Material and Methods :

Patients

The study comprised 337 consecutive gastric cancer patients treated at the Department of Surgery, Helsinki University Central Hospital, in 1983-1999. The patient material has been characterized before (1). In brief, 174 men and 163 women were included with a median age of 66 (range 30-87). Median follow-up of patients at study end was 12.7 years (range 4.7-20.8). Five-year overall survival was 29.4% (95% confidence interval (CI) 24.5-34.3). Approval of the study came from the local Ethics Committee and National Supervisory Authority of Welfare and Health. Survival data and cause of death were obtained prior to this study from patient records, the Population Registry, and Statistics Finland. All samples were reviewed by a gastrointestinal pathologist. Briefly, for antigen retrieval, slides were treated in a PreTreatment module (Lab Vision Corp., Fremont, CA, USA) in Tris-HCl buffer (pH 8.5) for 20 minutes at 98°C. Staining of sections was performed in Autostainer 480 (Lab Vision Corp.) using the Dako REAL EnVision Detection System, Peroxidase/DAB+, Rabbit/Mouse (Dako, Glostrup, Denmark).

Preparation of tumor tissue microarrays

Tumor samples were fixed in formalin, embedded in paraffin, and stored in the archives of the Department of Pathology, Helsinki University Central Hospital. Representative areas of each tumor were chosen from H&E stainings of the tumor samples. Three representative 0.6-mm cores from marked areas were obtained from each tumor with a tissue microarray instrument (Beecher Instruments, Silver Spring, MD, USA) as described.

In vivo mouse studies

Transgenic MMTV-neu mice were palpated twice a week for appearance and growth of mammary tumors and treatment with either vehicle or Chk1 inhibitor PF-477736 (15mg/kg)

was started when volume of tumors was approximately between 100-250 mm^3. Mice were treated intraperitoneally twice a day for three days and 24 hour after the last treatment mice were sacrificed and tumors were snap frozen in liquid nitrogen and stored at –80C for mRNA analysis. MMTV-neu mice were purchased from The Jackson Laboratory and the experiment was approved by the Regional State Administrative Agency for Southern Finland (ESLH-2009-00515/Ym-23 and 259/04.10.03/2012). To study CIP2A regulation by Chk1 in neuroblastoma NB1643 xenografts, CB17SC-M *scid*$^{-/-}$mice bearing NB-1643 tumors were randomized and once the tumor was ~200 mm^3 mice were treated with 10 mg/kg of PF-00477736 or vehicle control administered i.p. daily. Tumor samples (n = 2 per arm) were harvested following 48 hours of treatment (2 doses) and were snap frozen in liquid nitrogen and stored at –80C mRNA analysis. These animal studies were approved by the Children's Hospital of Philadelphia Institutional Animal Care and Use Committee. For both *in vivo* studies PF-477736 solution was prepared as previously described (2, 3).

Cell Cycle experiments

Both AGS and MKN-28 cells were blocked in early S-phase using established double Thymidine block (Fig. S3A) (4) and Thymidine-Aphidicolin block protocols (5). Cell were stained with Propidium iodide (1mg/ml stock, Sigma Aldrich, USA) and RNAse (0.5mg/ml, Roche Diagnostics) for 1 hour and different phases of cell cycle were obtained using the flow cytometer (FACS CantoII, BD Bioscience, USA) in accordance with the manufacturer's guidelines. Around 10000 events were recorded for each. Serum starvation experiments were done for the determination of CIP2a levels in proliferating and non-proliferating cells, 1x104 HeLa cells per well were seeded on a 96 well plate in quadruplicates and allowed to reattach to the plate. The cells were then washed with 1x PBS and fresh medium with or without FCS (10%) was added. After 48 hours cell nuclei were stained with 0,3 µg/ml Hoechst 33342 for

30 min at 37°C and 6 images per well were taken with an Olympus IX81 microscope and analysed using the Scan® Analysis Software from Olympus. Cells in late metaphase, ana- and telophase were counted manually to determine the mitotic index. To detect CIP2A protein levels, lysates from an equal number of cells were loaded on a SDS-PAGE gel and further analysed by Western Blot.

