

# Molecular Pathways: Emergence of Protein Kinase CK2 (CSNK2) as a Potential Target to Inhibit Survival and DNA Damage Response and Repair Pathways in Cancer Cells

Adam J. Rabalski<sup>1</sup>, Laszlo Gyenis<sup>1</sup>, and David W. Litchfield<sup>1,2</sup>

## Abstract

Protein kinase CK2 (designated CSNK2) is a constitutively active protein kinase with a vast repertoire of putative substrates that has been implicated in several human cancers, including cancer of the breast, lung, colon, and prostate, as well as hematologic malignancies. On the basis of these observations, CSNK2 has emerged as a candidate for targeted therapy, with two CSNK2 inhibitors in ongoing clinical trials. CX-4945 is a bioavailable small-molecule ATP-competitive inhibitor targeting its active site, and CIGB-300 is a cell-permeable cyclic peptide that prevents phosphorylation of the E7 protein of HPV16 by CSNK2. In preclinical models, either of these inhibitors exhibit antitumor efficacy. Furthermore, in combinations with chemotherapeutics such as cisplatin or gemcitabine, either CX-4945 or CIGB-300 promote synergistic induction of apoptosis. While CSNK2 is a regulatory partici-

pant in many processes related to cancer, its potential to modulate caspase action may be particularly pertinent to its emergence as a therapeutic target. Because the substrate recognition motifs for CSNK2 and caspases are remarkably similar, CSNK2 can block the cleavage of many caspase substrates through the phosphorylation of sites adjacent to cleavage sites. Phosphoproteomic strategies have also revealed previously underappreciated roles for CSNK2 in the phosphorylation of several key constituents of DNA damage and DNA repair pathways. Going forward, applications of proteomic strategies to interrogate responses to CSNK2 inhibitors are expected to reveal signatures for CSNK2 inhibition and molecular insights to guide new strategies to interfere with its potential to inhibit caspase action or enhance the susceptibility of cancer cells to DNA damage. *Clin Cancer Res*; 22(12); 2840–7. ©2016 AACR.

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## Learning Objectives

Upon completion of this activity, the participant should have a better understanding of the role of protein kinase CK2 (CSNK2) in cellular processes that underlie malignancy, including its capacity to interfere with caspase action and involvement in DNA repair pathways, and the biological rationale of CSNK2 inhibitors as promising candidates for novel therapeutic strategies.

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<sup>1</sup>Department of Biochemistry, Schulich School of Medicine & Dentistry, University of Western Ontario, London, Ontario, Canada. <sup>2</sup>Department of Oncology, Schulich School of Medicine & Dentistry, University of Western Ontario, London, Ontario, Canada.

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**Corresponding Author:** David W. Litchfield, Department of Biochemistry, Schulich School of Medicine & Dentistry, University of Western Ontario, London, Ontario N6A 5C1, Canada. Phone: 519-661-4186; Fax: 519-661-3175; E-mail: [litchfi@uwo.ca](mailto:litchfi@uwo.ca)

