Sulforaphane-Induced G2/M Phase Cell Cycle Arrest Involves Checkpoint Kinase 2 Mediated Phosphorylation of Cdc25C*

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Running Title: Activation of Chk2 in SFN-induced G2/M arrest

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SUMMARY

Previously, we showed that sulforaphane (SFN), a naturally occurring cancer chemopreventive agent, effectively inhibits proliferation of PC-3 human prostate cancer cells by causing caspase-9 and -8 mediated apoptosis. Here, we demonstrate that SFN treatment causes an irreversible arrest in G2/M phase of the cell cycle. Cell cycle arrest induced by SFN was associated with a significant decrease in protein levels of cyclin B1, Cdc25B and Cdc25C leading to accumulation of Tyr15 phosphorylated (inactive) cyclin-dependent kinase 1 (Cdk1). SFN-induced decline in Cdc25C protein level was blocked in the presence of proteasome inhibitor lactacystin, but lactacystin did not confer protection against cell cycle arrest. Interestingly, SFN treatment also resulted in a rapid and sustained phosphorylation of Cdc25C at Ser216 leading to its translocation from nucleus to the cytoplasm due to increased binding with 14-3-3β. Increased Ser216 phosphorylation of Cdc25C upon treatment with SFN was due to activation of checkpoint kinase 2 (Chk2), which was associated with Ser1981 phosphorylation of ataxia telangiectasia-mutated (ATM), generation of reactive oxygen species, and Ser139 phosphorylation of histone H2A.X, a sensitive marker for the presence of DNA double-strand breaks. Transient transfection of PC-3 cells with Chk2-specific small interfering RNA (siRNA) duplexes significantly attenuated SFN-induced G2/M arrest. HCT116 human colon cancer derived Chk2−/− cells were significantly more resistant to G2/M arrest by SFN compared with the wild type HCT116 cells. These findings indicate that Chk2-mediated phosphorylation of Cdc25C plays a major role in irreversible G2/M arrest by SFN. Activation of Chk2 in response to DNA damage is well documented, but the present study is the first published report to link Chk2 activation to cell cycle arrest by an ITC.
Epidemiological studies have revealed an inverse correlation between dietary intake of cruciferous vegetables and the risk for certain types of cancers including prostate cancer (1-5). Laboratory studies indicate that anticancer effect of cruciferous vegetables is due to isothiocyanates (ITCs) that exist as thioglucoside conjugates (glucosinolates) in a variety of edible plants including broccoli, cabbage, watercress and so forth (6-9). Cruciferous vegetable-derived organic ITCs are generated by hydrolytic cleavage of corresponding glucosinolates through catalytic mediation of myrosinases, which are released when the plant cells are damaged due to cutting or chewing (6-9). Sulforaphane (SFN) is one such ITC analogue that has received a great deal of attention not only because it is present in high concentrations in certain varieties of broccoli but also because of its potent anticancer activity (10-15). For example, oral administration of SFN [1-isothiocyanato-4-(methylsulfinyl)butane; CH$_3$-SO-(CH$_2$)$_4$-N=C=S] caused a statistically significant reduction in 9,10-dimethyl-1,2-benzanthracene-induced mammary tumor incidence and multiplicity in rats (11). SFN was shown to offer impressive and prolonged protection of human retinal pigment epithelial cells, keratinocytes and mouse leukemia cells against oxidative damage (13). Anti-oxidative effect of SFN was also observed in aortic smooth muscle cells from spontaneously hypersensitive rats (14). Significantly, SFN exhibited bactericidal activity against clinical isolates and antibiotic resistant strains of Helicobacter pylori, and inhibited benzo[a]pyrene-induced forestomach cancer in mice (15). Moreover, SFN was effective in eradicating Helicobacter pylori in human gastric xenografts implanted in nude mice (16). Mutagenicity of food-derived heterocyclic amines was inhibited by a SFN analogue in Ames Salmonella/reversion assay (17). SFN as well as its N-acetylcysteine conjugate administered during post-initiation period effectively reduced azoxymethane-induced
colonic aberrant crypt foci formation in rats (18). Modulation of carcinogen metabolism due to inhibition of cytochrome P450 dependent monooxygenases and/or induction of Phase II detoxification enzymes, such as glutathione transferases, is believed to be responsible for activity of SFN against chemically induced cancers (19-23).

Evidence is accumulating to indicate that SFN can inhibit proliferation of cancer cells in culture by causing apoptosis and/or cell cycle arrest (24-28). Growth suppressive effect of SFN has been observed against HT29 and LS-174 human colon cancer cells (24,25), PC-3 and LNCaP human prostate cancer cells (26,28), and Jurkat T-leukemia cells (27). Apoptosis induction by SFN in Jurkat T-leukemia cells (27) and HT29 human colon cancer cells (24) correlated with overexpression of Bax and/or down-regulation of Bcl-2. Recent studies from our laboratory have indicated involvement of both caspase-8 and caspase-9 pathways in apoptosis induction by SFN in PC-3 cells (28). In addition, we showed that growth of PC-3 xenograft in vivo is inhibited significantly upon oral administration of SFN at a concentration that may be generated through dietary intake of cruciferous vegetables (28). While considerable progress has been made toward our understanding of the mechanism of SFN-induced apoptosis, the sequence of events leading to cell cycle arrest in SFN-treated cells is poorly defined. For example, previous studies have shown that SFN treated HT-29 colon cancer cells and Jurkat T-leukemia cells are arrested in G2/M phase (24,27), which may be of importance in anti-carcinogenic effect of SFN, but no attempts were made to define the mechanism of the cell cycle arrest.

In the present study, we demonstrate that SFN treatment causes an irreversible G2/M phase cell cycle arrest in PC-3 cells that is associated with a marked decrease in the expression of key G2/M regulating proteins, including cyclin B1, cell division cycle 25B (Cdc25B) and Cdc25C. In addition, we provide evidence to indicate that cell cycle arrest in SFN treated PC-3
cells is caused by generation of reactive oxygen species and ataxia telangiectasia-mutated (ATM)/checkpoint kinase 2 (Chk2)-mediated phosphorylation of Cdc25C at Ser216. Phosphorylation of Cdc25C in SFN treated cells leads to its retention in the cytosol through increased binding with 14-3-3β. Although activation of ATM/Chk2 in response to DNA damage by ionizing radiation, UV light or interference with DNA replication is well documented (reviewed in Ref. 29), the present study is the first published report to link ATM/Chk2 with cell cycle arrest by an ITC class of dietary cancer chemopreventive agent.

**EXPERIMENTAL PROCEDURES**

**Reagents**- SFN (>99% pure) was purchased from LKT Laboratories (St. Paul, MN). F-12K Nutrient Mixture, penicillin/streptomycin antibiotic mixture, and serum were from GIBCO (Grand Island, NY), propidium iodide and 4′,6-diamidino-2-phenylindole (DAPI) were from Sigma (St. Louis, MO), RNaseA was from Promega (Madison, WI), lactacystin was from Calbiochem Biosciences (La Jolla, CA), λ-protein phosphatase was from New England Biolabs (Beverly, MA), and the reagents for electrophoresis were from Bio-Rad (Richmond, CA). Antibodies against Cdk1, Cdc25C, ubiquitin, Phospho-Cdc25C (Ser216), Chk2, phospho-Chk2 (Thr68), phospho-Chk1 (Ser345), ATM, α-tubulin, α-Ran and 14-3-3β were from Santa Cruz Biotechnology (Santa Cruz, CA), antibody against phospho-ATM (Ser1981) was from Rockland Immunochemicals (Gilbertsville, PA), antibodies against cyclin B1 and actin were from Oncogene Research Products (Boston, MA), antibody against phospho-H2A.X (Ser139) was from Upstate (Charlottesville, VA), antibody against Chk1 was from Cell Signaling Technology (Beverly, MA), antibody against Cdc25B was from BD PharMingen (San Diego, CA), and antibody against phospho-Cdk1 (Tyr15) was from Sigma (St. Louis, MO).
Cell Culture and Cell Cycle Analysis- Monolayer cultures of PC-3 cells were maintained in F-12K Nutrient Mixture (Kaighn’s Modification) supplemented with 7% (v/v) non-heat inactivated fetal bovine serum and antibiotics in a humidified atmosphere of 95% air and 5% CO₂. Other cell lines including 293 and HCT116-derived Chk2⁻/⁻ and Chk2⁺/+ were maintained in DMEM supplemented with 10% fetal calf serum and antibiotics. The effect of SFN on cell cycle distribution was determined by flow cytometry following staining of the cells with propidium iodide. Briefly, 5 x 10⁵ cells were seeded and allowed to attach overnight. The medium was replaced with fresh complete medium containing desired concentration of SFN. Stock solution of SFN was prepared in DMSO, and an equal volume of DMSO (final concentration 0.02%) was added to controls. After 24 h of incubation at 37⁰C, floating and adherent cells were collected, washed with phosphate buffered saline (PBS), and fixed with 70% ethanol. The cells were then treated with 80 μg/ml RNaseA and 50 μg/ml propidium iodide for 30 min. The stained cells were analyzed using a Coulter Epics XL Flow Cytometer. The Chk2⁺/+ and Chk2⁻⁻ HCT116 cells were treated with 20 μM SFN or DMSO for 48 h prior to analysis of cell cycle distribution.