PP2A Assays

The protein phosphatase 2A (PP2A) activity in cellular lysates was determined by measuring the generation of free phosphates from the Threonine phosphopeptide using the Malachite green based PP2A Immunoprecipitation Phosphatase Assay Kit as described by the manufacturer (Millipore, Upstate Biotechnology, and Temecula, CA). The cell lysates were prepared by scrapping the cells with the phosphatase extraction or lysis buffer containing 20 mM imidazole-HCl, 2 mM EDTA, 2 mM EGTA, pH 7.0, with 10 ug/ml each of aprotinin, leupeptin, pepstatin, 1 mM benzamidine, and 1mM PMSF. The lysates were then sonicated for 10s and then centrifuged at 2000 g for 5 min at 4°C. The PP2A Immunoprecipitation Phosphatase assay was then performed as per the instructions provided by the manufacturer in presence of a PP2A-specific reaction buffer supplied by the manufacturer with 750 uM of the phosphopeptide substrate. After 10 min of incubation at room temperature in malachite green phosphate detection solution, absorbance in the samples was measured at 650 nm using the Multiscan GO spectrophotometer (Thermo Scientific, Vantaa, Finland). To avoid variability in different experimental repeats due to differences in the amounts of immunoprecipitated PP2Ac, the phosphatase activity was normalized to the immunoprecipitated PP2Ac levels, as detected and quantified using immunoblotting.

Bioinformatics

Co-expression among the 17543 genes in the neuroblastoma database (28) was assessed using the Spearman's rank correlation coefficient, with a cut-off threshold of $|\rho|$ and $p < 10^{-10}$, where the statistical significance was approximated using the Student's *t*-distribution with $n-2$ degrees of freedom (n = 83 samples in the neuroblastoma database). A total of 196 and 511 genes satisfied this threshold for CIP2A and Chk1, respectively, with an overlap of 168 genes. A total of 1469 MYC target genes were extracted from the supplementary of a prior study (32), and 1411 of those could be identified in the neuroblastoma database. Enrichment of the 39 shared MYC targets in the sample of 168 genes was assessed using the cumulative function of the hypergeometric distribution with population parameters 17543 and 1411. Gene ontology pathway over-representation analysis for the 39 shared MYC targets was performed using the DAVID tools.

Transient transfections of plasmids and luciferase assay

3 x103 AGS cells were plated in each well of the 96-well plate on day one. Next day either -1802CIP2ALuc (6) or 5XJunLuc (7) luciferase reporter constructs were transfected using Fugene (Roche Diagnostics, IN, U.S.A) according to the manufacturer's directions. To normalize the luciferase activity, 80 ng of a control plasmid expressing Renilla luciferase sequence was co-transfected into the cells. Cells were then analyzed 48 h post-transfection using the Dual–Glo Luciferase Assay System (Promega, Madison, WI, U.S.A). Results are presented post normalization with the Renilla luciferase level.

Western blot analysis and Immunohistochemistry

Western blot experiments and preparation of tumor tissue microarrays and immunohistochemistry were done as described previously (1). Western blot antibodies have been listed in the supplementary material. The following antibodies were used for IHC stainings: the rabbit polyclonal CIP2A (1:3000)(8), Chk-1 (1:500; Santa Cruz

Biotechnology,Santa Cruz, CA), Claspin (1:100; Cell Signaling Technology, Inc., Danvers, MA) and mouse monoclonal MYC antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