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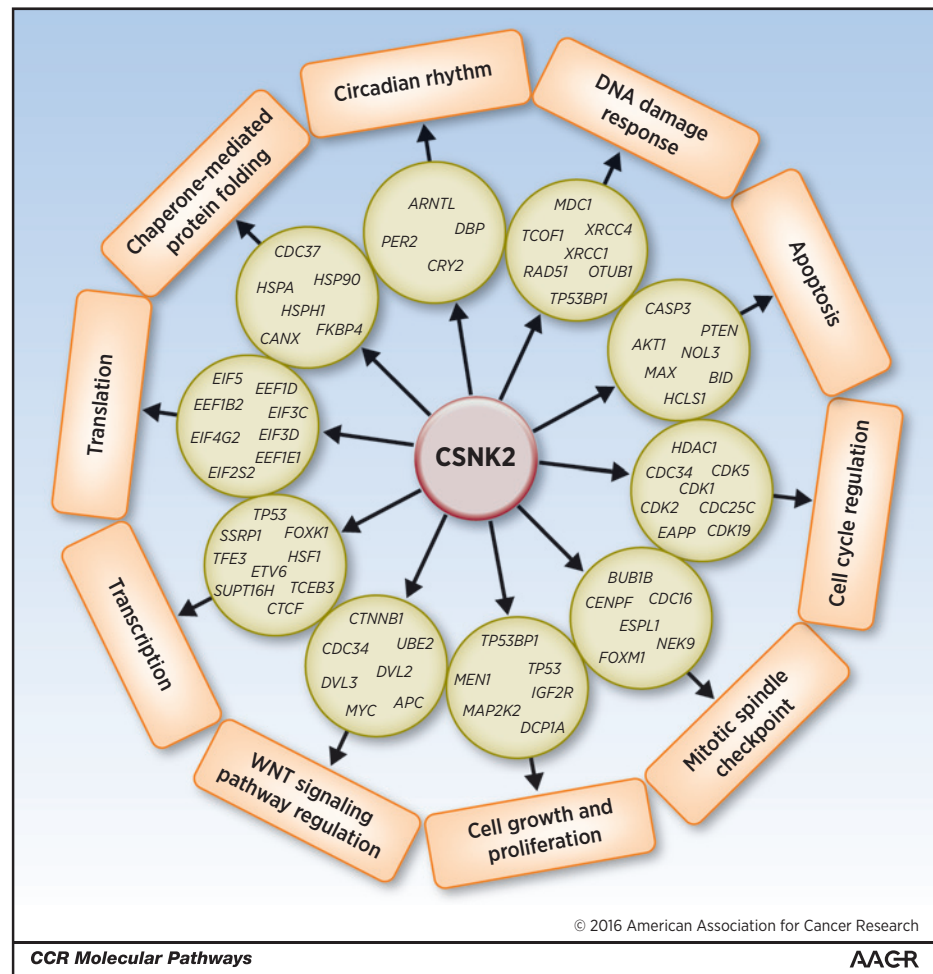
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## Background

Protein kinase CK2 (designated CSNK2) represents one small protein kinase family that has recently emerged as a potential therapeutic target based on alterations in its expression or activity in a number of cancers (Fig. 1; ref. 1). CSNK2 is composed of two closely related catalytic isoforms (CSNK2A1 or CSNK2A2) that both display catalytic activity in the presence or absence of its regulatory CSNK2B; subunit (see Supplementary Table S1 for definitions of the acronyms that appear throughout this article). The regulatory CSNK2B subunit is not essential for activity but can affect the ability of the catalytic subunits to phosphorylate certain

**Figure 1.**

CSNK2 has been implicated in a diverse array of biologic processes. This figure illustrates a selection of the biological processes in which CSNK2 has been implicated with examples of putative substrates or interacting proteins involved in each of these processes. These proteins were identified and analyzed using the following databases: GO: biological processes according to UniProt (52), Reactome (53), PHOSIDA (54), and PubMed (55).



