Chk1 Targeting Reactivates PP2A Tumor Suppressor Activity in Cancer Cells

Anchit Khanna1,2,10, Otto Kauko3,4,5, Camilla Böckelman6,7, Annika Laine3,4,5, Ilona Schreck8, Johanna I. Partanen4, Agnieszka Szwajda8, Stefanie Bormann9, Turker Bilgen3,11, Merja Helenius1, Yuba R. Pokharel3, John Pimanda10, Mike R. Russell12, Caj Haglund6, Kristina A. Cole12,13, Juha Klefström1, Tero Aittokallio8, Carsten Weiss9, Ari Ristimäki8,7, Tapio Visakorpi1, and Jukka Westermarck3,4

Abstract

Checkpoint kinase Chk1 is constitutively active in many cancer cell types and new generation Chk1 inhibitors show marked antitumor activity as single agents. Here we present a hitherto unrecognized mechanism that contributes to the response of cancer cells to Chk1-targeted therapy. Inhibiting chronic Chk1 activity in cancer cells induced the tumor suppressor activity of protein phosphatase protein phosphatase 2A (PP2A), which by dephosphorylating MYC serine 62, inhibited MYC activity and impaired cancer cell survival. Mechanistic investigations revealed that Chk1 inhibition activated PP2A by decreasing the transcription of cancerous inhibitor of PP2A (CIP2A), a chief inhibitor of PP2A activity. Inhibition of cancer cell clonogenicity by Chk1 inhibition could be rescued in vitro either by exogenous expression of CIP2A or by blocking the CIP2A-regulated PP2A complex. Chk1-mediated CIP2A regulation was extended in tumor models dependent on either Chk1 or CIP2A. The clinical relevance of CIP2A as a Chk1 effector protein was validated in several human cancer types, including neuroblastoma, where CIP2A was identified as an NMyc-independent prognostic factor. Because the Chk1–CIP2A–PP2A pathway is driven by DNA-PK activity, functioning regardless of p53 or ATM/ATR status, our results offer explanatory power for understanding how Chk1 inhibitors mediate single-agent anticancer efficacy. Furthermore, they define CIP2A-PP2A status in cancer cells as a pharmacodynamic marker for their response to Chk1-targeted therapy. Cancer Res; 73(22); 6757–69.

©2013 AACR.
treatments, Chk1 and DNA-PK have been recently identified to be required for tumor growth (13, 15–20). Notably, recent studies have shown that Chk1 activity is required for MYC-induced tumorigenesis (15, 16, 18). Moreover, even though Chk1 haploinsufficiency was shown to modestly promote tumorigenesis in combination with certain oncogenic lesions (17, 21, 22), recent studies provide genetic evidence that Chk1 may benefit tumor development (17, 23). Together with studies using either RNAi-based or chemical inhibition of Chk1 (13, 15–20), these results demonstrate that in the context of transformed cells, Chk1 activity promotes cell viability (13, 14, 24). However, even though induction of mitotic catastrophe explains cell-killing activity of Chk1 inhibitors when used in combination with DNA-damaging agents (13, 14, 24), mechanisms by which chronic Chk1 activity in unperturbed cancer cells promotes their viability and clonogenicity are very poorly understood. Moreover, even though ATR kinase is a well-established upstream activator of Chk1 when DNA replication is impeded (14), we do not understand the mechanisms driving chronic Chk1 activity in unperturbed human cancer cells.

In this study, we demonstrate that chronic Chk1 activity in unperturbed cancer cells promotes CIP2A transcription and thereby inhibits PP2A tumor suppressor activity. Furthermore, our data shows that CIP2A downregulation and subsequent increase in PP2A activity is essential for maximal inhibition of cancer cell viability and clonogenicity in response to Chk1 inhibition.

Materials and Methods

Cell viability assay

One day before transfection of siRNAs AGS, MKN-28 and MDA-MB231 cells were seeded in RPMI-1640 medium supplemented with 10% fetal calf serum at a density of 1 × 10^4 to 2 × 10^5 cells per well in 96-well plates. The cells were transfected with the following conditions: medium only, Lipofectamine 2000 reagent only, or 20 nmol/L of indicated siRNAs (with Lipofectamine 2000 reagent) in 200 μL of RPMI-1640 supplemented with 10% fetal calf serum. Subsequently, relative numbers of viable cells were measured by fluorescence at the 544 and 590 nm wavelengths in a FLUOstar OPTIMA Microplate Reader (BMG Labtech, Inc.), using the resazurin-based CellTiter-Blue Assay (Promega Corporation) according to the manufacturer’s instructions.