Immunoflourescence

AGS cells were seeded on sterilized slides or cover slips placed in each well of a 6-well plate overnight and the cells were then treated with the required chemicals or siRNAs for the desired time points. Subsequently the cover slips were placed in fresh sterilized wells and then washed with PBS before being fixed using 4% paraformaldehyde for 10 min at 37° C. Then after washing with PBS twice, cells were treated with 0.5% NP-40 for 5min at room temperature. This was followed by blocking the cells with 0.5% bovine serum albumin for 30 min. Cells were then exposed to the following primary antibody dilutions, CIP2A 1:250 (Santa Cruz Biotechnology, Santa Cruz, CA), phospho-Chk1-serine345 1:50 (Cell Signaling Technology, Inc., Danvers, MA), phospho-Chk1-serine317 1:50 (Cell Signaling Technology, Inc., Danvers, MA), Anti-phospho-Histone H2A.X(Ser139) 1:250 (Millipore, Upstate Biotechnology, Temecula, CA). After which the cells were washed with PBS 3 times and incubated in FITC-conjugated goat anti Rabbit IgG or anti-mouse IgG (Invitrogen–Molecular Probes, Eugene, Oregon, USA) for 1h at 37°C. Next cells were then stained with the Vectashield antifade solution (Vector Laboratories) containing 0.001 mol/L 4′,6-diamidino-2-phenylindole as counterstain and observed under Olympus BX50 epifluorescence microscope equipped with a charge-coupled device camera. Images were captured with the Image-Pro Plus 6.1 software (Media Cybernetics, Inc.) through each filter set. Quantitative automated microscopic analysis of cells was carried out with high-content imaging station ScanR (Olympus) equipped with inverted microscope IX81 and Hamamatsu ORCA-ER high sensitivity cooled CCDcamera (Hamamatsu Photonics K. K.). Four images / well were acquired with 10x magnification using specific filter sets for DAPI and Alexa594 (Semrock

Inc.) labels. Images were analysed with ScanR image analysis software. Cells were first segmented on the basis of DNA counterstaining (DAPI) by edge-finding algorithm and the average nuclear fluorescence intensities of DAPI and Alexa594 were measured. Clustered cell populations with high or low fluorescent intensity of Alexa594 were gated from two-dimensional scatter plots (Nuclear DAPI intensity vs. Nuclear Alexa594 intensity). Data presented displays percentage of cell population expressing high Nuclear Alexa 594 signal.

Cell culture

The following human cancer cell lines were used: AGS (gastric adenocarcinoma); MKN-28 (intestinal-type gastric adenocarcinoma); PC-3 (prostate adenocarcinoma); MDA-MB-231 (Breast adenocarcinoma) and HeLa (cervical carcinoma). All cell lines were obtained from American Type Culture Collection (ATCC) and grown under recommended conditions.

Soft agar and monolayer clonogenic assays

At 48 hours after transfection or transduction with shRNA lentiviruses,MKN-28 cells (1 × 104 per dish) were suspended in 1 mL of 0.25% agarose (GellyPhor; EuroClone Spa, Pero, Italy) supplemented with 2 mL complete RPMI-1640 culture medium (changed every third day). This suspension was layered over 1 mL of a base layer of 0.5% agarose in complete medium in six-well plates. After 8 or 12 days in agarose, cells were stained with Giemsa 1:20 in water (Sigma-Aldrich), the wells were scanned using the Surveyor Software (Objective Imaging Ltd.) with camera (Imaging Inc., Canada) attached to the Olympus IX71 (Olympus, Tokyo, Japan) microscope and colonies were counted by analysis with ImageJ Software (Wayne Rasband, National Institutes of Health, Betheseda, MD). Cell groupings that were greater than 1200 pixels in diameter with 3.2 × enlargements were counted as colonies. The time course dependent anchorage independent growth assay with the Chk1 inhibitor SB218078 was performed by seeding 200000 cells in a 6 well plate. On the following day the

medium was replaced with fresh growth medium containing 0,1 and 2.5uM of SB218078. After 24 hours of treatment 10000 cells/well were seeded in Costar-ultra low attachment 96-well plates (Corning Inc. U.S.A) in 5 replicates for each timepoint. Proliferation was then quantified at each time point by adding 10 ul of WST-1 reagent (Roche Diagnostics, Switzerland) in each well. After 1hour incubation at 37C, absorbance at 450nm was measured For colony formation assays, 2000 AGS cells in each well of a 6 well plate were seeded and transfected with respective siRNAs and grown for 8 days. Cells were then fixed with 3.7% formaldehyde and stained with 0.1% crystal violet solution in 10% ethanol. Area of colonies was measured using ImageJ 1.42q software (Wayne Rasband, National Institutes of Health, Betheseda, MD).