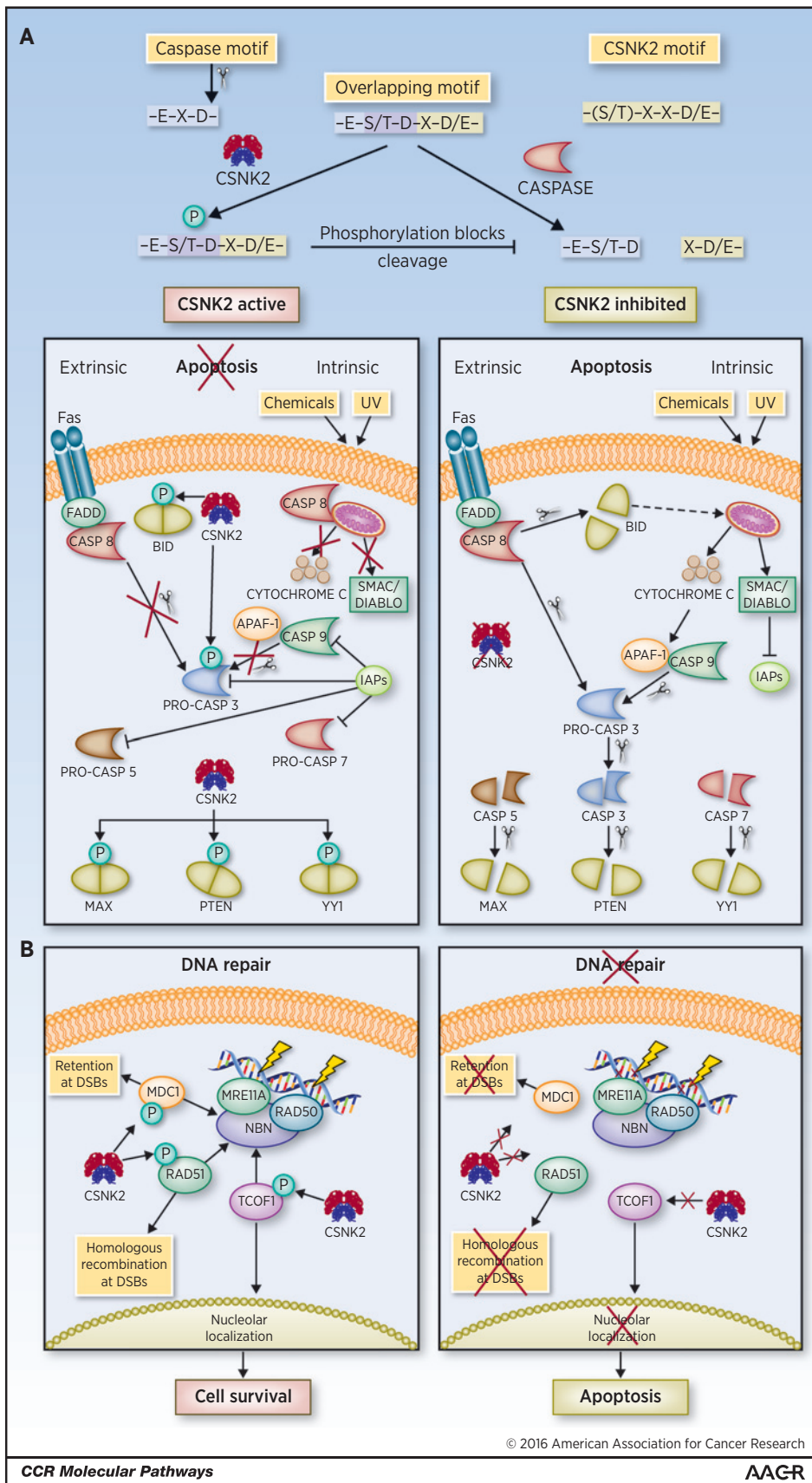
substrates (2). A recent analysis of transcript expression profiles for CSNK2A1, CSNK2A2, and CSNK2B in neoplastic tissues versus normal tissues deposited in the Oncomine database revealed varying degrees of altered expression of individual CSNK2 subunits, suggesting that deregulation of CSNK2 subunit expression profiles promotes cancer (3). To this point, the CSNK2-dependent phosphoproteome has not yet been fully elucidated, and the impact of alterations in CSNK2 on the phosphoproteome has not yet been systematically investigated. Nevertheless, it is evident both from an extensive body of literature and computational predictions of its substrates based on its consensus recognition motif (S/T-X-X-D/E/pS/pY) that CSNK2 is involved in a vast landscape of biological processes (Fig. 1), with more than 2,000 putative phosphorylation sites in the human proteome (4). While more comprehensive discussions of CSNK2 can be found elsewhere (5, 6), we highlight the involvement of CSNK2 in specific cellular processes, including its convergence with caspase pathways and its roles in DNA response and repair pathways, which have inspired ongoing efforts to exploit CSNK2 as a therapeutic target.