Western Blotting- After treatment with DMSO (control) or desired concentration of SFN for specified time interval, floating and attached cells were collected and lysed as described by us previously (28). Cell lysate was cleared by centrifugation at 14,000 rpm for 15 min. Lysate proteins were resolved by sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto PVDF membrane. The membrane was incubated in Tris buffered saline containing 0.05% Tween-20 and 5% (w/v) non-fat dry milk, and then exposed to desired primary antibody for 1 h at room temperature. Following treatment with appropriate secondary antibody, the immunoreactive bands were visualized using enhanced chemiluminescence method.
**Lactacystin Treatment**- Cells $(10^6)$ were plated, allowed to attach overnight, and treated with either DMSO (control) or $5 \mu M$ lactacystin for 2 h at $37^\circ C$. The cells were then exposed to $20 \mu M$ SFN for an additional 24 h. Cell lysates were prepared and subjected to immunoblotting for Cdc25C or ubiquitin. In a separate experiment, cells treated with DMSO (control), SFN alone, and lactacystin plus SFN were processed for determination of cell cycle distribution as described above.

**Immunoprecipitation**- PC-3 cells were treated with DMSO or $20 \mu M$ SFN for 4 and 24 h, washed twice with ice-cold PBS, and lysed as described above. Aliquots containing 200 µg of lysate protein were incubated overnight at $4^\circ C$ with 10 µg of anti-14-3-3β antibody. Protein A-agarose (50 µl, Santa Cruz Biotechnology) was subsequently added to each sample, and the incubation was continued for an additional 3 h at $4^\circ C$ with gentle shaking. The immunoprecipitates were subjected to SDS-PAGE followed by immunoblotting using anti-Cdc25C antibody as described above.

**Microscopic Analysis for Nuclear/Cytoplasmic Distribution of Cdc25C**- PC-3 cells (2 x $10^4$) were grown on coverslips, and allowed to attach overnight. Cells were then exposed to DMSO or $20 \mu M$ SFN for 4 or 24 h at $37^\circ C$, washed with PBS, and fixed with 4% paraformaldehyde for 30 min at room temperature. After blocking for 45 min with normal goat serum, cells were treated for 1 h at room temperature with anti-Cdc25C antibody (1:200 dilution with PBS containing 1% bovine serum albumin). After washing, cells were treated with Alexa Fluor 488 secondary antibody for 1 h at room temperature and counter stained with nuclear dye SYTOX® green (Molecular Probes, Eugene, OR). Slides were mounted and examined under a fluorescence microscope (Olympus Fluoview) at 60x magnification.
Preparation of Nuclear and Cytoplasmic Fractions- Nuclear and cytoplasmic fractions from control (DMSO treated) and SFN treated (20 µM for 4 h) PC-3 cells were prepared as described previously (30). Briefly, cells were harvested by scraping and rinsed twice in ice-cold PBS. The cells were then swollen in ice-cold hypotonic lysis buffer (20 mM HEPES, pH 7.1, 5 mM KCl, 1 mM MgCl₂, 10 mM N-ethylmaleimide, 0.5 mM phenylmethyl sulfonyl fluoride (PMSF), 5 µg/ml pepstatin-A, 2 µg/ml chymostatin, 5 µg/ml leupeptin, 5 µg/ml aprotinin, 5 µg/ml antipain) for 10 min. The cells were lysed by 20 strokes in a Dounce homogenizer, and the nuclei were cleared by centrifugation (400x g, 10 min). Following this step, the supernatant (cytosolic fraction) was concentrated, and stored at -80°C. The nuclear extract was prepared using the same lysis buffer and stored at -80°C prior to Western blot analysis for Cdc25C. The blot was stripped and re-probed with α-tubulin or α-Ran antibodies to ensure equal protein loading as well as to rule out cross-contamination of cytoplasmic and nuclear fractions.

Chk2 Kinase Assay- Approximately 10⁶ cells were plated at a confluency of ~70% and exposed to SFN (20 µM, 4 h) or IR (10 Gy, 1 h). Cells were collected, and Chk2 kinase activity was measured by immunoprecipitation kinase assay using GST-Cdc25C as a substrate as described previously (31). Briefly, control (DMSO treated) and SFN treated cells were lysed in 1 x lysis buffer containing 20 mM Tris/HCl (pH 7.5) containing 150 mM NaCl, 5 mM EDTA, 0.5% NP40, 1 mM NaF, 1mM DTT, 1 mM Na-vanadate, and 1 mM leupeptin and aprotinin. Lysates were cleared, adjusted for equal protein content, and 5 µl of monoclonal anti-Chk2 antibody was added and the mixture was incubated for 2 h. Protein-A/G beads (15 µl) were added and the incubation was continued for an additional 1 h. The immune-complexes were washed three times with 1 x lysis buffer containing 500 mM NaCl and twice with 1 x lysis buffer containing 100 mM NaCl. The immune-complexes were suspended in 25 µl kinase buffer (25
mM HEPES, pH 7.5, containing 50 mM NaCl, 5 mM MnCl₂, 0.5 mM EDTA, 5 mM DTT, 2.5 mM PMSF) and incubated with 0.5 μg of GST-Cdc25C in the presence of 50 μCi of γ-²³²P-ATP and 10 μM cold ATP. Reaction mixtures were incubated at 30°C for 25 min and the reaction was terminated by the addition of an equal volume of sample buffer. The proteins were resolved by SDS-PAGE, transferred onto Immobilon-P membrane, and the radiolabeled proteins were visualized by autoradiography. Following autoradiography, the membrane was probed with anti-Chk2 antibody to confirm equal protein loading. To determine if SFN interacts with Chk2 to affect its kinase activity, SFN (5 or 20 μM) was directly added to the kinase reaction mixture containing Chk2 immunoprecipitated from control or IR exposed cells.

**siRNA Transfection-** RNA interference of Chk2 was performed using a 21 base pair (including a 2-deoxynucleotide overhang) siRNA duplexes purchased from Dharmacon (Lafayette, CO). The coding strand for Chk2 siRNA was GAACCUGAGGACCAAGAACdTdT. For transfection, PC-3 cells were seeded in 6-well plates, and transfected at 30% confluency with siRNA duplexes (200 nM) using Oligofectamine (Invitrogen, Carlsbad, CA) according to the manufacturer’s recommendations. Cells treated with Oligofectamine (mock) or transfected with a control non-specific siRNA duplex VIII (Dharmacon, Catalogue # D-001206-08-20; ACUCUAUCUGCACGCUGACuu) were used as controls for direct comparison. After 24 h of transfection, cells were treated with DMSO or SFN (20 μM) for 24 h. Both floating and adherent cells were collected, washed with PBS and processed for analysis of cell cycle distribution or immunoblotting.