Chemical Inhibitors

The following chemical inhibitors were used: 4, 5-Dimethoxy-2-nitrobenzaldehyde (DNA-Dependent Protein Kinase Inhibitor (DNA-PKi), MERCK, Germany), PF-477736 (Chk1 inhibitor, Tocris Bioscience, UK), SB218078 and GÖ6976 (Chk1 inhibitors, Calbiochem, Germany), ATM/ATR Kinase inhibitor (CGK 733, MERCK, Germany).

RNAi

The HP validated siRNAs were obtained from Qiagen Sciences (Germantown, MD). Either of the following double-stranded oligonucleotides was transiently transfected into respective cell lines. Cells at 30% – 50% confluency in a six-well plate were transfected with the siRNA in antibiotic free medium using RNAiMAX Reagent (Invitrogen, Carlsbad, CA) using the reverse transfection method provided by the manufacturer.

Scrambled (Scr.) or Control siRNA - 5′-UAACAAUGAGAGCACGGCTT-3′

CIP2A.1-5′-CUGUGGUUGUGUUUGCACUTT-3′

CIP2A.2- 5′-ACCAUUGAUAUCCUUAGAATT-3′

Chk1.1 - 5′- AAC UGA AGA AGC AGU CGC AGU TT-3'

Chk1.2 - 5'- AAG AAA GAG AUC UGU AUC AAU TT-3'

Chk1.3 – 5'- CCC GCA CAG GUC UUU CCU UAU TT -3'

Claspin.1 - 5'- GAGGACGTAATTGATGAAGTA-3'

Claspin.2 – 5' – CACGGAAATGCCATGGCACTA-3'

DNA-PK – 5'- TTCGGCTAACTCGCCAGTTTA-3'

ATM.1: 5'-AACAUACUACUCAAAGACAUU-3'

ATM.2: 5'-UGGUGCUAUUUACGGAGCU -3'

ATR.1 : 5'-AACCUCCGUGAUGUUGCUUGA-3'

ATR.2 : 5'-CGAGACUUCUGCGGAUUGC -3'

PPP2R2A: 5'-CUGCAGAUGAUUUGCGGAUdTdA--3'

Quantitative Real Time Polymerase chain Reaction (qRT-PCR)

Total mRNA was extracted from cells or MMTV-neu tumors using the RNeasy kit (Qiagen, Valencia, CA) and converted to cDNA by using the M-MLV Reverse Transcriptase, RNaseH Minus, Point Mutant cDNA synthesis kit (Promega Corporation, Madison, WI). cDNAs were subjected to quantitative real-time polymerase chain reaction (PCR) by using the Light Cycler (Roche Diagnostics, Mannheim, Germany) and SYBR Green PCR Master Mix kit (Roche Diagnostics) and Taqman (Applied Biosystems). Primer sequences (Sigma- Proligo, St Louis, MO) used for PCR of CIP2A were as follows: human CIP2A forward, 5′-

