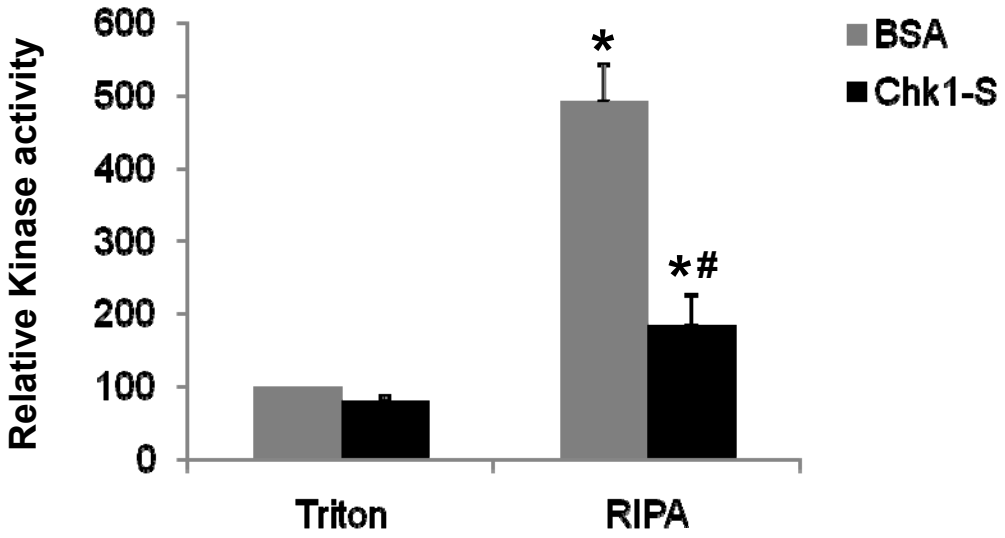


Fig. S11. Increase of Chk1 kinase activity after washing of Chk1 immunoprecipitates with RIPA buffer: effect of adding exogenous Chk1-S. HEK293 cell lysate was collected with 1% Triton X-100 buffer for immunoprecipitation using Chk1 antibodies. The immunoprecipitates were then washed with the Triton X-100 buffer or the more stringent RIPA buffer. After the washes, the immunoprecipitates were incubated with purified Chk1-S or albumin prior to kinase activity assay as described in Methods. *, $p<0.05$ vs. the Triton X-100 wash conditions. #, $p<0.05$ vs. the RIPA wash plus BSA condition. The results show that washing with the RIPA buffer increased the kinase activity of the Chk1 immunoprecipitates and this increase was abrogated by adding exogenous Chk1-S.