**Measurement of Reactive Oxygen Species (ROS)-** Generation of intracellular ROS was examined by flow cytometry using 6-carboxy-2’,7’-dichlorodihydrofluorescein diacetate, di(acetoxymethyl ester) (H₂DCFDA; Molecular Probes, Eugene, OR). Briefly, PC-3 cells (0.5 x
10^5) were plated in T25 flasks, and allowed to attach overnight. The cells were first exposed to 20 μM SFN for 0, 30 min, 1 h or 3 h (37°C), and then treated with 5 μM H2DCFDA for 30 min at 37°C. Subsequently, the cells were collected by trypsinization, washed twice with PBS, and analyzed for DCF fluorescence using a Coulter Epics XL Flow Cytometer.

*Immunohistochemistry for Histone H2A.X Phosphorylation*- PC-3 cells (10^5) were grown on coverslips, and allowed to attach overnight. Cells were then exposed to DMSO (control) or 20 μM SFN for 1 and 2 h or 20 μM etoposide for 2 h (positive control) at 37°C. Cells were then washed with PBS, and fixed with 2% paraformaldehyde overnight at 4°C. Subsequently, the cells were permeabilized with 0.1% Triton-X100 for 15 min at room temperature, washed with PBS, and incubated with normal goat serum [1:20 in PBS containing 0.5% (w/v) bovine serum albumin and 0.15% (w/v) glycine (BSA buffer)] for 45 min at room temperature. After washing with BSA buffer, cells were treated with antibodies against phospho-H2A.X (Ser139) (2 μg/ml) for 1 h at room temperature. Cells were then washed with BSA buffer, and incubated with 2 μg/ml goat anti-mouse Alexa Fluor 568 antibody (Molecular Probes, Eugene, OR) for 1 h at room temperature and counter stained with 10 ng/ml DAPI. Slides were mounted and examined under a fluorescence microscope at 40x (objective lens) magnification.

**RESULTS**

*SFN Treatment Caused Irreversible G2/M Phase Arrest in PC-3 Cells*- We showed previously that SFN exhibits highly significant activity against proliferation of PC-3 cells in a concentration-dependent manner (28). In the present study, we used this cell line as a model to determine if the inhibition of cell proliferation is due to perturbations in the cell cycle progression. Effect of SFN on cell cycle distribution was determined using a flow cytometer.
following staining of the cells with propidium iodide, and the data are shown in Fig. 1. A 24 h exposure of PC-3 cells to 20 μM SFN resulted in a statistically significant increase in G2/M phase cells that was accompanied by a decrease in G0/G1 phase cells (Fig. 1). At 40 μM SFN concentration, the G2/M phase arrest was abolished but a significant increase in cells with sub-G0/G1 DNA content was evident indicating apoptosis induction. The sub-G0/G1 fraction was minimal in DMSO treated controls as well as in cells treated with 20 μM SFN (Fig. 1).

Cell cycle checkpoints are activated to ensure orderly and timely completion of critical events such as DNA replication and chromosome segregation. Activation of checkpoints in response to DNA damage, inhibition of DNA replication or disruption of the mitotic spindles leads to cell cycle arrest to allow time for repair of damage, but in case of severe damage cell cycle arrest leads to apoptosis. To determine if the cell cycle arrest induced by SFN was reversible, cells were first exposed to DMSO (control) or 20 μM SFN for 16 h, and then either processed for analysis of cell cycle distribution (Figs. 2 A and B) or washed and cultured in drug-free fresh complete media for an additional 24 h prior to cell cycle distribution analysis (Figs. 2C and D). Consistent with the results shown in Fig. 1, SFN treatment caused a significant increase in the fraction of cells with G2/M DNA content that was accompanied by a decrease in G0/G1 phase cells (compare panels A and B in Fig. 2). Cell cycle distribution was not altered when DMSO treated cells were cultured in fresh complete medium for an additional 24 h (compare panels A and C in Fig. 2). Interestingly, culture of SFN treated cells (16 h exposure to 20 μM SFN) in drug-free media for 24 h resulted in a significant increase in sub-G0/G1 apoptotic cells (Fig. 2D). The fraction of cells with sub-G0/G1 DNA content was minimal in cells treated with DMSO or SFN for 16 h or in DMSO treated cells that were subsequently cultured in fresh complete medium for 24 h. The increase in sub-G0/G1 fraction in SFN treated cells cultured in

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drug-free medium was not accompanied by a decrease in G2/M phase cells, instead was associated with a decline in G0/G1 phase cells (Fig. 2). Taken together, these results indicated that SFN-induced G2/M cell cycle arrest was irreversible and not a secondary effect.

Effect of SFN on Levels of Proteins That Regulate G2/M Transition- Eukaryotic cell cycle progression is regulated by sequential activation of Cdks, whose activity is dependent upon their association with regulatory cyclins (32-34). A complex between cyclin-dependent kinase 1 (Cdk1; also known as p34Cdc2) and cyclinB1 is important for entry into mitosis in most organisms (32-34). While phosphorylation of Cdk1 at Thr161 is essential for full activation of Cdk1/cyclinB1 kinase complex, reversible phosphorylations at Thr14 and Tyr15 of Cdk1 suppress its kinase activity (33,34). Dephosphorylation of Thr14 and Tyr15 of Cdk1, and hence activation of Cdk1/cyclinB1 kinase complex is catalyzed by dual specificity phosphatases Cdc25B and Cdc25C, and this reaction is believed to be a rate-limiting step for entry into mitosis (32,33,35). To gain insights into the mechanism of cell cycle arrest upon treatment with SFN, levels of cyclinB1, Cdk1, Cdc25B and Cdc25C protein were compared by immunoblotting using lysates from control and SFN treated cells, and representative blots are shown in Fig. 3A. In comparison with control, level of cyclinB1 was reduced by about 56% and 90% in SFN treated cells at 4 and 16 h time points, respectively. Interestingly, SFN-induced decline in cyclinB1 protein level was partially blocked at 24 h time point (about 67% reduction compared with the control) indicating a biphasic response, which was observed in two independent experiments. While protein level of Cdk1 was not significantly altered by SFN treatment, level of Cdc25C protein was reduced by about 41%, 75% and 83% in cells treated with SFN for 4, 16 and 24 h, respectively, compared with control cells. A marked decrease in Cdc25B protein level (>60% reduction compared with control) was also evident in SFN treated cells at 16 and 24 h time
points (Fig. 3A). These results indicated that SFN-induced G2/M phase cell cycle arrest in PC-3 cells was associated with a marked decline in the protein levels of cyclinB1, Cdc25B and Cdc25C, but not Cdk1.

**SFN Treatment Enhanced Phosphorylation of Cdk1 (Tyr15) and Cdc25C (Ser216)**

Because Cdc25B and Cdc25C, whose level was reduced markedly in SFN treated cells, play critical roles in dephosphorylation of Cdk1 (35), we hypothesized that SFN treatment might lead to accumulation of Tyr15 phosphorylated (inactive) Cdk1. We examined this possibility by immunoblotting using an antibody specific for phospho-Cdk1 (Tyr15). As shown in Figure 3B, Tyr15 phosphorylation of Cdk1 was increased by >200% in SFN treated cells at 16 and 24 h time points compared with the control.

The function of Cdc25C is negatively regulated by phosphorylation at Ser216, which creates a binding site for 14-3-3 (36,37). Binding of Cdc25C with 14-3-3 prevents nuclear localization of this dual-specificity phosphatase (36,37). Therefore, we examined the effect of SFN on Ser216 phosphorylation of Cdc25C. As can be seen Figure 3B, the level of Ser216 phosphorylated Cdc25C was significantly higher (between 150 and 300% increase over control) in SFN treated cells compared with control. Increased Ser216 phosphorylation of Cdc25C over control was evident as early as 1 h after SFN treatment and persisted for the duration of the experiment (24 h post-treatment; Fig. 3B).