CTGGTGAGATAATCAGCAATTT-3′, human CIP2A reverse, 5′-CGAAACATT CATCAGACTTTTCA-3′, mouse CIP2A forward, 5'-GAACAGATAAGGAAAGAGTTGAGCA-3' and mouse CIP2A reverse 5'-ACCTTCTAATTGAGCCTTGTGC-3'. Transcript levels were normalized to levels of ß -actin expression, which were determined by PCR of the same samples using the following primers; human ß-actin forward, 5′-CGAGCACAGAGCCTCGCCTTTGC-3′, human ß-actin reverse 5′-CATAGGAATCCTTCTGACCCATG-3', mouse ß-actin forward 5'-TGGCTCCTAGCACCATGAAGA-3' and mouse ß-actin reverse 5'-GTGGACAGTGAGGCCAGGAT-3'. For CIP2A realtime RT-PCR analysis from neuroblastoma xenografts, two micrograms of DNAase treated total RNA from tumor samples were oligodT primed, reverse transcribed and amplified with Taqman Assays on Demand on an ABI 7900 (Applied Biosystems, Foster City, CA, USA). mRNA expression of CIP2A (Hs00405413_m1) was determined using absolute quantification with standard curves from serial dilutions of BE2C neuroblastoma cell line cDNA, and expressed relative to the geometric mean of three housekeeping genes, GAPDH (Hs99999905_m1), HPRT (Hs02800695_m1) and TBP (Hs00920497_m1).

References.

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Supplemetary Figure Legends:

Figure S1. Expression analysis of Chk1 and CIP2A in human malignancies.

A. Statistically significant overexpression of Chk1 mRNA in patients of gastric intestinal type of adenocarcinoma vs. normal gastric mucosa. Data was obtained from Oncomine database (https://www.oncomine.org).

B. Significant correlation between Chk1 and CIP2A mRNA expression in human ovarian cancer tissue samples. The ovarian data was obtained by using Genesapiens database (https://www.genesapiens.org)

C. Significant correlation between Chk1 (CHEK1) and CIP2A (KIAA1524) mRNA expression in human colon cancer tissue samples. The colon data from Oncomine database (https://www.oncomine.org).

D, E. Kaplan-Meier curves demonstrating a significant decrease in relapse free survival in neuroblastomas expressing high levels of Chk1 (D) or CIP2A (E).

Figure S2. Chk1 and Claspin (CLSPN) positively regulate CIP2A protein expression in human cancer cells

A. Inhibition of p-S216-CDC25C protein expression by indicated small molecule Chk1 kinase inhibitors or by Chk1 shRNA in indicated cell lines.

B. Inhibition of pS345-Chk1 and CIP2A levels by SB218078 (1uM) in AGS cells

C. Inhibition of CIP2A protein expression by either two different Claspin siRNAs in AGS cells 72h post transfection.

D-F. Inhibition of CIP2A protein expression by either of the Chk1 siRNAs in the indicated cancer cell lines 72 hr post-transfection (HeLa; Cervical cancer; MDA-MB-231; Breast cancer, PC-3; Prostate cancer).

Figure S3. Effect of cell cycle on CIP2A expression.

A. Scheme of events for inducing a double-Thymidine block in AGS and MKN-28 cells.

B. Analysis of cell cycle phases in unsynchronized and synchronized (early S-phase block) AGS and MKN-28 cells post double-Thymidine block.

C. CIP2A mRNA measurement in synchronized (using a double Thymidine block) versus unsynchronized control population in AGS and MKN-28 cells respectively.

D. CIP2A mRNA measurement in synchronized (using a Thymidine-Aphidicolin block) versus unsynchronized control population in AGS cells.

E. CIP2A protein expression in synchronized (using a Thymidine-Aphidicolin block) versus unsynchronized control population in AGS cells.

F-H. Serum starvation of HeLa cells does not regulate CIP2A expression. (F) Numbers of cells after culture in the absence (-) or presence of FCS (+) for 48 hours. (G) Mitotic index of the HeLa cells after culture in the absence (-) or presence of FCS (+) for 48 hours. (H) CIP2A and Actin expression from equal number of cells after culture in the absence (-) or presence of FCS (+) for 48 hours.