MCF10A MycER culture and cell-cycle analysis

MCF10A MycER cells were cultured as described previously (25) in human mammary epithelial cell basal growth media MCDB 170 (US Biological) with supplements. For cell-cycle analysis, the cells were seeded on glass coverslips and allowed to attach overnight. Next day, normal growth medium was replaced with medium lacking EGF and insulin and the cells were starved for 24 hours in the presence or either dimethyl sulfoxide (DMSO) or Chk1 inhibitor SB218078. To activate MycER, 4-OHT (or ethanol as vehicle control) was added to the cells, which were fixed 24 hours later with 4% paraformaldehyde and subjected to immunostaining with antibody against proliferation marker Ki67 (Zymed, rabbit polyclonal). The immunostained cells were analyzed by immunofluorescence microscopy and the percentages of cells expressing Ki67 were scored from each assay.

In vitro kinase assays

For in vitro kinase assays, MKN-28 cells were homogenized in ice-cold lysis buffer. Beads were removed by centrifugation, and DNA-PKcs was immunoprecipitated from the supernatants using the DNA-PKc (G4) antibody (Santa Cruz Biotechnology). Kinase assay was then carried out for 15 minutes at 30°C by mixing 30 μL of immunoprecipitated sample, Chk1 (100 ng; c8870; Sigma) and ATP (50 μmol/L final concentration) and samples were incubated for 15 minutes at 30°C. Then 30 μL of 2× sample buffer was added and samples heated for 5 minutes at 95°C and then immunoblotted for phospho-Chk1-serine345 (Cell Signaling Technology, Inc.), Chk1 rabbit polyclonal (Santa Cruz Biotechnology), and DNA-PKcs antibodies.

Statistical analysis

Overall survival was calculated from date of diagnosis to death. Associations between protein expression of the studied biomarkers (CIP2A, Chk-1, c-MYC, and Claspin) were assessed by Fisher exact test according to the Monte Carlo method at the 99% confidence level (IBM SPSS Statistics, version 19.0 for Mac; SPSS, Inc., an IBM Company). All statistical tests were two-sided. Student t test was two-sided.

Additional materials and methods used in this article are provided in the Supplementary Data.

Results

Coexpression and prognostic role of CIP2A and Chk1 in human malignancies

CIP2A is involved in gastric cancer progression (12). Analysis of tissue microarray of human gastric carcinoma specimens for Chk1 and for Claspin, the latter being a Chk1 scaffold protein that promotes Chk1 activity (14), demonstrated that Chk1 and Claspin were expressed in 162 of 195 (83.2%) and 163 of 199 (81.9%) specimens, respectively (Fig. 1A and B). Moreover, a statistically significant association between Chk1 and Claspin expression levels was observed (Fig. 1B). Cancer selective expression of Chk1 mRNA in gastric carcinoma versus normal samples was validated using the Oncomine database (Supplementary Fig. S1A). Notably, CIP2A expression was significantly associated with expression of both Chk1 and Claspin (Fig. 1C and D). In addition, a significant association between Chk1 and CIP2A expression was detected at the mRNA level in human ovarian and colon cancer tissue samples (Supplementary Fig. S1B and S1C). On the basis of recent identification of Chk1 as a drug target candidate in human neuroblastomas (20), we mined genomic analysis of 88 neuroblastoma samples (26) for expression of both Chk1 and CIP2A. Similar to gastric, ovarian, and colon cancers (Fig. 1C and D and Supplementary Fig. S1B and S1C), a statistically significant correlation was observed between the mRNA expression of...
Figure 1. Coexpression and prognostic role of CIP2A and Chk1 in human malignancies. A, paraffin block sections of gastric cancer specimens from patients ($n = 223$) were subjected to immunohistochemistry analysis using antibodies against Chk1 and Claspin (CLSPN). B, statistical analysis of association between Chk1 and Claspin immunopositivity in gastric cancer specimens. C, immunohistochemical analysis of Chk1, Claspin, and CIP2A protein expression in gastric cancer specimens ($n = 223$). D, statistical analysis of association between Chk1, Claspin, and CIP2A immunopositivity in gastric cancer specimens. E, statistically significant correlation between Chk1 and CIP2A mRNA expression in human neuroblastomas. F, Kaplan–Meier curves demonstrating a significant decrease in overall survival of neuroblastoma patients with high Chk1 and CIP2A mRNA expression. G, expression of CIP2A mRNA in MYCN amplification positive and negative neuroblastoma patients. H, correlation coefficient between Chk1 and CIP2A expression in MYCN amplification positive and negative neuroblastoma patients.
Chk1 and that of CIP2A in neuroblastomas (Fig. 1E). Importantly, in addition to their coexpression, Kaplan–Meier analysis for both Chk1 and CIP2A demonstrated a significant decrease in overall (Fig. 1F) and relapse-free (Supplementary Fig. S1D and S1E) survival in neuroblastoma patients with high expression of either of these genes. Importantly, CIP2A expression and relationship between Chk1 and CIP2A in neuroblastoma is independent on NMYC amplification status, a known major prognostic factor in this disease (Fig. 1G and H). These results indicate that low CIP2A expression could be used as a novel NMYC-independent marker of neuroblastoma patients with favorable prognosis. Together, these results demonstrate coexpression of Chk1 and CIP2A in several human cancer types and indicate that understanding of the functional relevance of their coexpression would be of clinical importance.