Next, we determined if SFN-induced decline in Cdc25C protein level (Fig. 3A) involved ubiquitin-proteasome system because arsenic-induced G2/M phase cell cycle arrest was shown to be due to ubiquitin/proteasome-mediated degradation of Cdc25C (38). We addressed this question by determining the effect of lactacystin, a specific inhibitor of proteasome, on SFN-induced decline in Cdc25C protein as well as on cell cycle arrest. The decline in Cdc25C protein
level upon treatment with SFN was nearly completely blocked in the presence of lactacystin (Fig. 3C). The blot was probed with anti-ubiquitin antibody to determine if Cdc25C was ubiquitinated. Indeed, high molecular weight polyubiquitin conjugates were evident in the lane containing lysate from cells treated with SFN and lactacystin but not in control lysate (Fig. 3C). To our surprise, however, lactacystin treatment did not protect against SFN-induced G2/M arrest (Fig. 3D). These results indicated that, in our model, the Cdc25C protein level per se did not influence cell cycle arrest caused by SFN.

SFN Promoted Translocation of Cdc25C from Nucleus to the Cytoplasm- Because Ser216 phosphorylation of Cdc25C creates a binding site for 14-3-3 (36,37), we examined the effect of SFN on binding of Cdc25C with 14-3-3. The lysate proteins from control and SFN treated cells (20 μM for 4 or 24 h) were immunoprecipitated using anti-14-3-3β antibody and the immunocomplex was analyzed for the presence of Cdc25C by immunoblotting. As can be seen in Fig. 4A, SFN treatment resulted in increased binding of Cdc25C with 14-3-3β at 4 and 24 h time points. These results suggested that SFN treatment might lead to translocation of Cdc25C from nucleus to cytoplasm due to increased binding with 14-3-3β. We examined this possibility by immunohistochemistry, and the data are shown in Fig. 4B. Cells were treated with DMSO (control) or 20 μM SFN for 4 or 24 h, and then stained with anti-Cdc25C antibody (red) or nucleic acid binding dye SYTOX® green (green). In DMSO treated control cells, Cdc25C was localized in the cytoplasm (red staining surrounding SYTOX® green stained nuclei) as well as in the nucleus (brown-black staining in nucleus). In contrast, nuclei of the cells treated with SFN for 24 h were brightly stained with SYTOX® green indicating translocation of Cdc25C from nucleus to the cytoplasm. In agreement with the results of immunoblotting indicating a decline in Cdc25C protein level in SFN treated cells (Fig. 3A), a marked decrease in Cdc25C
immunostaining (red fluorescence around SYTOX® green stained nuclei) was observed in SFN treated cells (Fig. 4B). SFN-induced decline in Cdc25C protein level was more pronounced at 24 h than at 4 h time point after treatment.

Cytoplasmic accumulation of Cdc25C upon treatment with SFN was confirmed by biochemical fractionation of cytoplasmic and nuclear fractions from control (DMSO treated) and SFN treated (20 μM for 4 h) cells followed by immunoblotting using anti-Cdc25C antibody, and the results are shown in Fig. 4C. A 4 h time point was selected to minimize influence of SFN-induced decline in Cdc25C protein level. In DMSO treated control, the intensity of Cdc25C immunoreactive band was significantly higher in the lane corresponding to nuclear fraction than in the cytoplasmic fraction. Treatment of cells with SFN resulted in a decrease in nuclear Cdc25C signal intensity with a concomitant increase in cytoplasmic Cdc25C signal intensity (Fig. 4C). The blot was stripped and re-probed with anti-α-tubulin and anti-α-Ran antibodies to determine cross-contamination, if any, of the nuclear and cytoplasmic fractions and to ensure equal protein loading (Fig. 4C). The results confirmed that SFN treatment, indeed, promoted translocation of Cdc25C from nucleus to the cytoplasm.

**SFN Treatment Increased Thr68 Phosphorylation of Checkpoint Kinase 2 (Chk2)**

Several kinases including checkpoint kinase 1 (Chk1) and Chk2 have been implicated in Ser216 phosphorylation of Cdc25C (36, 39-41). Chk1 and Chk2 are intermediaries of DNA damage checkpoints and activated by phosphorylation on Ser345/Ser317 and Thr68, respectively (42-46). We therefore examined whether SFN treatment affects phosphorylation of Chk1 or Chk2. Representative immunoblots for total and phospho-Chk2 (Thr68) showed increased Thr68 phosphorylation of Chk2 over control that was evident as early as 1 h after SFN treatment and persisted for the duration of the experiment (Fig. 5A). The level of Chk2 protein was not affected
by SFN treatment. SFN treatment neither affected Chk1 protein level nor its phosphorylation (data not shown). The kinase activity of Chk2 was determined in the lysates prepared from control (DMSO treated) and SFN treated cells (20 μM for 4 h) using GST-Cdc25C as a substrate. As can be seen in Fig. 5B, the Chk2 kinase activity was significantly higher in SFN treated cells than in control cells. ATM is an upstream kinase implicated in phosphorylation and hence activation of Chk2 (46). Immunoblotting using an antibody specific for phospho-ATM (Ser1981) revealed increased phosphorylation of ATM in SFN treated (20 μM, 4 h) cells. Lysate from 293 cells exposed for 1 h to 10 Gy IR was used as a positive control (Fig. 5C).

To eliminate the possibility that SFN may directly interact with Chk2 protein to affect its kinase activity, we exposed 293 cells to 10 Gy IR for 1 h (to activate Chk2) prior to preparation of lysates and immunoprecipitation of Chk2 protein. SFN (5 or 20 μM) was then added to the kinase reaction mixture containing immunoprecipitated Chk2, and the kinase activity was determined using GST-Cdc25C as a substrate following a 25 min incubation in the presence of SFN. As shown in Fig. 5D, addition of SFN to the kinase reaction mixture did not have any appreciable effect on Chk2 kinase activity (Fig. 5D).

To determine if activation of Chk2 persisted even after drug removal, the cells were first treated with DMSO or 20 μM SFN for 4 h, washed with PBS, and then cultured in drug-free fresh complete medium for 0, 2, 8 or 24 h. The cells were harvested and processed for immunoblotting for phospho-Chk2 (Thr68), and the results are shown in Fig. 5E. The Thr68 phosphorylation of Chk2 was evident for up to 24 h after SFN removal.

*SFN-Induced Phosphorylation of Chk2 Is Not Specific for PC-3 Cells*- To rule out a possibility that activation of Chk2 in SFN treated PC-3 cells was a cell line-specific effect, we examined 293 cells for SFN-induced Chk2 activation. Similar to the results obtained in PC-3
cells, SFN caused activation of Chk2 at 1, 16 and 24 h time points (Fig. 5F). Additionally, SFN-induced phosphorylation of Cdc25C (Ser216) and accumulation of Tyr15 phosphorylated Cdk1 was also observed in 293 cells. While IR and UV induced phosphorylation of Chk1, SFN failed to activate Chk1 (Fig. 5F). These results clearly indicated that SFN-induced activation of Chk2 was not unique to the PC-3 cell line.

siRNA Mediated Down-regulation of Chk2 Partially Blocked SFN-induced G2/M Arrest

To experimentally verify the role of Chk2 in SFN-induced cell cycle arrest, we used siRNA technology to suppress Chk2 protein expression. As can be seen in Fig. 6A, transfection of a siRNA targeted to Chk2 suppressed Chk2 protein expression by >70% in comparison with mock control. Expression of Chk2 was not affected in cells transfected with a non-specific control siRNA (Fig. 6A). Chk2 siRNA transfected cells and control cells (mock and control siRNA transfected cells) were then treated with SFN and their cell cycle distribution was assessed after 24 h (Fig. 6B). Treatment of control siRNA transfected cells with SFN (20 μM for 24 h) resulted in about 2.2-fold increase in G2/M phase cells (Fig. 6B). Similar effect of SFN on cell cycle distribution was observed in mock-transfected cells. SFN-induced G2/M block was partially but statistically significantly attenuated in cells transfected with Chk2 siRNA (Fig. 6B).