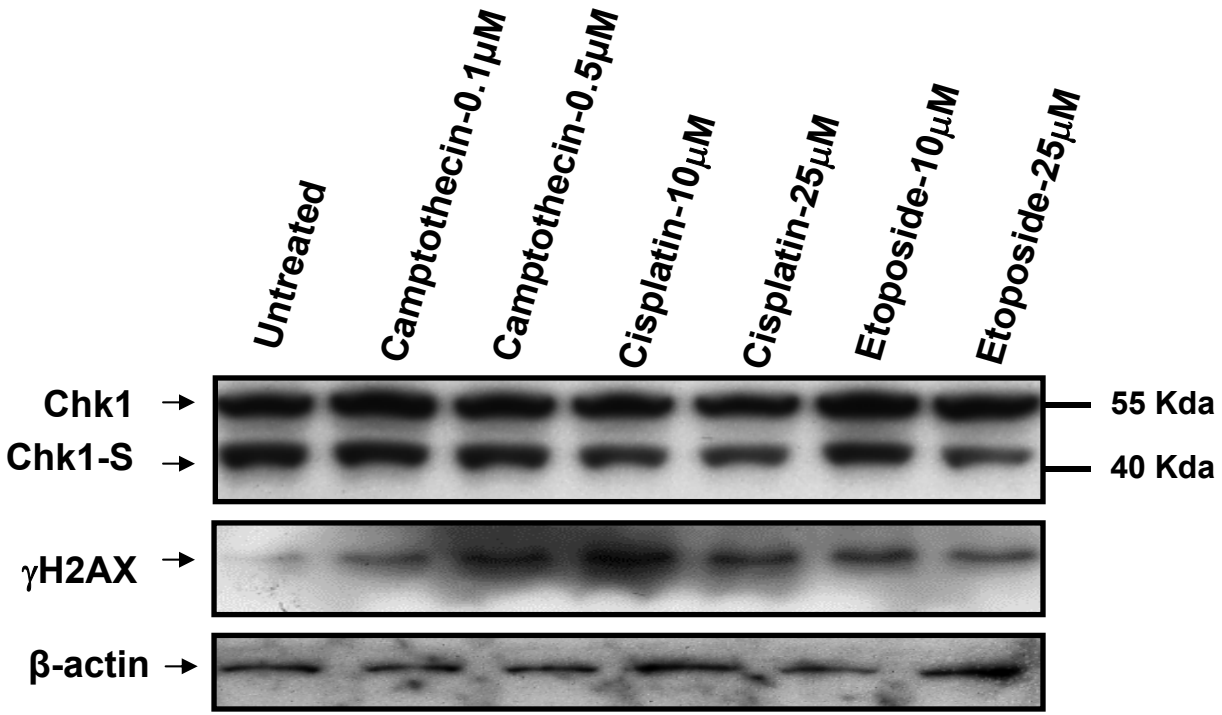


Fig. S 12. Chk1-S expression does not change during DNA damage. U2OS cells were treated with cisplatin, etoposide, or camptothecin for 2 hours. Total cell lysates were used for immunoblot analysis. The results show that Chk1-S expression is not changed during DNA damage. H2AX phosphorylation (γH2AX) confirmed the DNA damage response during treatment.

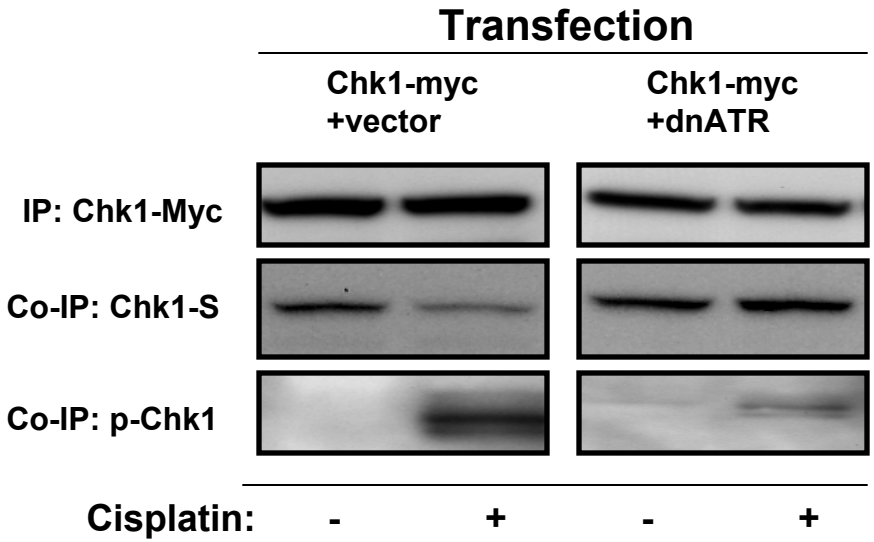


Fig. S 13. Chk1-S interaction with Chk1 is disrupted during cisplatin-induced DDR. HEK293 cells were co-transfected with Chk1-Myc and empty vector or dnATR. The cells were treated with 40μM cisplatin for 2 hours to collect cell lysate for immunoprecipitation using anti-Myc antibodies followed by immunoblot analysis. The results suggest that Chk1/Chk1-S interaction is disrupted during cisplatin treatment in an ATR- and Chk1 phosphorylation-dependent manner.