Chk1 activity promotes CIP2A expression in tumors

To determine whether coexpression of Chk1 and CIP2A is because of their functional effects on each other, both proteins were depleted from gastric cancer cells using 2 independent siRNAs. Although CIP2A depletion did not affect Chk1 expression, inhibition of Chk1 either by siRNA (Fig. 2A–C and F) or by small molecule inhibitors (SB218078 and Gt treatment. Indeed, CIP2A mRNA expression was decreased by xenografts were treated with the Chk1 inhibitor PF-477736, in vivo promotes CIP2A expression also using xenograft model (20). To confirm that Chk1 activity promotes CIP2A expression also in vivo, in a model that is dependent on Chk1 activity, mice carrying neuroblastoma xenografts were treated with the Chk1 inhibitor PF-477736, and CIP2A mRNA expression was studied 48 hours after treatment. Indeed, CIP2A mRNA expression was decreased by 45% in neuroblastoma tumors in vivo by PF-477736 compared with vehicle control (Supplementary Fig. S4B). Again, analysis of control genes expression from the same tumor samples indicated that Chk1 inhibition did not result in general inhibition of transcription (Supplementary Fig. S4C). We recently demonstrated that CIP2A hypomorph mouse model displays reduced MMTV-neu–induced mammary tumorigenesis (7). CIP2A expression was positively regulated by Chk1 also in this CIP2A-dependent tumor model, as Chk1 inhibition by systemic PF-477736 treatment inhibited CIP2A mRNA expression in MMTV-neu mammary tumors (Fig. 2H). Together these results demonstrate inhibition of CIP2A expression by Chk1-targeted cancer therapy in vivo.

Inhibition of CIP2A expression defines cellular response to Chk1 inhibition

Importantly, depletion of either Chk1 or Claspin decreased the viability of AGS cells (Fig. 3A; Supplementary Fig. S4E). In concert with p53 independent role for Chk1 in regulation of CIP2A expression (Fig. 2A and B; Supplementary Fig. S2D–S2F), Chk1 RNAi inhibited also viability of 2 p53 mutant cancer cell lines, MKN-28 (gastric cancer; Fig. 3B), and MDA-MB-231 (breast cancer; Fig. 3C). Inhibition of both Chk1 and Claspin also inhibited colony growth of AGS cells (Fig. 3D and E). Moreover, the growth of AGS cell colonies was dependent on Chk1 kinase activity, as demonstrated by experiments using 2 small molecule inhibitors of Chk1 (Fig. 3F). Finally, the anchorage-independent growth of gastric cancer cells was also significantly decreased by 2 different small hairpin RNAs specific for Chk1 (Fig. 3G) and by small molecule Chk1 inhibitor SB218078 (Supplementary Fig. S4F and S4G). Therefore, Chk1 activity supports viability and clonogenicity of the same cell lines in which it promotes CIP2A expression, and in which CIP2A promotes cell viability and tumor growth (Supplementary Fig. S4D; refs. 4, 9, 12).

Notably, exogenous expression of CIP2A (Fig. 4B and Supplementary Fig. S4H) increased AGS cell viability in scrambled siRNA-transfected cells, and rescued in large both inhibition of CIP2A levels and cell viability in Chk1 siRNA treated cells (Fig. 4A and B). Statistical analysis of difference in relative cell viability between cells transfected with either control plasmid, or with CIP2AFlag, demonstrated that CIP2A protein expression levels define the response of these cells to RNAi-mediated Chk1 inhibition (Fig. 4A). These findings were again replicated in 2 p53 mutant cell lines (Fig. 4C–E; Supplementary Fig. S4I). Importantly, in addition to short-term cell viability effects, exogenous CIP2A expression also significantly inhibited Chk1 and Claspin siRNA effects in a clonogenicity assay in AGS cells (Fig. 4F).