Effect of Chk2 down regulation on SFN-induced phosphorylation of Chk2 and Cdc25C was also examined, and the results are shown in Fig. 7. The SFN-induced Thr68 phosphorylation of Chk2 was relatively more pronounced in the mock control (about 4-fold increase over DMSO control) than in Chk2 siRNA transfected cells (about 1.9-fold increase over DMSO control; Fig. 7, top panel). Likewise, Ser216 phosphorylation of Cdc25C was relatively more pronounced in mock-transfected cells (about 4-fold increase) than in the cells transfected with Chk2 siRNA (about 2-fold). Consistent with the results in Fig. 3, treatment of mock-transfected cells with
SFN resulted in about 90% decrease in Cdc25C protein level. In contrast, a decrease of only about 50% in Cdc25C protein level was observed when Chk2 siRNA transfected cells were treated with SFN (Fig. 7, bottom panel). Two important conclusions can be drawn from data in Fig. 7. First, it is clear that down-regulation of Chk2 protein level reduces SFN-induced phosphorylation of both Chk2 and Cdc25C. Furthermore, inhibition of Chk2 and Cdc25C phosphorylation seems to stabilize Cdc25C protein suggesting that Ser216 phosphorylation of Cdc25C may regulate its degradation.

Immunoblotting for phospho-Cdc25C (Fig. 7, middle panel) revealed the presence of additional bands with reduced electrophoretic mobility in the lane containing lysate protein from SFN treated cells but not control cells. The phospho-Cdc25C immunoreactive bands with reduced electrophoretic mobility were not observed if the lysate was treated with λ-protein phosphatase prior to immunoblotting (data not shown). These results indicated that the slower migrating bands were phosphorylated forms of Cdc25C.

**HCT116-derived Chk2−/− Cells Were Significantly More Resistant to SFN-induced G2/M Arrest Than Wild Type Cells** - Role of Chk2 in cell cycle arrest by SFN was further investigated using HCT116-derived Chk2−/− and Chk2+/+ human colon cancer cells. As can be seen in Fig. 8A, the G2/M blockade induced by SFN was relatively more pronounced in Chk2+/+ HCT116 cells than in the HCT116-derived Chk2−/− cells. Consistent with the results in PC-3 and 293 cells, SFN treatment (20 μM for 24 h) caused phosphorylation of Chk2 (Thr68) and accumulation of Tyr15 phosphorylated Cdk1 in Chk2+/+ cells (Fig. 8B). These observations provided additional support to the conclusion that Chk2 plays an important role in SFN-induced cell cycle arrest. Because SFN caused a significant increase in fraction of cells with G2/M DNA content in both Chk2−/− and
Chk2+/+ HCT116 cells (Fig. 8A), compensatory Chk2-independent mechanisms are likely to contribute to the cell cycle arrest in Chk2−/− HCT116 cells.

Data shown in Fig. 7 suggested that inhibition of Cdc25C phosphorylation in SFN treated cells by siRNA-based knockdown of Chk2 could stabilize Cdc25C protein. We further explored this possibility by determining the effect of SFN on level and Ser216 phosphorylation of Cdc25C in Chk2−/− and Chk2+/+ cells, and the results are shown in Fig. 8C. The decline in Cdc25C protein level upon treatment with SFN was relatively more pronounced in Chk2+/+ cells (about 89% reduction in Cdc25C protein level in SFN treated cells compared with DMSO control; compare lanes 1 and 2 in Fig. 8C, top panel) than in HCT116-derived Chk2−/− cells (about 24% reduction in SFN treated cells compared with DMSO control; compare lanes 3 and 4 in Fig. 8C, top panel). Likewise, SFN-induced Ser216 phosphorylation of Cdc25C was much higher in Chk2+/+ cells (about 7-fold increase over DMSO control; compare lanes 1 and 2 in Fig. 8C, bottom panel) than in HCT116-derived Chk2−/− cells (about 1.7-fold increase over DMSO control; compare lanes 3 and 4 in Fig. 8C, bottom panel). While these observations suggest that Ser216 phosphorylation of Cdc25C may regulate its degradation, additional studies are needed to experimentally verify this possibility. The contribution of Cdc25C protein degradation to SFN-induced cell cycle arrest is insignificant since G2/M blockade by SFN was insensitive to lactacystin treatment.

**SFN Treatment Generated ROS and Promoted Ser139 Phosphorylation of Histone H2AX**

SFN is an electrophilic molecule capable of reacting with cellular nucleophiles including glutathione (47), which raised the question whether SFN generates ROS to cause DNA damage. ROS generation in SFN treated cells was monitored by flow cytometry using H2DCFDA, which is hydrolyzed by non-specific cellular esterases and oxidized in the presence of ROS (48). As shown in Fig. 9A, SFN treatment (20 μM) resulted in a time-dependent increase in fluorescence
indicating ROS generation. Since ROS can directly cause DNA damage as well as oxidize nucleotides that can be converted to double-strand breaks during DNA replication (49,50), we examined whether SFN treatment caused DNA double-strand breaks by measuring Ser139 phosphorylation of H2A.X, which has emerged as a sensitive marker for the presence of DNA double-strand breaks (51). Phosphorylation of H2A.X in SFN treated cells was clearly evident at 2 h time point as determined by immunoblotting using phospho-specific H2A.X antibody (Fig. 9B). Immunohistochemical analysis further confirmed Ser139 phosphorylation of H2A.X in SFN treated as well as in etoposide treated (positive control) PC-3 cells (Fig. 9C). Collectively, these results indicated that SFN treatment caused ROS-mediated DNA double-strand breaks to activate ATM-Chk2.

**DISCUSSION**

We have shown previously that SFN effectively inhibits proliferation of PC-3 human prostate cancer cells by causing caspase-8 and caspase-9 mediated apoptosis, and that the growth of PC-3 xenografts in nude mice is significantly retarded upon oral administration of SFN (28). Moreover, inhibition of PC-3 xenograft growth was observed at a dose of SFN that can be generated through dietary intake of cruciferous vegetables. These results prompted us to further examine the mechanism by which SFN inhibits proliferation of cancer cells. Data presented herein indicate that SFN treated PC-3 cells are irreversibly arrested in G2/M phase of the cell cycle. Cell cycle arrest in SFN treated cells was accompanied by a marked decline in the level of cyclinB1, Cdc25B and Cdc25C. It is reasonable to postulate that SFN treatment may affect activity of Cdk1/cyclinB1 kinase not only by reducing complex formation due to a reduction in the level of cyclinB1 protein but also by causing accumulation of Thr14/Tyr15 phosphorylated...
(inactive) Cdk1 due to a decline in the level of Cdc25B and Cdc25C proteins. Indeed, Western blotting using anti-phospho-Cdk1 antibody revealed a significant increase in the level of Tyr15 phosphorylated Cdk1 in SFN treated cells. SFN-induced decline in Cdc25C protein level in PC-3 cells was nearly fully blocked in the presence of proteasome inhibitor lactacystin. Interestingly, lactacystin-mediated restoration of Cdc25C protein level did not significantly affect SFN-induced cell cycle arrest. These results suggested that, in our model, the level of Cdc25C protein does not influence G2/M arrest by SFN.

Cell cycle arrest in the presence of SFN has been demonstrated previously in other cellular models, but the results are inconsistent. For example, a net increase in the percentage of G2/M phase cells upon treatment with SFN was observed in HT29 human colon cancer cells as well as in Jurkat leukemia cells (24,27). The mechanism of G2/M arrest was not thoroughly investigated in any of the above studies, but an increase in the level of cyclinA and cyclinB protein was reported in SFN treated HT29 cells (24). In contrast, Chiao et al. (26) showed that treatment of LNCaP human prostate cancer cells with 3-50 \( \mu \)M SFN resulted in an enrichment of G1 phase cells. While the reasons for this discrepancy are not yet clear, the inconsistency could be due to differences in genetic background of the cells. For example, PC-3 cells do not require androgen for growth and lack functional p53, whereas LNCaP cells are androgen responsive and contain wild type p53. It would be of interest to determine if p53 status influences SFN-induced cell cycle arrest.