#### Convergence of CSNK2 with caspase pathways

CSNK2 is generally considered to be a constitutively active enzyme (i.e., its catalytic subunits do not require activating phosphorylation, association with its regulatory subunit, or

the presence of second messengers to be catalytically active), which is expressed at higher levels in many forms of human cancer (1, 3, 7, 8). CSNK2 has also been shown to be essential for viability and to enhance cancer cell survival associated with inhibition of apoptosis. These observations are particularly intriguing when considering the remarkable resemblance of the consensus recognition motif of CSNK2 with that of caspases (Fig. 2A) that cleave substrates at aspartic acid residues during the progression of apoptosis. In fact, several published examples (Fig. 2A) show that caspase substrates, including Bid, which promotes apoptotic progression when it is cleaved, can be phosphorylated by CSNK2 at sites adjacent to caspase cleavage sites. Because phosphorylation adjacent to caspase cleavage sites blocks cleavage, these observations suggest that increased phosphorylation by CSNK2 could promote cell survival by inhibiting caspase action (9–14).

Building on these observations, a combined computational and biochemical approach revealed an extensive repertoire of proteins with overlapping CSNK2 and caspase recognition motifs suggesting that CSNK2 could have widespread impact on caspase action (15). In addition to the identification of many proteins known to be caspase substrates, these studies also demonstrated that CSNK2 could phosphorylate CASP3



**Figure 2.** Protein kinase CSNK2 plays pivotal roles in the control of cell survival and responses to DNA damage. A, on the basis of the remarkable similarity of the consensus recognition motifs for phosphorylation by CSNK2 (S/T-D-X-X-D/E) and cleavage by caspases (E-X-D), it is evident that there are many cellular proteins where CSNK2 phosphorylation could occur adjacent to caspase cleavage sites to block their cleavage by caspases (15). As described in the text, the proteins shown here represent caspase substrates including Bid, which promotes apoptotic progression when it is cleaved, and CASP3, where CSNK2 phosphorylation adjacent to a caspase cleavage site has been demonstrated to block its activation (9, 10, 12–15). B, proteins shown here are involved in DNA damage repair, which requires phosphorylation by CSNK2 to execute respective DNA repair processes. A recurring theme with the illustrated proteins (i.e., MDC1, RAD51, and TCOF1) is the ability of CSNK2 to promote phosphorylation-dependent interactions with the FHA domain of the NBN component of the MRN complex (21–24, 27). As described in the text, the potential of CSNK2 to block caspase action in the manner shown in A has inspired efforts to use CSNK2 inhibitors to render cancer cells more susceptible to apoptosis. Similarly, the involvement of CSNK2 in DNA damage responses shown in B has motivated the evaluation of CSNK2 inhibitors for enhancing the vulnerability of cancer cells to DNA-damaging agents. DSB, double-stranded break.

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to prevent its cleavage by upstream caspases activated by intrinsic and extrinsic apoptotic stimuli (Fig. 2A). Collectively, these findings demonstrate that CSNK2 has the potential to inhibit caspase action at both the level of caspase activation and cleavage of downstream substrates. Furthermore, these findings raise the prospect that the higher (i.e., pathologic) levels of CSNK2 that are observed in cancer cells could result in the pathologic rewiring of caspase pathways to promote cell survival. Interestingly, further characterization of the relationship between CSNK2 and caspases revealed intriguing isoform specificity. In this respect, CASP3 appears to be preferentially phosphorylated by CSNK2A2 rather than CSNK2A1, despite the fact that these two isoforms of CSNK2 have very similar enzymatic characteristics (16). Furthermore, CSNK2B attenuated phosphorylation, suggesting that misregulation of CSNK2 subunits in cells could be an additional mechanism to affect the regulation of apoptosis (12). The capacity of CSNK2 to promote cancer cell survival *in vitro* was also revealed in a recent large-scale screen via RNA interference knockdown and cDNA overexpression of kinases in DLD-1 colorectal adenocarcinoma cells in which overexpression of CSNK2A1 promoted resistance to TNFSF10 as determined by flow cytometry using antibodies against cleaved PARP and cleaved CASP3 (17). Overall, while CSNK2 is only one of many constituents that intersect caspase pathways, its ability to modulate caspase action through phosphorylation of caspase substrates (potentially including caspases themselves) represents one possible mechanism by which CSNK2 might be exploited to neutralize cancer cell survival.