As an independent approach to demonstrate relevance of CIP2A regulation for therapeutic response to Chk1 inhibition, we assessed Chk1 inhibitor effects on CIP2A expression and viability regulation in an immortalized human prostate cell line PNT2 (30) that we have recently identified as a rare cell line, whose viability is not inhibited by CIP2A-targeted siRNA (unpublished results). Interestingly, as compared with one of the CIP2A-dependent cell lines, AGS, inhibition of Chk1 activity in PNT2 cells did not result in inhibition of CIP2A expression.
Figure 2. Chk1 activity promotes CIP2A expression both in vitro and in vivo. A, effect of Chk1 and CIP2A siRNAs on protein expression of CIP2A, Chk1, and PR65 in AGS gastric cancer cells 72-hour posttransfection. B, inhibition of CIP2A protein expression by Chk1 siRNAs in MKN-28 gastric cancer cell line 72-hour posttransfection. C, immunofluorescent stainings of AGS cells with DAPI and CIP2A antibodies 72-hour posttransfection with scrambled (Scr.), Chk1.1, or Chk1.2 siRNAs. D, inhibition of CIP2A mRNA expression in AGS cells by two different small molecule inhibitors of Chk1 after 24-hour treatment. E, effect of small molecule inhibitors of Chk1 on CIP2A protein expression in AGS cells after 24-hour treatment. F, effect of scrambled (Scr.) and two different CIP2A and Chk1 siRNAs on CIP2A mRNA expression in AGS cells. G, activity of either CIP2A (−1802bpCIP2ALuc) or AP-1 (5xJunLuc) promoter luciferase reporter in AGS cells treated either with DMSO or with Chk1 inhibitors G6976 (1 μmol/L) and SB218078 (1 μmol/L) for 24 hours. E–G, error bars indicate ± SD of three independent experiments. H, systemic treatment with Chk1 inhibitor PF-477736 for 72 hours inhibits CIP2A mRNA expression in vivo in MMTV-neu transgenic mouse mammary tumors. Shown is mean ± SEM; Student t test was used to obtain the statistical significance value. Number of tumors analyzed for each treatment is shown in parenthesis.
Moreover, PNT2 cells were significantly more resistant to inhibition of cell viability in response to Chk1 inhibitor treatment (Fig. 4H). Together, these results strongly indicate inhibition of CIP2A expression as a decisive mechanism whether cell viability is impaired in response to Chk1 inhibition.

**Chk1 inhibition reactivates PP2A tumor suppressor activity**

CIP2A interacts with PP2A and inhibits its tumor suppressor phosphatase activity toward several phosphoprotein targets, including MYC, AKT, DAPK, and E2F1 (4–7, 12). Consequently, siRNA-mediated inhibition of CIP2A, Chk1, or...
Claspin expression reactivated PP2A phosphatase activity in AGS cells (Fig. 5A). Importantly, in cells in which CIP2A downregulation by Chk1 inhibitor SB218078 was prevented by exogenous CIP2A expression, SB218078 treatment rather decreased than increased PP2A activity (Fig. 5B). These results further demonstrate that CIP2A expression levels define cellular response to Chk1 inhibition.

To verify the PP2A activity assay results, and to examine functional effects of PP2A reactivation in Chk1-inhibited cells, expression of serine 62 phosphorylated MYC, the prototypic target for CIP2A-regulated PP2A activity (4, 10, 12) was studied after depletion of Chk1, Claspin, and CIP2A. Transfection of Chk1, Claspin, or CIP2A siRNA resulted in inhibition of expression of both CIP2A and serine 62 phosphorylated MYC (Fig. 5C;...
Figure 5. Chk1 inhibition reactivates PP2A tumor suppressor activity. A, depletion of Chk1, Claspin, or CIP2A induces PP2A activity in AGS cells. B, PP2A activity in pcDNA3.1 or CIP2A Flag transfected AGS cells treated with SB218078 (1 μmol/L; 48 hours) as indicated. C, Chk1 depletion inhibits CIP2A protein expression and of serine 62 phosphorylated MYC in AGS cells 72 hours posttransfection. D, Western blot analysis of SB218078 effects (1 μmol/L, 24 hours) on serine 62 phosphorylation of MYC-ER fusion protein (approx. 100 kDa) or endogenous MYC protein (approx. 65 kDa) in tamoxifen-treated MCF-10 cells stably transfected with MYC-ER. E, serum-starved MYC-ER expressing MCF-10A cells, pretreated for 24 hours with either DMSO or SB218078 (1 μmol/L), were induced to MYC-mediated proliferation by tamoxifen treatment. Quantitation of Ki67 positive cells demonstrates requirement of Chk1 activity for MYC-induced proliferation. F, Venn diagram displaying overlap of genes that are significantly associated with either CIP2A or Chk1 or MYC expression in human neuroblastomas (n = 168), P < 10−10, hypergeometric distribution. G, real-time PCR analysis of SKP2 mRNA expression from AGS cells transfected with CIP2A and Chk1 siRNA for 72 hours. H and I, inhibition of CIP2A target PP2A B-subunit PPP2R2A expression by siRNA rescues Chk1 siRNA effects on the AGS cell colony growth. Shown are mean values + SD of representative results from three independent experiments (Student t test).
Supplementary Fig. S5A). However, neither Chk1 nor CIP2A siRNA inhibited MYC mRNA expression, confirming posttranscriptional regulation of MYC (Supplementary Fig. S5B). We have previously shown that CIP2A expression levels positively correlate with MYC protein expression in gastric cancer (12). Importantly, in the same tumor material, also Chk1 and nuclear MYC protein were found to be coexpressed (Supplementary Table S1).