Activity of Cdc25C is negatively regulated by phosphorylation at Ser216, which creates a binding site for 14-3-3 (36,37). The binding with 14-3-3 hinders nuclear accumulation of Cdc25C, which is required for activation of Cdk1/cyclinB kinase complex in the nucleus (36,37). Therefore, phosphorylation of Cdc25C on Ser216 represents an important regulatory mechanism
by which cells delay or block mitotic entry under normal conditions as well as in response to DNA damage. In our model, SFN treatment caused an increase in Ser216 phosphorylation of Cdc25C that was evident as early as 1 h after treatment and persisted for the duration of the experiment (24 h after SFN treatment). We also observed an increase in binding of Cdc25C with 14-3-3β in SFN treated cells when compared with control (Fig. 4A). Immunohistochemistry and immunoblotting confirmed that SFN treatment promotes cytoplasmic sequestration of Cdc25C (Figs. 4B and C). Because cell cycle arrest by SFN was not significantly affected upon restoration of the Cdc25C protein level, cytoplasmic translocation of Cdc25C due to increased binding with 14-3-3β appears to be the main mechanism of cell cycle arrest by SFN in our model.

Chk1 and Chk2, which are important intermediaries of DNA damage checkpoint pathways, are implicated in Ser216 phosphorylation of Cdc25C. Chk1 and Chk2 are activated in response to DNA damage by ionizing radiation and/or UV light and by interference with DNA replication. Chk1 is activated by ATR [Ataxia telangiectasia mutated (ATM) and Rad3-related protein kinase], whereas ATM-dependent phosphorylation at Thr68 leads to activation of Chk2. The results of the present study indicate that increased Ser216 phosphorylation of Cdc25C in SFN treated cells is associated with ATM-dependent activation of Chk2. Phosphorylation of ATM (Ser1981) and Chk2 (Thr68) was very low in DMSO treated control PC-3 cells but increased rapidly and dramatically upon treatment with SFN (Fig. 5). The time course for phosphorylation of Cdc25C upon treatment with SFN mirrored that of Chk2 phosphorylation (Fig. 3B and 5A). The kinase assays using immunoprecipitated Chk2 clearly indicated an increase in Chk2 kinase activity in SFN treated cells compared with the control. Moreover, transfection of PC-3 cells with Chk2 specific siRNA not only reduced
expression of Chk2 protein but also caused a marked decrease in SFN-induced phosphorylation of both Chk2 (Thr68) and Cdc25C (Ser216) (Fig. 7). SFN-induced G2/M arrest was partially but statistically significantly attenuated in Chk2 siRNA transfected cells (Fig. 6B). SFN-induced cell cycle arrest in Chk2 siRNA transfected cells was only partially blocked probably because Chk2 siRNA did not fully eliminate Chk2 protein expression. Such a finding raises another possibility that Chk2-independent mechanisms may also contribute to SFN-induced cell cycle arrest. Consistent with this possibility, SFN-induced G2/M phase arrest was observed in Chk2 null cells but significantly more so than in the wild type HCT116 cells. Nonetheless, the results of the present study clearly indicate that Chk2 protein level affects sensitivity of cells to SFN-induced G2/M arrest.

It is widely accepted that activation of checkpoints in response to DNA damage leads to cell cycle arrest but in case of severe damage the cell cycle arrest leads to apoptotic cell death. The effects of SFN are compatible with this model. SFN treatment caused generation of ROS that was associated with increased phosphorylation of H2A.X at Ser139 suggesting presence of DNA double-strand breaks. Results shown in Fig. 2 indicated a significant enrichment of G2/M phase cells upon treatment with SFN with very little cells in the sub-G0/G1 phase (apoptotic cells). The induction of cell cycle arrest in the absence of cell death clearly shows that cell cycle arrest is not a secondary event rather it leads to apoptosis since culture of SFN treated (G2/M arrested) cells in drug free media led to a >5-fold increase in sub-G0/G1 fraction (Fig. 2D).

SFN-induced decline in Cdc25C protein level was blocked by about 50% in Chk2 siRNA transfected cells (Fig. 7, bottom panel) in comparison with mock-transfected cells. Because Chk2 depletion also led to a reduction in SFN-induced phosphorylation of Cdc25C, it is possible that Ser216 phosphorylation of Cdc25C regulates its degradation. Results in HCT116-derived Chk2−/−
cells also support this possibility since SFN-induced decline in Cdc25C protein level was relatively more severe in Chk2+/+ than in HCT116-derived Chk2−/− cells (Fig. 8C). It is important to mention that Chk1 dependent phosphorylation of Cdc25A has been shown to regulate its stability (52). Specifically, these investigators showed that loss of Chk1 resulted in the accumulation of a hypophosphorylated form of Cdc25A protein, and Chk1-deficient cells failed to degrade Cdc25A after ionizing radiation treatment (52).

A fundamental question, which remains unanswered, is how SFN treatment causes DNA damage to activate ATM/Chk2. One possibility is that SFN directly reacts with the nucleophilic sites in DNA to cause damage, which is probable because SFN is a highly electrophilic molecule capable of reacting with nucleophiles such as GSH (47). Alternatively, SFN treatment may cause transient oxidative stress and subsequent DNA damage due to its reaction with cellular antioxidant GSH. Even though further studies are needed to systematically explore the above mentioned possibilities, data presented in this paper demonstrate an increase in ROS upon treatment of PC-3 cells with SFN.

In conclusion, the results of the present study indicate that SFN treated PC-3 cells are irreversibly arrested in G2/M phase due to ROS mediated activation of ATM/Chk2 leading to Ser216 phosphorylation and cytoplasmic sequestration of Cdc25C.

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REFERENCES


FOOTNOTES

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1The Abbreviations used are: ITCs, isothiocyanates; SFN, sulforaphane; Cdk1, cyclin-dependent kinase1; Cdc25B, cell division cycle 25B; Cdc25C, cell division cycle 25C; Chk, checkpoint kinase; PBS, phosphate buffered saline; SDS-PAGE, sodium dodecyl-sulfate polyacrylamide gel electrophoresis; ROS, reactive oxygen species; H2DCFDA, 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate, di(acetoxymethyl ester); ATM, ataxia telangiectasia-mutated; ATR, ATM- and Rad3-related; BSA buffer, PBS containing 0.5% (w/v) bovine serum albumin and 0.15% (w/v) glycine.
FIGURE LEGENDS

Fig. 1. **Effect of sulforaphane (SFN) on cell cycle distribution in PC-3 cells.** PC-3 cells were treated with DMSO (control) or SFN (20 or 40 μM) for 24 h. Both floating and attached cells were collected, and processed for analysis of cell cycle distribution as described under “Experimental Procedures”. Representative histograms for cell cycle distribution in control and SFN treated cells are shown. Data in the table are mean ± SE (n=3). *Significantly different compared with DMSO treated control, \( P < 0.05 \) by Student’s \( t \)-test.

Fig. 2. **Sulforaphane (SFN)-induced G2/M arrest in PC-3 cells is irreversible.** PC-3 cells were treated with DMSO (control) or 20 μM SFN for 16 h. Subsequently the cells were either processed for analysis of cell cycle distribution (panel A and B) or cultured in drug-free fresh complete medium for an additional 24 h prior to analysis of cell cycle distribution (panels C and D). Data in the table are mean ± SE (n=3). *Significantly different at \( P < 0.05 \) by one-way ANOVA followed by Bonferroni’s Multiple Comparison Test.