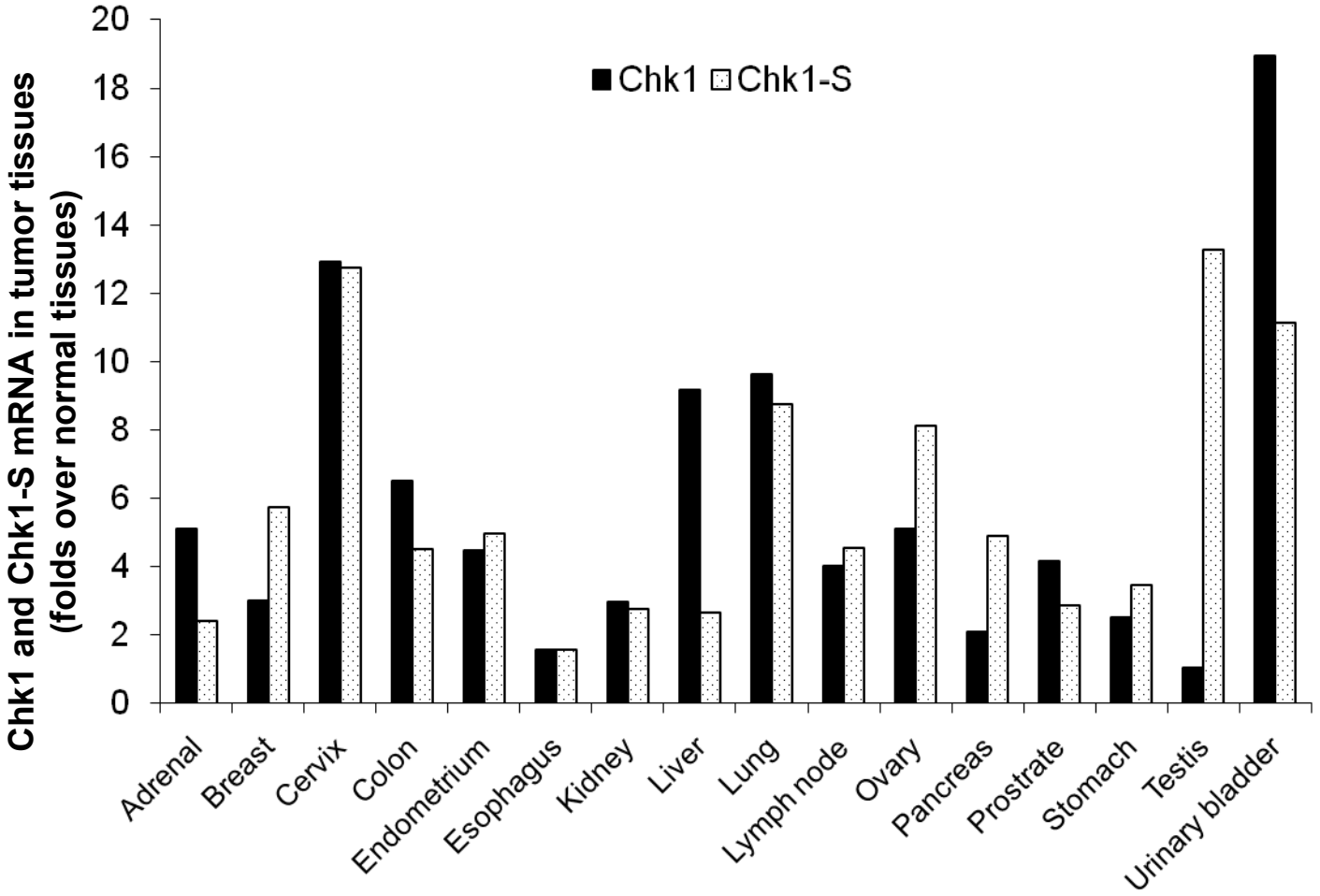


Fig. S 14. Relatively high Chk1 and Chk1-S expression in human cancer tissues. A cDNA panel of multiple human cancer and normal tissue samples was analyzed for Chk1 and Chk1-S mRNA expression by real-time PCR analysis of. The signal of cancer samples was normalized with that of the matched normal tissues to indicate fold changes in gene expression. (n=3-7 and 9-20 for each normal and cancer tissue type).