To examine whether Chk1 activity contributes to MYC-mediated proliferation, we used conditional MYC-ER expressing MCF-10A cells that after serum starvation are driven to MYC-induced cell-cycle progression by nuclear translocation of MYC-ER fusion protein in response to tamoxifen treatment (25). Similar to endogenous MYC, serine 62 phosphorylation of MYC-ER was also inhibited by SB218078, along with CIP2A downregulation (Fig. 5D), whereas in accordance with posttranslational mechanism of MYC regulation by CIP2A, total MYC-ER expression was not affected (Supplementary Fig. S5C). Intriguingly, SB218078-elicited inhibition of CIP2A expression and MYC serine 62 phosphorylation was accompanied by total loss of MYC-induced proliferation of growth factor deprived cells, as measured by the number of Ki67 and histone 3 phosphorylation (ser-10) positive cells, 24 hours after tamoxifen application (Fig. 5E; Supplementary Fig. S5D).

To further validate the assumption that Chk1 promotes MYC activity through CIP2A, we identified sets of genes that are coexpressed with CIP2A or Chk1 (absolute Spearman correlation $r, P < 10^{-10}$, $t$-distribution) in the neuroblastoma database (26), and assessed whether MYC target genes would be particularly enriched among genes that associated with CIP2A and Chk1. The CIP2A and Chk1 associated expression profiles (Supplementary Table S2) were highly positively correlated ($r, P < 10^{-11}$), which further implies a functional link between them. Importantly, MYC target genes comprised almost one-fourth of the 168 genes that were associated with CIP2A and Chk1 and thus showed very significant enrichment (39 shared MYC targets, $P < 10^{-10}$, hypergeometric distribution; Fig. 5F). Among the Chk1 and CIP2A correlating MYC targets, SKP2 is an independently validated direct MYC target that mediates MYC’s proliferative activity (31, 32). Significant inhibition of SKP2 gene expression was validated in AGS cells transfected with both Chk1 and CIP2A siRNAs (Fig. 5G).

We recently showed that depletion of PP2A complex B subunit PPP2R2A reversed CIP2A RNAi effects on proliferation, and on regulation of MYC target genes (8). Therefore, to finally link CIP2A, PP2A, and MYC to Chk1-regulated clonogenicity, we assessed whether depletion of PPP2R2A could reverse effects of Chk1 RNAi. Indeed, cells that were depleted of PPP2R2A (Supplementary Fig. S5E) were relatively resistant to inhibition of colony growth by Chk1 siRNA (Fig. 5H and I).

Identification of DNA-PK as upstream mediator of Chk1 serine 345 phosphorylation and CIP2A expression in unperturbed cancer cells