Fig. 3. **Effects of sulforaphane (SFN) on levels and phosphorylation of proteins involved in the regulation of G2/M transition.** **A.** Immunoblotting for cyclinB1, Cdk1, Cdc25B and Cdc25C using lysates from control and SFN treated PC-3 cells. PC-3 cells were treated with 20 μM SFN for the indicated times. Both floating and attached cells were collected and used for preparation of cell lysate. Blots were stripped and re-probed with anti-actin antibody to ensure equal protein loading. Immunoblotting for each protein was performed two or more times using independently prepared lysates, and the results were comparable. **B.** Immunoblotting for phospho-Cdk1 (Tyr15) and phospho-Cdc25C (Ser216) using lysates from control and SFN treated PC-3 cells. Immunoblotting for each protein was performed two or more times using independently prepared lysates, and the results were comparable. Blots were stripped...
and re-probed with anti-actin antibody to ensure equal protein loading. C, Effect of proteasome inhibitor lactacystin on SFN-induced decline in Cdc25C protein level. PC-3 cells were treated with DMSO or 20 μM SFN in the absence or presence of 5 μM lactacystin. Cell lysates were prepared and subjected to immunoblotting using anti-Cdc25C antibody to determine its protein level or anti-ubiquitin antibody to determine presence of high molecular weight polyubiquitin conjugates or anti-actin antibody to ensure equal protein loading. D, Effect of lactacystin on SFN-induced cell cycle arrest. PC-3 cells were treated with DMSO (control) or SFN (20 μM) for 24 h in the absence or presence of 5 μM lactacystin prior to processing for analysis of cell cycle distribution. Data are mean ± S.E. (n= 3). *Significantly different compared with control, P < 0.05, by Student’s t-test.

Fig. 4. Effect of sulforaphane (SFN) on binding of Cdc25C with 14-3-3β, and on nuclear/cytoplasmic distribution of Cdc25C. A, Effect of SFN on binding of Cdc25C with 14-3-3β. Lysate proteins (200 μg) from control and SFN treated cells (20 μM for 4 or 24 h) were used for immunoprecipitation using anti-14-3-3β antibody followed by immunoblotting for Cdc25C. B, Immunohistochemical analysis for nuclear/cytoplasmic distribution of Cdc25C in control (DMSO treated) and SFN treated cells. Cells were treated with DMSO (control) or 20 μM SFN for 4 or 24 h, and then stained with anti-Cdc25C antibody (red) or SYTOX® green (green). In DMSO treated control cells, Cdc25C was localized in the cytoplasm (red staining surrounding SYTOX® green stained nuclei) as well as in the nucleus (brown-black staining in nuclei). The nuclei of SFN treated cells were brightly stained with SYTOX® green, especially at 24 h time point, indicating translocation of Cdc25C from nucleus to the cytoplasm. C, Immunoblotting for Cdc25C using nuclear and cytoplasmic fractions prepared from control (DMSO treated) and SFN treated cells (20 μM for 4 h). Blots were probed with anti-α-tubulin
(middle) and anti-α-Ran (lower) antibodies to normalize for equal protein loading as well as to rule out cross-contamination of the nuclear and cytoplasmic fractions.

Fig. 5. Effect of sulforaphane (SFN) on ATM and Chk2 activation in PC-3 and/or 293 cells. A, Immunoblotting for effect of SFN on protein level and Thr68 phosphorylation of Chk2. PC-3 cells were cultured in the presence of 20 μM SFN for the indicated time periods. The blots were stripped and re-probed with anti-actin antibody to ensure equal protein loading. B, Effect of SFN on Chk2 kinase activity. PC-3 cells were treated with DMSO (control) or 20 μM SFN for 4 h. Chk2 was immunoprecipitated from the lysates of control and SFN treated cells, and the kinase activity was determined using GST-Cdc25C as a substrate. The membrane was probed with anti-Chk2 antibody to ensure equal protein loading. C, Effect of SFN on Ser1981 phosphorylation of ATM. Lysates from PC-3 cells treated with DMSO or SFN (20 μM for 4 h) were subjected to immunoblotting using an antibody specific for phospho-ATM (Ser1981). Lysate from 293 cells exposed for 1 h to 10 Gy IR was included as a positive control. Equal protein loading was confirmed by re-probing the membrane with anti-ATM antibody (lower panel). D, Chk2 kinase activity following addition of SFN directly to the kinase reaction mixture. 293 cells were exposed to 10 Gy IR for 1 h prior to preparation of cell lysate and immunoprecipitation using anti-Chk2 antibody. SFN (5 or 20 μM) was directly added to the kinase reaction mixture containing immunoprecipitated Chk2, and the incubation was carried out for 25 min. Chk2 kinase activity was determined using GST-Cdc25C as a substrate. The membrane was probed with anti-Chk2 antibody to ensure equal protein loading. E, SFN-induced activation of Chk2 persists even after drug removal. PC-3 cells were treated with DMSO or 20 μM SFN for 4 h, washed and cultured in drug-free fresh complete medium for 0, 2, 8 or 24 h prior to harvesting. Cell lysates were prepared and subjected to immunoblotting using phospho-
Chk2 (Thr68) antibody. Blot was stripped and re-probed with anti-actin antibody to confirm equal protein loading. *F*, Effect of SFN on phosphorylation of Chk2 (Thr68), Chk1 (Ser345), Cdc25C (Ser216) and Cdk1 (Tyr15) in 293 cells. 293 cells were treated with 20 μM SFN for the indicated time periods. Lysates from 293 cells exposed to IR or UV were used as positive controls. The blot was probed with anti-Cdk1 antibody to ensure equal protein loading.

Fig. 6. **Effect of siRNA-based depletion of Chk2 protein on sulforaphane (SFN)-induced G2/M arrest.** *A*, Immunoblotting for Chk2 using lysates from cells transfected with Chk2 targeting siRNA or control transfected cells (mock or control siRNA). Blots were stripped and re-probed with anti-actin antibody to ensure equal protein loading. *B*, Representative histograms for effect of SFN treatment (20 μM for 24 h) on cell cycle distribution in PC-3 cells transfected with Chk2 specific siRNA and in control transfectants (mock and control siRNA transfected cells). The fraction of G2/M phase cells following treatment with SFN was significantly higher in control transfectants (mock and control siRNA transfected) than in the cells transfected with Chk2 targeting siRNA (*P* < 0.05 by Student’s *t*-test).

Fig. 7. **Effect of siRNA-based Chk2 protein depletion on sulforaphane (SFN)-induced phosphorylation of Chk2 (Thr68) and Cdc25C (Ser216).** Control (mock transfected) and Chk2 siRNA transfected PC-3 cells were treated with DMSO or 20 μM SFN for 24 h, harvested, and processed for immunoblotting using antibodies against phospho-Chk2 (Thr68), phospho-Cdc25C (Ser216) and Cdc25C. Blots were stripped and re-probed with antibodies against actin to ensure equal protein loading. Experiment was repeated twice using independently prepared lysates, and the results were comparable.

Fig. 8. **Effect of sulforaphane (SFN) on cell cycle distribution, and phosphorylation of Chk2 (Thr68) and Cdc25C (Ser216) in HCT116-derived Chk2⁻/⁻ and Chk2⁺/⁺ cells.** *A*, The
Chk2<sup>−/−</sup> (empty bars) and Chk2<sup>+/+</sup> (solid bars) cells were treated with DMSO (control) or 20 μM SFN for 48 h prior to analysis of cell cycle distribution. Data are mean ± SE (n=3). *The fraction of G<sub>2</sub>/M phase cells was statistically significantly different between control and SFN treatment groups for both Chk2<sup>−/−</sup> and Chk2<sup>+/+</sup> cells, and between SFN treated Chk2<sup>−/−</sup> and Chk2<sup>+/+</sup> cells by one-way ANOVA (P < 0.05) followed by Bonferroni’s Multiple Comparison Test. 

**B,** Effect of SFN on phosphorylation of Chk2 (Thr68) and Cdk1 (Tyr15) in Chk2<sup>−/−</sup> and Chk2<sup>+/+</sup> cells. Lysates from control (DMSO treated) and SFN treated Chk2<sup>−/−</sup> and Chk2<sup>+/+</sup> HCT116 cells were subjected to immunoblotting using antibodies specific for phospho-Chk2 (top) or phospho-Cdk1 (middle). The blot was probed with anti-Cdk1 antibody (bottom) to confirm equal protein loading. Lane 1, lysate from DMSO treated Chk2<sup>+/+</sup> cells; lane 2, lysate from SFN treated Chk2<sup>+/+</sup> cells; lane 3, lysate from DMSO treated Chk2<sup>−/−</sup> cells; and lane 4, lysate from SFN treated Chk2<sup>−/−</sup> cells.