#### CSNK2 and DNA damage response and repair pathways

Another manner in which CSNK2 may contribute to the regulation of cancer cell survival that might be exploited for intervention is through its involvement in DNA damage response (DDR) and DNA repair pathways (Fig. 2B). In this respect, recent large-scale phosphoproteomic analyses of G361 melanoma cells and GM00130 B lymphocyte cells treated with the radiomimetic drug neocarzinostatin or with ionizing radiation to induce DNA double-stranded breaks (DSB), respectively, demonstrated a dynamic response in motifs preferential for CSNK2 phosphorylation in many proteins. These results reveal a previously underappreciated role for CSNK2 as an important mediator in the cellular response to DSBs (18, 19). Other indications that CSNK2 is involved in DDR and DNA repair pathways include the demonstration that CSNK2A1 localizes to perinuclear structures whereas CSNK2A2 becomes nuclear in response to DSBs caused by ionizing radiation (20). Some of the CSNK2 substrates that are involved in DDR are scaffold proteins such as mediator of DNA damage checkpoint 1 (MDC1), where phosphorylation of serine-aspartate-threonine repeats regulates retention of the MRE11A-RAD50-NBN (MRN) complex at DSBs through interactions with Forkhead-associated (FHA) domains in the NBN component of the MRN complex (21, 22). Another critical interaction for NBN is colocalization to the nucleolus that is dependent on the phosphorylation of TCOF1 by CSNK2 at threonine 210, independent of MRE11A (23, 24). In terms of regulating processes tasked with repairing damaged DNA, the phosphorylation of XRCC1 by CSNK2 is required for single-strand break repair (25), and phosphorylation of XRCC4 is necessary for

nonhomologous end joining repair (26). In homologous recombination, phosphorylation of RAD51 by PLK1 at serine 14 primes the phosphorylation of threonine 13 by CSNK2, regulating binding to the NBN component of the MRN complex and facilitating recruitment of RAD51 to damaged DNA (27). In another recent study, it was determined that the phosphorylation of a deubiquitylase OTUB1 by CSNK2 is necessary for its nuclear accumulation to promote the formation of TP53BP1 repair foci at DSBs (28). While undoubtedly many details regarding its roles in DDR and DNA repair pathways remain to be elucidated, these examples show that CSNK2 has an important role in several discrete aspects of these processes. A recurring theme that is highlighted by these examples is that phosphorylation by CSNK2 promotes phosphorylation-dependent interactions such as those involving the FHA domain of the NBN component of the MRN complex. Overall, the widespread involvement in these processes could be relevant to the development of strategies that neutralize the advantages or exploit the unique vulnerabilities of cancer cells.

### Clinical-Translational Advances

As CSNK2 is implicated in a number of fundamental biological processes and found at varying levels of deregulation in human cancers, widespread interest has been expressed in targeting this kinase therapeutically (1). While there are many inhibitors described in the literature (29), our discussion focuses on two molecules, CX-4945 and CIGB-300, that have advanced to clinical trials.

#### CX-4945: An ATP-competitive CSNK2 inhibitor in clinical trials

CX-4945 was developed by Cylene Pharmaceuticals, Inc. and is a potent and selective ATP-competitive inhibitor ( $K_i = 0.38$  nmol/L for CSNK2A1 and CSNK2A2 with an  $IC_{50}$  of 1 nmol/L) with no extensive inhibitory activity against a panel of 238 kinases (30, 31). Characterization of its *in vitro* effects revealed varying degrees of activation of caspase-3 and caspase-7 in cancer cells with no detectable change of caspase-3/7 activity in normal cells. Interestingly, CX-4945 elicited differential effects on the cell cycle with BT-474 breast cancer cells arresting in G<sub>2</sub>-M transition and BxPC-3 prostate cancer cells arresting in G<sub>1</sub> (31). *In vitro* studies employing CX-4945 in combination with gemcitabine or cisplatin revealed enhanced antiproliferative effects in A2780 and SKOV-3 ovarian cancer cells. Antiproliferative activity was 23% to 38% higher than Bliss additivity when CX-4945 was added to cells after treatment with gemcitabine or cisplatin. These treatments were also accompanied by decreased phosphorylation of the CSNK2 substrates XRCC1 and MDC1 and accumulation of single-stranded and double-stranded DNA breaks (32). These data are in agreement with the observations of CSNK2 localizing to the nucleus in response to DSBs and for the role of CSNK2 in the DDR and DNA repair mechanisms (20, 25, 33, 34). Currently CX-4945 is in phase I/II clinical trials in the United States, South Korea, and Taiwan for the treatment of cholangiocarcinoma in combination with gemcitabine and cisplatin (NCT02128282). The aim of this trial is to determine its maximum tolerable dose in patients followed by a randomized phase II assessment using CX-4945 in combination with gemcitabine and cisplatin versus the standard of care.

