Journal of Cellular Biochemistry 104:1181–1191 (2008)

# Involvement of the Role of Chk1 in Lithium-Induced G2/M Phase Cell Cycle Arrest in Hepatocellular Carcinoma Cells

**Xiao-Ming Wang, Jiao Li, Xiao-Cheng Feng, Qiong Wang, Dong-Yin Guan, and Zong-Hou Shen***

Department of Biochemistry and Molecular Biology, Shanghai Medical College, Fudan University, 130 Dong an Road, Shanghai 200032, China

**Abstract** Lithium, a therapeutic agent for bipolar disorder, can induce G2/M arrest in various cells, but the mechanism is unclear. In this article, we demonstrated that lithium arrested hepatocellular carcinoma cell SMMC-7721 at G2/M checkpoint by inducing the phosphorylation of cdc2 (Tyr-15). This effect was p53 independent and not concerned with the inhibition of glycogen synthase kinase-3 and inositol monophosphatase, two well-documented targets of lithium. Checkpoint kinase 1 (Chk1), a critical enzyme in DNA damage-induced G2/M arrest, was at least partially responsible for the lithium action. The lithium-induced phosphorylation of cdc2 and G2/M arrest was abrogated largely by SB218078, a potent Chk1 inhibitor, as well as by Chk1 siRNA or the over-expression of kinase dead Chk1. Furthermore, lithium-induced cdc25C phosphorylation in 7721 cells and in vitro kinase assay showed that the activity of Chk1 was enhanced after lithium treatment. Interestingly, the increase of Chk1 activity by lithium may be independent of ataxia telangiectasia mutated (ATM)/ATM and Rad3-related (ATR) kinase. This is because no elevated phosphorylation on Chk1 (Ser-317 and Ser-345) was observed after lithium treatment. Moreover, caffeine, a known ATM/ATR kinase inhibitor, relieved the phosphorylation of cdc2 (Tyr-15) by hydroxyurea, but not that by lithium. Our study's results revealed the role of Chk1 in lithium-induced G2/M arrest. Given that Chk1 has been proposed to be a novel tumor suppressor, we suggest that the effect of lithium on Chk1 and cell cycle is useful in tumor prevention and therapy. J. Cell. Biochem. 104: 1181–1191, 2008. © 2008 Wiley-Liss, Inc.

**Key words:** lithium; cell cycle arrest; Chk1; cdc2; cdc25C

Lithium has been used in the treatment of bipolar mood disorder for decades [Singh et al., 2004]. To date, several mammalian enzymes are known targets of lithium-including inositol monophosphatase (IMPase), glycogen synthase kinase-3 (GSK-3), and several phosphomonoesterases; however, the mechanisms underlying its actions remain unclear [Phiel and Klein, 2001].

Pharmacological doses of lithium cause the stabilization of bipolar disorder, developmental defects, and increased neutrophil production [Boggs and Joyce, 1983; Kao and Elinson, 1998]. Cell proliferation can be affected by lithium, either positively or negatively [Smits et al., 1999; Ohteki et al., 2000], depending on the cell types. Although the lithium-induced attenuation of cell proliferation in normal or neoplastic cells has been known for a long time [Huot et al., 1972], the possible preventive nature of lithium in human cancer development was only reported recently [Cohen et al., 1998]. This study revealed that mental patients with lithium treatment have lower cancer prevalence than the general population. A significant inverse relationship was noted between cancer development and lithium dose. Moreover, recent studies showed that the inhibition of cell proliferation by lithium is associated with the interference of cell cycle progression. Smits et al. [1999] have observed a G2/M cell cycle arrest through the inhibition of cdc2 activation in various transformed (P19

Grant sponsor: National Natural Science Foundation of China; Grant number: 30370343.

*Correspondence to: Zong-Hou Shen, Department of Biochemistry and Molecular Biology, Shanghai Medical College, Fudan University, 130 Dong-An Road, Shanghai 200032, China. E-mail: zhshen@shmu.edu.cn

Received 3 July 2007; Accepted 11 December 2007

DOI 10.1002/jcb.21693

© 2008 Wiley-Liss, Inc.

embryonal carcinoma, U2OS osteosarcoma, etc.) or immortalized (NIH3T3) cell lines after lithium treatment. The similar G2/M transition block induced by lithium is also observed in bovine aortic endothelial cells, accompanied by the stabilization of p53 [Mao et al., 2001]. Furthermore, the inhibition of the astrocytes growth by lithium is associated with G2/M arrest [Yarden et al., 2002]. These evidences suggest that G2/M checkpoint is a critical event in lithium actions of cell proliferation.