Figure S4. *In vitro* and *in vivo* regulation of CIP2A mRNA expression by Chk1 and effect on cancer cell viability and anchorage independent growth by Chk1, Claspin and CIP2A

A. Inhibition of CIP2A mRNA expression in MKN-28 stably transfected with Chk1 shRNAs expressing lentivirus.

B. Relative CIP2A mRNA expression in neuroblastoma xenografts *in vivo* in PF-477736 (Chk1 inhibitor) treated mice versus vehicle.

C. Relative mRNA expression of GAPDH in neuroblastoma xenografts *in vivo* in PF-477736 (Chk1 inhibitor) treated mice versus vehicle.

D. Inhibition of cell viability by CIP2A siRNAs 6 days post transfection in PC-3 (prostate cancer) cell line.

E. Effect of two different Claspin siRNAs on AGS cell viability 6 days post transfection**.**

F. Effect of SB218078 on anchorage independent viability of AGS cells. 10000cells/well treated for 24h with SB218078 were seeded in Costar-ultra low attachment 96-well plates in 5 replicates for each time point. Anchorage-independent viability was then quantified at each time point by CTG assay.

G. Effect of SB218078 on anchorage independent viability of MKN-28 cells. 10000cells/well treated for 24h with SB218078 were seeded in Costar-ultra low attachment 96-well plates in 5 replicates for each time point. Anchorage-independent viability was then quantified at each time point by CTG assay.

H. Immunofluorescence analysis demonstrating overexpression of CIP2A in CIP2A Flag transfected AGS cells.

I. Western blot showing CIP2A overexpression in MDA-MB231 cells transfected with control (pcDNA3.1) and CIP2A overexpressing vector (CIP2A Flag) post 72h of transfection with scrambled or Chk1 siRNA.

Figure S5. Regulation of MYC and cell cycle by Chk1-CIP2A pathway and effect of DNA-PK on CIP2A and pS345-Chk1 levels independent of ATM/ATR

A. Effect of Claspin and CIP2A siRNAs on expression of CIP2A and phospho-MYC-serine 62 in AGS cells 72h post transfection.

B. Effect of Scr., Chk1 and CIP2A siRNA on MYC and CIP2A mRNA expression 72h post transfection. Shown is the average of two independent experiments + S.D.as error bars.

C. Effect of SB218078 on total MYC-ER levels. Total MYC-ER expression was studied 24 hours after treatment with SB218078 in the absence or presence of Tamoxifen. Notably, short-term Chk1 inhibition did not inhibit total MYC-ER expression even though it inhibited MYC-ER transactivation as indicated by decreased expression of endogenous MYC in the tamoxifen+SB218078 treated samples.

D. Quantitation of Histone H3 positive cells demonstrates that SB218078, abrogates the increase in mitotic MCF10-MYC-ER cells upon activation of MYC-ER in comparison to the control (DMSO). Shown is mean values + S.D., of three independent experiments. Students t-test was used obtain the statistical significance value.

E. Effect of PPP2R2A, Chk1 and their combination on PPP2R2A and Actin protein levels relative to the control (Scr.) in AGS cells.

F. Immunoflourescent stainings of AGS cells with DAPI and phosphoChk1-serine345 antibodies 72h post transfection with scrambled (Scr.) or two different Chk1 siRNAs.

G. Effect of ATM/ATR inhibitor (400nM) and Camptothecin (1uM) on pChk1-S345 and pChk1-S317 in AGS cells.

H. Quantification of phosphoChk1-serine 345 levels in ATM/ATR inhibitor (400nM) treated AGS cells at indicated time points.

I. Expression of CIP2A, LaminB, ATM, ATR and Chk1 proteins in HeLa cells transfected with nothing (-), Scr. (control) and siRNAs targeting both ATM and ATR.

J. Immunofluurescent stainings of AGS cells with DAPI and DNA-PK antibodies 72h post transfection with scrambled (Scr) or DNA-PK siRNA.

K. Inhibition of CIP2A protein expression by DNA-PK specific siRNA 72h post transfection in AGS cells