Upon acutely induced DNA-damage, Chk1 is phosphorylated on both serines 317 and 345 via ATM/ATR-mediated mechanisms (14, 22). However, the mechanisms supporting serine 345 phosphorylation in cancer cells in the absence of DNA-damaging agents are not yet understood. Importantly, a recent study demonstrated a fundamental difference between the biological consequences of phosphorylation of Chk1 on either serine 317 or 345 (33). In agreement with the requirement of Chk1 serine 345 phosphorylation for cell viability in unperturbed conditions (33), in exponentially growing AGS cells, Chk1 was constitutively phosphorylated on serine 345, whereas serine 317 phosphorylation was hardly detected under these conditions (Fig. 6A). Constitutive serine 345 Chk1 staining was specific, as demonstrated by the loss of positive staining for phosphorylated serine 345 on Chk1 in cells transfected with 2 individual siRNAs targeting Chk1 (Supplementary Fig. S5F). When excessive DNA damage was induced by camptothecin treatment, however, ATM/ATR-dependent phosphorylation of serine 317 at Chk1 was observed (Fig. 6A). In line with previously published results (14, 22), induction of DNA damage by camptothecin also increased Chk1 serine 345 phosphorylation, and this DNA-damage–induced serine 345 phosphorylation of Chk1 was sensitive to ATM/ATR inhibition (Supplementary Fig. S5G). However, although camptothecin-induced phosphorylation of both serines 317 and 345 of Chk1 were effectively blocked by treatment with chemical ATM/ATR inhibitor (Fig. 6A; Supplementary Fig. S5G), neither Chk1 serine 345 phosphorylation (Fig. S5H), expression of CIP2A, or expression of serine 62 phosphorylated MYC (Fig. 6B) were affected by treatment with the ATM/ATR inhibitor in unperturbed cells. Furthermore, CIP2A expression was not affected by siRNA-mediated inhibition of ATM/ATR expression in either MKN-28 (Fig. 6C) or HeLa cells (Supplementary Fig. S5I). Together these results confirm involvement of different regulatory mechanisms of serine 345 phosphorylation of Chk1 between unperturbed cells, and cells in which excessive DNA damage has been induced. Furthermore, these results strongly indicate that constitutive Chk1 serine 345 phosphorylation, and Chk1-mediated regulation of CIP2A, occurs via an ATM/ATR-independent mechanism.

Therefore, we examined whether a different DDR kinase was involved. The DNA-dependent protein kinase (DNA-PK) transduces DNA-damage responses and directly phosphorylates Chk2 (34), making it a plausible candidate to mediate also Chk1 phosphorylation. Importantly, DNA-PKc inhibition, by either the specific small molecule inhibitor DMNB(DNA-PKI), or by DNA-PKc specific siRNAs (Supplementary Fig. S5J), inhibited expression of serine 345 phosphorylated Chk1 (Fig. 6D). Moreover, in an in vitro kinase assay, DNA-PKc immunoprecipitated from MKN-28 cells was capable of phosphorylating recombinant Chk1 on serine 345, identifying DNA-PKc as Chk1 serine 345 kinase (Fig. 6E). Inhibition of DNA-PK by either DNA-PKI or by RNAi inhibited the expression of CIP2A (Fig. 6F; Supplementary Fig. S5K) and, similar to inhibition of Chk1 (Fig. 5C) and CIP2A (Supplementary Fig. S5A; refs. 4, 8, 10, 12), DNA-PKc inhibition also inhibited the expression of serine 62 phosphorylated MYC (Fig. 6G). Together these results indicate that in unperturbed cancer cells, DNA-PKc activity supports Chk1 serine 345 phosphorylation and CIP2A expression. However, our data does not exclude other unidentified active
Figure 6. DNA-PKc acts as an upstream mediator of Chk1 serine 345 phosphorylation and CIP2A expression in unperturbed cancer cells. A, immunofluorescent stainings of unperturbed and camptothecin-(400 nmol/L for 24 hours) and/or ATM-ATR inhibitor-(400 nmol/L for 24 hours) treated AGS cells with indicated antibodies and DAPI. γ-H2AX staining was used to demonstrate camptothecin-induced double-stranded DNA breaks.

B, Western blot analysis of CIP2A and phospho serine 62 MYC expression levels in AGS cells treated either with indicated concentrations of ATM/ATR inhibitor (ATM/ATRi) or with Chk1 inhibitor SB218078 (1 μmol/L) for 48 hours. C, expression of CIP2A, PCNA, ATM, ATR, and Chk1 proteins in MKN-28 cells cotransfected with 2 independent siRNAs targeting both ATM and ATR, 72-hour posttransfection. D, immunofluorescent stainings of AGS cells with DAPI and phospho-Chk1-Serine-345 antibody after either transfection of DNA-PK targeting siRNA or treatment with DNA-PK inhibitor (DNA-PKi; 10 μmol/L for 48 hours).

E, DNA-PKc immunoprecipitated from exponentially growing MKN-28 cells was used in in vitro kinase assay with recombinant Chk1 protein as a substrate. Chk1 protein amounts and serine 345 phosphorylation was studied from kinase reaction by Western blotting.