**C,** Effect of SFN on Ser216 phosphorylation and protein level of Cdc25C in Chk2<sup>−/−</sup> and Chk2<sup>+/+</sup> cells. Lysates from control (DMSO treated) and SFN treated Chk2<sup>−/−</sup> and Chk2<sup>+/+</sup> HCT116 cells were subjected to immunoblotting using antibodies against phospho-Cdc25C (Ser216) and total Cdc25C. The blots were probed with anti-actin antibody to ensure equal protein loading. Lane 1, lysate from DMSO treated Chk2<sup>+/+</sup> cells; lane 2, lysate from SFN treated Chk2<sup>+/+</sup> cells; lane 3, lysate from DMSO treated Chk2<sup>−/−</sup> cells; and lane 4, lysate from SFN treated Chk2<sup>−/−</sup> cells.

**Fig. 9. Effect of sulforaphane (SFN) on ROS generation and phosphorylation of histone H2A.X (Ser139) in PC-3 cells.** 

**A,** Flow cytometric analysis for ROS generation using H2DCFDA in PC-3 cells treated with 20 μM SFN for the indicated time periods. Note a progressive time-dependent increase in fluorescence intensity in the presence of SFN. The experiment was repeated, and the results were comparable. 

**B,** Immunoblotting for phospho-H2A.X (Ser139). PC-3 cells were cultured in the presence of 20 μM SFN for the indicated time
periods. The blot was stripped and probed with anti-actin antibody to ensure equal protein loading. Lysate from PC-3 cells treated with 20 μM etoposide for 2 h was included as a positive control (last lane). C, Immunofluorescence analysis for phospho-histone H2A.X (γ-H2A.X) in control (DMSO treated), etoposide treated (20 μM for 2 h), and SFN treated (20 μM for 1 or 2 h) PC-3 cells. Note a time-dependent increase in phospho-H2A.X (Ser139) immunostaining in SFN treated cells. The staining was negligible in control cells, but was clearly evident in etoposide treated (positive control) PC-3 cells.
Figure 1

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| 0        | 3 ± 0.3   | 56 ± 0.7 | *      | 25 ± 0.5 |*
| 20       | 4 ± 0.3   | 35 ± 0.4 | *      | 38 ± 2.0 |*
| 40       | 24 ± 0.7  | 34 ± 0.7 | 15 ± 0.3 | 25 ± 1.0 |
**Figure 2**

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*Significant differences.*

**Graphs:**
- **A** 16h DMSO
- **B** 16h SFN (20 µM)
- **C** 16h DMSO → 24h drug free
- **D** 16h SFN (20 µM) → 24h drug free

**Legend:**
- G₀/G₁
- S
- G₀/M

**Note:**
- Sub-G₀/G₁
- G₀/G₁
- S
- G₀/M

**Reference:**
- [www.jbc.org](http://www.jbc.org)
Figure 3

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C. Cdc25C

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<th>Protein</th>
<th>Ub</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cdc25C</td>
<td></td>
</tr>
<tr>
<td>Actin</td>
<td>44</td>
</tr>
</tbody>
</table>

D. % G2/M

<table>
<thead>
<tr>
<th>SFN (20 µM)</th>
<th>Lact. (5 µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* indicates significance.
Figure 4

A

IP-14-3-3β
IB-Cdc25C
KDa
Cdc25C
0 4 24
Time (Hours)

B

Control 20 µM SFN

4h

24h

C

Nuclear  Cytoplasmic
SFN (20 µM) - +  - + KDa
Cdc25C
α-Tubulin
α-Ran

55
51
30
Figure 5

A. 20 µM SFN

<table>
<thead>
<tr>
<th></th>
<th>Time (Hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-Chk2</td>
<td>0 1 4 16 24</td>
</tr>
<tr>
<td>Actin</td>
<td>0 1 4 16 24</td>
</tr>
<tr>
<td>Chk2</td>
<td>0 1 4 16 24</td>
</tr>
<tr>
<td>Actin</td>
<td>0 1 4 16 24</td>
</tr>
</tbody>
</table>

B. 20 µM SFN

<table>
<thead>
<tr>
<th></th>
<th>SFN (20 µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-Chk2</td>
<td>- + - + - +</td>
</tr>
</tbody>
</table>

C. p-ATM (Ser1981)

<table>
<thead>
<tr>
<th></th>
<th>ATM</th>
</tr>
</thead>
<tbody>
<tr>
<td>SFN (20 µM)</td>
<td>- - - +</td>
</tr>
<tr>
<td>IR (10Gy)</td>
<td>- - + +</td>
</tr>
</tbody>
</table>

D. 32P-GST-Cdc25C

<table>
<thead>
<tr>
<th></th>
<th>Chk2</th>
</tr>
</thead>
<tbody>
<tr>
<td>SFN (µM)</td>
<td>0 0 5 20</td>
</tr>
<tr>
<td>IR (10Gy)</td>
<td>- + + +</td>
</tr>
</tbody>
</table>

E. Culture time after SFN removal

<table>
<thead>
<tr>
<th></th>
<th>0h 2h 8h 24h</th>
</tr>
</thead>
<tbody>
<tr>
<td>SFN (20 µM)</td>
<td>- + - + - +</td>
</tr>
<tr>
<td>p-Chk2</td>
<td>61 61 61 61</td>
</tr>
<tr>
<td>Actin</td>
<td>44 44 44 44</td>
</tr>
</tbody>
</table>

F. 20 µM SFN

<table>
<thead>
<tr>
<th></th>
<th>0 1 16 24 IR UV</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-Chk2</td>
<td>61</td>
</tr>
<tr>
<td>p-Chk1</td>
<td>55</td>
</tr>
<tr>
<td>p-Cdc25C</td>
<td>55</td>
</tr>
<tr>
<td>p-Cdk1</td>
<td>34</td>
</tr>
<tr>
<td>Cdk1</td>
<td>34</td>
</tr>
</tbody>
</table>
Figure 6

A

<table>
<thead>
<tr>
<th></th>
<th>Mock</th>
<th>Control siRNA</th>
<th>Chk2 siRNA</th>
<th>KDa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chk2</td>
<td></td>
<td></td>
<td></td>
<td>61</td>
</tr>
<tr>
<td>Actin</td>
<td></td>
<td></td>
<td></td>
<td>44</td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th></th>
<th>DMSO</th>
<th>20 µM SFN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mock</td>
<td>G₂/M-20%</td>
<td>G₂/M-37%</td>
</tr>
<tr>
<td>Control siRNA</td>
<td>G₂/M-17%</td>
<td>G₂/M-38%</td>
</tr>
<tr>
<td>Chk2 siRNA</td>
<td>G₂/M-17%</td>
<td>G₂/M-26%</td>
</tr>
<tr>
<td>SFN (20 µM)</td>
<td>Mock</td>
<td>Chk2 siRNA</td>
</tr>
<tr>
<td>-------------</td>
<td>------</td>
<td>------------</td>
</tr>
<tr>
<td>p-Chk2</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Actin</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>p-Cdc25C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Actin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cdc25C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Actin</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 8

A

% G1/M phase

SFN concentration (µM)

B

Chk2+/+  Chk2+/+

Chk2-/−  Chk2-/−

SFN (20 µM)

p-Chk2  p-Chk2

Cdk1  Cdk1

KDa

C

Chk2+/+  Chk2+/+

Chk2-/−  Chk2-/−

SFN (20 µM)

Cdc25C  Cdc25C

Actin  Actin

p-Cdc25C  p-Cdc25C

KDa

1  2  3  4

1  2  3  4
Sulforaphane-induced G2/M phase cell cycle arrest involves checkpoint kinase 2 mediated phosphorylation of Cdc25C

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