GSK-3 and IMPase are two well-documented targets of lithium in mammalian cells. Lithium can directly inhibit GSK-3 by competing with $Mg^{2+}$ [Klein and Melton, 1996; Ryves and Harwood, 2001], which leads to the signaling of the Wnt pathway through stabilization of β-catenin, and is assumed to be involved in the effects of embryonic development [Hedgepeth et al., 1997; Sinha et al., 2005]. Besides β-catenin, a number of cell cycle regulatory proteins including p53, p21, and cyclin D are also modified by GSK-3 [Diehl et al., 1998; Rossig et al., 2002; Kulikov et al., 2005]. In addition, lithium has been proposed to impede G-protein-coupled signaling cascades through the depletion of intracellular inositol by direct inhibition of IMPase [Hallcher and Sherman, 1980]. Until now, however, whether the inhibition of GSK-3 and IMPase is related to the cell cycle arrest by lithium is not known.

Cell cycle checkpoints play pivotal roles in the regulation of critical events, such as DNA replication and chromosome segregation [Sancar et al., 2004]. Cell cycle checkpoints are mainly regulated by cyclin-dependant kinases (Cdk). In response to DNA stress, p53 induced p21 expression exerts an important role in G1/S checkpoint by inactivating Cdk2. On the other hand, G2/M transition is largely dependent on cyclin B1/cdc2 (Cdk1) activity [Nyberg et al., 2002; Sancar et al., 2004]. The activity of cyclin B1/cdc2 complex is regulated immediately, at least in part, by the positive regulator cdc25C and the two negative regulators Weel and Myt1. The protein kinase Wee1 phosphorylates cdc2 on Tyr-15, and Myt1 phosphorylates cdc2 on Thr-14 and Tyr-15, leading to an inactive form of cyclin B1/cdc2 complex [Watanabe et al., 1995; Liu et al., 1997]. The dual specificity phosphatase, cdc25C, dephosphorylates cdc2 on these residues, thus leading to the activation of cyclin B/cdc2 complex [Gautier et al., 1991]. This dephosphorylation/activation is an essential step for the onset of mitosis. Cdc25C is negatively regulated by the phosphorylation of its Ser-216 residue during interphase or in response to DNA damage. The phosphorylation of this residue creates a binding site for 14-3-3 proteins, which is believed to be responsible for the nuclear export of cdc25C, and therefore induces a sustained phosphorylation on cdc2 and G2/M arrest [Peng et al., 1997; Karlsson-Rosenthal and Millar, 2006].

Two checkpoint kinases, Chk1 and Chk2, have been recently identified in humans and are shown to phosphorylate cdc25C on Ser-216. Chk1, a serine/threonine kinase, is activated by the phosphorylation on Ser-345 and Ser-317 through the DNA damage-activated ataxia telangiectasia mutated (ATM)/ATM and Rad3-related (ATR) kinases. Activated Chk1 inhibits the cdc25C by phosphorylating Ser-216 to promote its association with 14-3-3 proteins. The Chk2 kinase is also activated by ATM/ATR kinases, but its role is probably more important in DNA damage-induced apoptosis than in cell cycle arrest [Bartek and Lukas, 2003; Sancar et al., 2004]. Modifications in the G2/M checkpoint function are common in human tumors [Stewart et al., 2003]. For example, ATM mutations occur in ataxia telangiectasia, a familial disease that is associated with an elevated incidence of leukemia, lymphoma, and breast cancer [Khanna, 2000]. Mutations of Chk2 and Chk1 also arise in human cancers. Chk2 mutations are reported in human lung cancer [Liu et al., 2000], whereas Chk1 mutations are observed in human colon and endometrial cancers [Bertoni et al., 1999].

In the present study, we show that lithium inhibits hepatocellular carcinoma cell SMMC-7721 proliferation by inducing G2/M cell cycle arrest, independent of GSK-3 repression and inositol depletion. Moreover, our study's results reveal that Chk1, a critical kinase in G2/M checkpoint, at least partially accounts for the effect of lithium. This effect of lithium may be useful in tumor prevention and therapy.

## MATERIALS AND METHODS

### Reagents

Lithium chloride, etoposide, hydroxyurea, SB216763, and inositol were purchased from Sigma (St Louis, MO). Nocodazole and SB218078 were from Calbiochem (La Jolla, CA).