F, CIP2A protein expression in AGS cells treated for 48 hours with ATM/ATR inhibitor (400 nmol/L) or DNA-PK inhibitor (10 μmol/L). G, CIP2A protein expression and phospho-MYC-serine 62 levels in MKN-28 cells treated for 48 hours with DNA-PK inhibitor at indicated concentrations. Shown are representative results from 2 independent experiments.
signaling mechanisms that may contribute to Chk1 serine 345 phosphorylation and activity in unperturbed cancer cells.

Discussion

New generation small molecule inhibitors of Chk1 demonstrate single-agent therapeutic activity in several preclinical cancer models (13, 15, 16, 18–20). However, most of our current knowledge related to regulation and functional role of Chk1 has been acquired from experiments in which DNA damage has been induced acutely in combination with Chk1 inhibition (14, 22, 24, 35). When Chk1 is inhibited in this context, inability to repair the damaged DNA leads to cellular crisis and massive apoptosis (14, 24). This synthetic lethal interaction between DNA damage induction and Chk1 inhibition have served as the dogmatic model to explain nononcogene addiction of malignant cells to Chk1 activity (14, 24, 35). However, our understanding of the mechanisms, which contribute to single-agent activity of Chk1-targeted therapies in cancer cells with continuous Chk1 phosphorylation, is very limited.

In this study, we reveal hitherto unrecognized downstream mechanism by which chronic Chk1 activity promotes cancer cell viability and clonogenicity. Our results demonstrate that inhibition of Chk1 activity inhibits expression of human oncoprotein CIP2A, which in turn results in reactivation of PP2A tumor suppressor activity (Fig. 7). Importantly, results of several experiments demonstrate that CIP2A expression levels define cellular response to Chk1 inhibition in vitro. CIP2A dependency of Chk1-regulated phenotypes was evidenced both at the level of regulation of PP2A activity (Fig. 5B) and cell viability (Fig. 4A–F). As a proof-of-principle functional target for this newly identified pathway, we show that both MYC serine 62 phosphorylation and MYC activity are regulated by Chk1 and CIP2A (Fig. 5C–G). General applicability of Chk1-dependent regulation of CIP2A expression and the functional relevance of this regulation was confirmed by using several cell lines derived from different types of human cancers irrespective of p53 status. Importantly, in addition to in vitro data, we also present significant evidence indicating that Chk1–CIP2A–MYC pathway functions in vivo in tumors. These data include demonstration of coexpression of Chk1, CIP2A, and MYC in human tumors as well as prognostic role of both Chk1 and CIP2A in the same tumor type. Moreover, we show that among the genes whose expression is significantly associated with Chk1 and CIP2A expression in human tumors, MYC target genes are significantly overpresented. Finally, we show that in vivo tumors that are dependent on either Chk1 or CIP2A expression, inhibition of Chk1 activity by small molecules in clinical development results in inhibition of CIP2A transcription. In future, it would be of great interest to use genetically modified mouse models to assess the degree by which PP2A inhibition contributes to in vivo tumor response to single-agent Chk1 inhibition. It is anticipated that high CIP2A expression, or loss of PP2A B-subunit PPP2R2A observed recently in human breast cancer (36), would induce relative resistance to Chk1 inhibitors because of lack of induction of PP2A tumor suppressor activity.

Our results strongly indicate that constitutive serine 345 phosphorylation of Chk1 promotes CIP2A expression and cancer cell viability in unperturbed conditions. Association of Chk1 serine 345 phosphorylation with increased cell viability is strongly supported in the recent study by Bunz and colleagues demonstrating that, although serine 317 phosphorylation of Chk1 is not relevant to cell viability or proliferation in unperturbed conditions, mutated serine 345 did not support viability (33). Moreover, a recent study showed that constitutive phosphorylation of serine 345 was observed in human neuroblastoma cell lines derived only from high-risk primary tumors (20). Consequently, only the cell lines expressing serine 345 phosphorylated Chk1 were highly sensitive to small molecule Chk1 inhibitors also used in this study (20). Association of high Chk1 serine 345 phosphorylation with increased malignancy of cancer cells perfectly corroborates the findings that CIP2A expression associates with increased tumor grade and poor

![Figure 7](image-url)
Patient prognosis in most of the studied human cancer types (4, 7, 10, 12).

The upstream mechanisms promoting serine 345 phosphorylation in unperturbed cells have been elusive. Even though ATR kinase mediates serine 345 phosphorylation of Chk1 under acute DNA damage conditions (refs. 14, 22; Supplementary Fig. 5S5), inhibition of ATR did not affect expression of CIP2A or serine 62 phosphorylated MYC (Fig. 6B and C; Supplementary S5J and S5K). Instead, we identify another member of the phosphatidylinositol 3-kinase-related kinase family of DNA damage response kinases, DNA-PK, as a strong candidate for mediating Chk1 serine 345 phosphorylation in unperturbed conditions. We also show that DNA-PK inhibition either by RNAi or by small molecule inhibitor recapitulates Chk1-dependent regulation of CIP2A expression and MYC serine 62 phosphorylation (Fig. 6F and G; Supplementary S5J and S5K). Altogether, these results provide novel insights for understanding the upstream mechanisms of chronic Chk1 activity in unperturbed cancer cells.