### CIGB-300: A CSNK2 inhibitory peptide in clinical trials

An alternative strategy for targeting CSNK2 emerged from a screen performed with a phage display library to identify cyclic peptides that bind to a peptide encoding amino acids 28-38 of the E7 protein of human papillomavirus 16 (HPV16). This screen yielded a cyclic peptide (termed P15) that blocked phosphorylation of recombinant HPV-16 E7 protein *in vitro* (35). When fused to a cell-permeable peptide derived from the HIV-Tat protein, the resulting peptide, designated CIGB-300, induced caspase activation in TC-1 cells. Further characterization of CIGB-300 was performed in human trials to investigate safety and tolerability in female patients with cervical cancer, which resulted in 75% of all patients in all treatment groups having reduced total lesion areas, with 90% having partial response and 19.4% showing complete response in total lesion area reduction (36). CIGB-300 also displays efficacy *in vitro* in chronic lymphocytic leukemia cell (CLL) lines (including MEC1, WaC3D5, JVM3, and MO1043), producing IC<sub>50</sub> values between 27 and 38 μmol/L with 72-hour treatment at 30 μmol/L resulting in less than 40% cell viability (37). Antitumor activity of CIGB-300 in mouse xenograft models of transplanted with MO1043 cells was also effective, as mean tumor volume decreased more than 50% relative to control treatment. (37). Other *in vitro* investigations, using combinations of CIGB-300 with chemotherapeutic agents including paclitaxel, doxorubicin, cisplatin, and 5-fluorouracil in lung and cervical cancer cell models, show promise for further clinical investigation (38). Paclitaxel displayed the strongest synergism with CIGB-300 in NCI-H125 and SiHa cell lines, with 5-fold less peptide being required to achieve an antiproliferative effect level of 94% in NCI-H125 cells. With the same combination setting, the concentration of paclitaxel could be reduced more than 3,000-fold without compromising the final antiproliferative effect (38). Ongoing trials for optimization of CIGB-300 treatment and delivery approaches highlight its utility as an alternative to small-molecule ATP-competitive inhibitors for targeting CSNK2 in cancer (39).

### Emerging prospects for combinatorial treatments involving CSNK2 inhibition

CX-4945 has been used in combination with other agents and has shown promise for targeting a number of malignancies. For example, CX-4945 has been studied *in vitro* in combination with DNA-damaging agents in acute myeloid leukemia cell lines and patient-derived cells (40). In another study of hematologic malignancies, CX-4945 showed a significant decrease in cell viability of patient-derived CLL cell lines *in vitro* (41). When CX-4945 was combined with GS-1101 (PI3KD inhibitor), ibrutinib (BTK inhibitor), or fludarabine, synergistic responses were observed in CLL cell lines that were not observed in the unpaired treatments with combination indices (using the Chou-Talalay method) of 0.46, 0.56, and 0.30 for CX-4945 and GS-1101, ibrutinib and fludarabine, respectively. Combinations of CX-4945 and bortezomib in *in vitro* assays using patient-derived cells from multiple myeloma and mantle cell lymphoma also enhanced mitochondria-dependent apoptosis associated with suppression of NFKB1 and STAT3 target genes leading to 50% reduction of *NOS2* and *BCL2* gene expression (42). In other *in vitro* cell models of cancer such as A431 epidermal carcinoma cells and H2170 lung cancer cells, combining erlotinib and CX-4945 resulted in suppression of AKT1 phosphorylation in addition to downregulation of phosphorylation sites on AKT1 and MTOR substrates (43). Combined

treatment resulted in a 3-fold increase in apoptosis as determined by caspase-3 and caspase-7 activity assays in comparison with treatment with CX-4945 or erlotinib alone. This enhanced anti-proliferative effect was also demonstrated in mouse xenograft models, with the greatest reduction in tumor volume resulting from combined treatment in both A431 and H2170 xenograft mice (43).