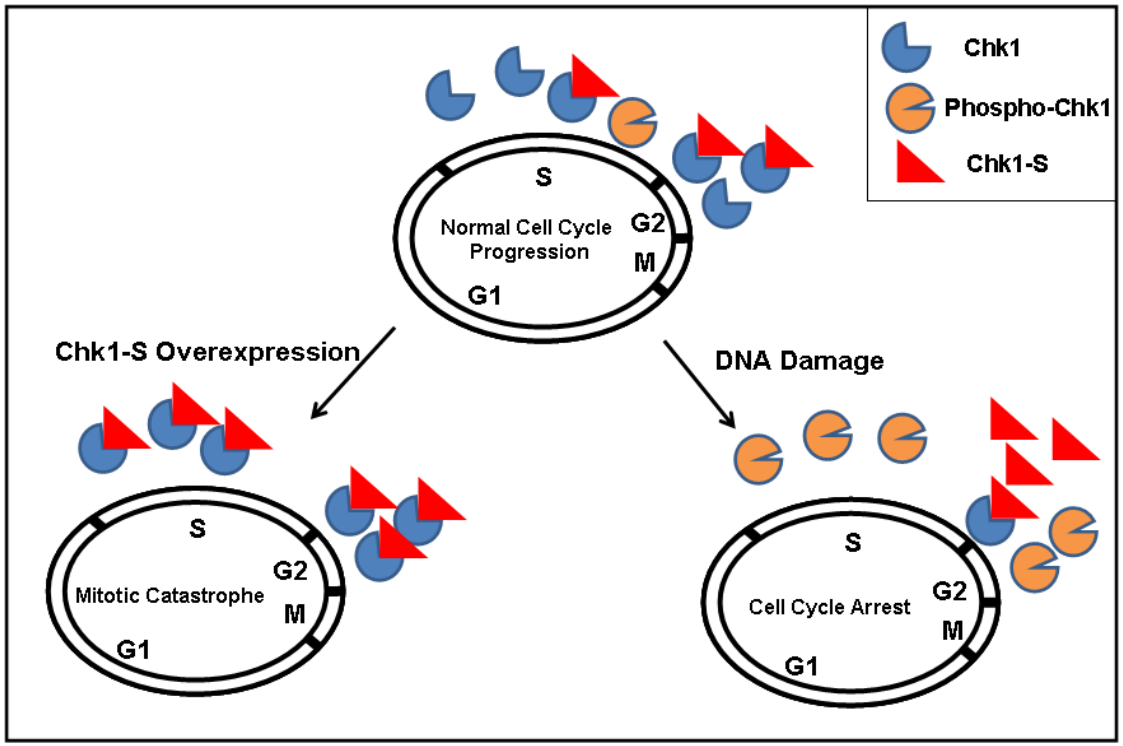


Fig. S 15. Chk1 regulation by Chk1-S in cell cycle and DNA damage response. In the S-phase of normal cell cycle, Chk1 expression increases and some Chk1 molecules are phosphorylated preventing Chk1-S binding, resulting in high Chk1 activity to keep the cell in S phase till the completion of DNA replication. In G2 phase, Chk1-S expression increases and Chk1 phosphorylation decreases promoting Chk1/Chk1-S interaction and reducing Chk1 activity, leading to G2/M transition and mitotic entry. Under conditions of Chk1-S overexpression, Chk1-S sequesters Chk1 and diminishes its kinase activity during S phase, resulting in pre-mature mitotic entry leading to mitotic catastrophe. During DNA damage, Chk1 is phosphorylated resulting in decreased Chk1-S binding, increased Chk1 activity, and G2/M cell cycle arrest.