PP2A inactivation is a universal characteristic of cancer cells (1–5). Thereby, identification of novel link between DNA damage signaling and PP2A inactivation in malignant cells fosters our general understanding of constitutively active signaling circuits in cancer cells (Fig. 7). Considering the plethora of phosphoproteins regulated by PP2A (37), we postulate that in addition to MYC serine 62 phosphorylation, other oncogenic PP2A targets will be identified in future to be regulated by Chk1-driven CIP2A expression in human malignancies. Supporting this, MYC-independent but PP2A-dependent CIP2A target mechanisms and proteins have been recently identified (5–8). Therefore, our data does not exclude that other CIP2A-regulated PP2A targets than MYC would be involved in inhibition of cell viability in Chk1-inhibited cancer cells (Fig. 7). As a matter of fact, function of CIP2A as an inhibitor of broad-specificity serine/threonine phosphatase complex PP2A (37) most likely explains how expression status of only one gene, CIP2A, may define functional outcome of Chk1 inhibition in unperturbed cancer cells in vitro.

Clinically, results of this work may help in understanding the molecular basis of poor response of cancer patients to Chk1 inhibitor monotherapy. Moreover, our results indicate that assessment of transcriptional effects of Chk1 inhibition may unveil novel biomarkers of tumor response to Chk1 inhibitors. However, based on our results, it is plausible that reactivation of PP2A, by targeting of CIP2A, or by other means, would be therapeutically beneficial in cancers that are dependent on chronic Chk1 activity.

Disclosure of Potential Conflicts of Interest
A. Khanna has ownership interest (including patents). J. Westermarck has ownership interest (including patents) in patents related to CIP2A RNAi therapy. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Conception and design: A. Khanna, C. Weiss, T. Visakorpi, J. Westermarck
Development of methodology: A. Khanna, O. Kauko, T. Attokallo
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Khanna, O. Kauko, C. Böckelman, A. Laine, I. Schreck, J.J. Partanen, S. Bormann, T. Bälgén, M.A. Helenius, Y. Pokharel, J.E. Pimanda, M. Russel, C.H. Haglund, K.A. Cole, J. Klefström, A. Ristimäki
Writing, review, and/or revision of the manuscript: A. Khanna, O. Kauko, C. Böckelman, A. Laine, C.H. Haglund, J. Klefström, T. Attokallo, C. Weiss, A. Ristimäki, T. Visakorpi, J. Westermarck
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A. Khanna, J.E. Pimanda, J. Klefström, A. Ristimäki
Study supervision: C. Weiss, T. Visakorpi, J. Westermarck

Acknowledgments
The authors thank Drs. D. Bohmann, T. Mäkelä, and J. Ivaska for critical reading of the manuscript. Dr. B. Gillespie is greatly thanked for his critical advice during this project. Drs. M. Anna and M. Nykter are greatly acknowledged for their help in bioinformatics analysis. Dr. E. Chan is acknowledged for CIP2A antibody and Dr. H. Van Dam for SYLVJLac; construct. C. Chang and C. Painter are acknowledged for their advice about in vivo use of Chk1 inhibitor.

Grant Support
This work was supported by Academy of Finland (grants 8217676, 122546, and 137687), Foundation for Finnish Cancer Institute, Finnish Cancer Associations, Sigrid Juselius Foundation, The Finnish Funding Agency for Technology and Innovation (TEKES), NIH (grant SK00CA136979), and by competitive research funding from Tampere University Hospital district.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received April 17, 2013; revised August 7, 2013; accepted August 29, 2013; published OnlineFirst September 26, 2013.

References


Chk1 Targeting Reactivates PP2A Tumor Suppressor Activity in Cancer Cells

Anchit Khanna, Otto Kauko, Camilla Böckelman, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-13-1002

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2013/09/26/0008-5472.CAN-13-1002.DC1

Cited articles
This article cites 37 articles, 11 of which you can access for free at:
http://cancerres.aacrjournals.org/content/73/22/6757.full#ref-list-1

Citing articles
This article has been cited by 5 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/73/22/6757.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link
http://cancerres.aacrjournals.org/content/73/22/6757.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.