Because resistance is commonly observed with both chemotherapy and targeted therapy involving kinase inhibitors, inhibition of CSNK2 has also been explored as a strategy for overcoming both *de novo* resistance and acquired resistance. Analysis of several multidrug-resistant cell lines revealed that CX-4945 sensitized cells to vinblastine and doxorubicin, resulting in reduction of cell viability by 50% when treating with vinblastine and a 2-fold increase in doxorubicin accumulation in resistant cells (44). These results indicate that CSNK2 inhibition may have utility for overcoming *de novo* resistance.

There is also mounting evidence to suggest that CSNK2 inhibition may be effective to combat adaptive resistance. Investigation of imatinib-resistant chronic myeloid leukemia (CML) cell lines revealed 2-fold higher protein and activity levels of CSNK2, along with increased localization of CSNK2A1 and CSNK2B to the cytoplasm of imatinib-resistant cells in comparison with imatinib-sensitive cells (45). Interestingly, treatment with CX-4945 abrogated CSNK2A1 and BCR-ABL1 interaction as determined by co-immunoprecipitation and glycerol gradient sedimentation leading to the induction of apoptosis in resistant cell lines at concentrations that failed to promote PARP cleavage in imatinib-sensitive CML. Furthermore, treatment of imatinib-resistant CML cells with CX-4945 or siRNA targeting CSNK2A1 promoted higher sensitivity to lower concentrations of imatinib, suggesting that the kinome had adapted in response to Bcr-Abl inhibition by imatinib, leading to increased dependence on CSNK2 activity (45). In an *in vitro* cell model of T-cell acute lymphoblastic leukemia, CX-4945 downregulated the unfolded protein response as determined by HSPA5 expression and caused increased ERN1, phosphorylated EIF2S1, and DDIT3, indicating endoplasmic reticulum stress. When paired with thapsigargin or temsirolimus the synergistic cytotoxicity was further potentiated in these cell-based assays (46). More recently, analysis of acquired resistance in triple-negative breast cancer using SUM149 and SUM159 cell lines *in vitro* revealed that resistance to the BET bromodomain inhibitor JQ1 was associated with increased binding of BRD4 to MED1 in a bromodomain-independent manner and dependent on BRD4 phosphorylation by CSNK2A1, which keeps transcriptional regulation of genes promoting cellular proliferation active (47). The use of CSNK2 inhibitor or protein phosphatase PPP2CA activators such as perphenazine decreased phosphorylation of BRD4, suggesting a rationale for this combination for tackling BET bromodomain inhibitor resistance in triple-negative breast cancer. Collectively, these observations raise the prospect that CSNK2 inhibitors could be used to enhance the effectiveness of other interventions.

### Emerging considerations: Phosphoproteomic and kinome profiling and characterization of signaling pathway "rewiring" after small-molecule inhibition

With recent advances in mass spectrometry-based proteomics and the increased sophistication of computational approaches, it is now possible to explore cellular responses to kinase inhibition

on a proteome-wide scale. In addition to identification of direct targets of individual kinases within cells, phosphoproteomic studies are also revealing new relationships between different kinases or kinase-mediated pathways within the complex and intricate regulatory networks that orchestrate cellular processes. For example, in a thyroid cancer cell line harboring the BRAF V600E mutation, phosphoproteomic analysis following short-term treatment (15 or 30 minutes) with vemurafenib (PLX4032) or the MAP2K1/2 inhibitor selumetinib (AZD6244) revealed increased phosphorylation of numerous sites. Interestingly, approximately 50% of the upregulated sites conformed to the CSNK2 consensus motif, suggesting that CSNK2 activity is affected by inhibition of MAPK signaling (48). Building on that observation, combination of CX-4945 with vemurafenib demonstrated a synergistic effect in reducing cell viability by 60% in V600E mutant thyroid cancer cells and in mutant melanoma cell lines. In another global phosphoproteomic study, CSNK2 phosphorylation sites had increased phosphorylation site stoichiometry in gefitinib-resistant PC9 lung cancer cells in comparison with gefitinib-sensitive cells, in addition to having higher phosphorylation stoichiometry than MAPK- or EGFR-regulated phosphorylation sites (49). Collectively, results from these large-scale phosphoproteomics studies imply that CSNK2 may have an important role in drug resistance mechanisms and offer new insights regarding pathways that may be regulated or influenced by CSNK2.

As a companion to phosphoproteomic workflows, complementary proteomic strategies have been devised to monitor kinome responses (e.g., simultaneously profiling the activity of hundreds of kinases) when cells are treated with kinase inhibitors (50). For example, multiplexed inhibitor beads (designated MIBs) have been used as a means to capture and infer the activity of hundreds of active kinases (i.e., the active kinome) from cell extracts. With triple-negative breast cancer cells and mouse models, this approach revealed activation of receptor tyrosine kinases such as PDGFRB and DDR1 in response to the MAP2K inhibitors selumetinib or trametinib, which in turn activated downstream kinases capable of activating the MAPK pathway through an alternative route to circumvent inhibition (51). Based on these observations, it is evident that the combined application of proteomic and phosphoproteome strategies will reveal how cells respond to specific drugs leading to insights regarding compensatory changes that contribute to resistance to kinase inhibitors and the promise of combinations that might be devised to effectively attenuate both primary and compensatory pathways. In this respect, applications of proteomic and phosphoproteomic strategies for profiling CSNK2 inhibitors would undoubtedly be invaluable in assessing the prospect of resistance to CSNK2 inhibition and revealing the relationships of CSNK2 with other constituents of the regulatory kinase networks.

## Conclusions and Future Perspectives

At this stage, our understanding of how CSNK2 contributes to malignancy remains far from complete. Nevertheless, based

on its capacity to interfere with caspase action to promote cell survival, it can be envisaged that CSNK2 inhibitors could neutralize the survival advantage of cancer cells. In a similar respect, as CSNK2 is also involved in the DDR and DNA repair pathways, it is anticipated that inhibition of CSNK2 could enhance the vulnerability of cancer cells to interventions that target these processes. To this point, two distinct CSNK2 inhibitors, namely CX-4945 and CIGB-300, have advanced to clinical trials. However, judging from the published literature, patent databases, and abstracts from meetings such as the Annual Meeting of the American Association for Cancer Research, it is apparent that many additional CSNK2 inhibitors may be on the horizon (29). It is also pertinent to consider how the emergence of systematic proteomic and phosphoproteomic workflows promise to guide clinical applications of CSNK2 inhibitors by revealing prognostic signatures for the identification of malignancies that may be suitable for CSNK2-targeted intervention, biomarkers for monitoring inhibitor action, and adaptive responses that are elicited in response to CSNK2 inhibitors. Overall, despite concerns that its ubiquitous expression and widespread roles in biological processes would render CSNK2 unsuitable as a target for intervention, a striking number of preclinical studies as well as initial reports from clinical trials show very promising results. Looking to the future, it can be imagined that CSNK2 inhibitors could be an important addition to the arsenal of targeted agents that are exploited to neutralize the advantages of cancer cells, deployed to enhance their vulnerabilities, or used in combinations with other drugs to overcome or anticipate resistance that continues to limit the utility of many current therapeutic regimens.

## Authors' Contributions

**Conception and design:** A.J. Rabalski, L. Gyenis, D.W. Litchfield  
**Writing, review, and/or revision of the manuscript:** A.J. Rabalski, L. Gyenis, D.W. Litchfield  
**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** L. Gyenis  
**Study supervision:** D.W. Litchfield  
**Other (figure creation):** L. Gyenis

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