Antibodies against p53, p21, cdc2, cyclin B1, and cdc25C were purchased from Lab Vision (Fremont, CA). Antibodies against Chk1, phospho-cdc2 (Tyr-15), phospho-cdc25C (Ser-216), phospho-Chk1/2 Antibody kit, and Chk1 siRNA kit were from Cell Signaling Technology (Beverly, MA). Horseradish peroxidase-conjugated secondary antibodies and antibodies against β-catenin, protein A/G agarose, PCNA, and GAPDH were from Santa Cruz Biotechnology (Santa Cruz, CA). Kinase dead Chk1 plasmids (D130A) were generous gifts from Drs. Kum Kum Khanna (Post Office Royal Brisbane Hospital, Australia) and David Gillespie (Beatson Institute for Cancer Research, UK).

### Cell Culture and MTT Assay

The human hepatocellular carcinoma cell lines, SMMC-7721 (7721), HepG2, and Hep3B, obtained from the cell bank of the Chinese Academy of Science, were grown in DMEM medium containing 10% fetal bovine serum at 37°C in a 5% $CO_2$ incubator.

MTT assay: 7721 cells were incubated with various concentrations of LiCl, NaCl, and KCl for 72 h in 96-well plates. After culturing, cell proliferation was evaluated by measuring the mitochondria-dependent conversion of the tetrazolium salt, MTT (Sigma), to a colored formazan product as previously described [Ding et al., 2001].

### Cell Cycle Analysis

The distribution of cell cycle was assayed by flow cytometric DNA analysis of propidium iodide-labeled cells. Briefly, 7721 cells were plated in six-well plates and were grown to 70–80% confluence prior to treatment with LiCl or other agents for the indicated doses and time. After trypsinization, the cells were collected and fixed in ice-cold 70% ethanol at −20°C overnight. The cells were then washed twice with ice-cold PBS, resuspended in PBS containing 100 U/ml RNase A, incubated at 37°C for 30 min, stained with propidium iodide (20 μg/ml), and analyzed using a Becton Dickinson FACS cytometer (MountainView, CA). To determine the mitotic index, the cells were stained with DAPI (20 μg/ml), and the percentage of mitotic cells was counted using Olympus fluorescence microscopy (Tokyo, Japan).

### Cellular Protein Preparation and Western Blot Analysis

Whole cell extracts were prepared by lysing cells with $1 \times$ SDS lysis buffer containing 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol, 10 mM NaF, 2 mM $Na_3VO_4$, and 5 μg/ml leupeptin. Lysates were then collected by scraping and then they were cleared by centrifugation at 4°C; the supernatants were collected for further analysis. Nuclear extracts were prepared following the method described previously [Gao et al., 2005] with slight modifications. For Western blot analysis, 50 μg of proteins were loaded onto 10% SDS-polyacrylamide gels for electrophoresis and then were transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% milk for 2 h at room temperature and then were incubated overnight with primary antibodies at 4°C. Following washing and incubation with horseradish peroxidase-conjugated secondary antibody (1:1,000) for 2 h at room temperature, the membranes were washed, detected by a Pierce chemiluminescent substrate (Rockford, IL), and exposed to X-ray film.

### In Vitro Chk1 Kinase Assay

To monitor the activity of endogenous Chk1, $1 \times 10^6$ 7721 cells were untreated or treated with 2 mM HU or different doses of LiCl for 12 h. The cells were lysed in MCLB2 (50 mM Tris–HCl [pH 7.5], 2 mM DTT, 5 mM EDTA, 0.5% Nonidet P-40, 100 mM NaCl, 1 mM $Na_3VO_4$, 2 mM PMSF, 5 μg/ml leupeptin, 20 mM NaF). Clarified lysates, representing 2 mg of total cellular protein, were incubated with 2 μg of Chk1 antibody (SC-7898, Santa Cruz, CA) overnight and then with protein A/G agarose for 2 h at 4°C. The precipitates were washed twice with MCLB2 and twice with incomplete kinase buffer (50 mM Tris–HCl [pH 7.5], 1 mM DTT, 10 mM $MgCl_2$). Then 50 μl kinase reaction buffers were carried out in the presence of incomplete kinase buffers containing 10 mM ATP, 10 μCi of [γ-$^{32}$P]ATP, and 5 μg of CHKtide Substrate Peptide (12–373, Millipore, Billerica, MA). The reaction mixtures were incubated at 30°C for 25 min, and 40 μl aliquot were transferred onto the center of a 2 cm by 2 cm P81 paper. The papers were washed three times with 0.75% phosphoric acid, once with acetone, then were transferred into

scintillation vials containing 5 ml scintillation cocktail, and quantitated by liquid scintillation counting. Relative Chk1 kinase activity was measured by comparing CPM of samples to CPM of control.

## RESULTS

### Lithium Inhibits 7721 Cells Proliferation and Induces G2/M Cell Cycle Arrest

Cell proliferation can be affected by lithium, either positively or negatively [Smits et al., 1999; Ohteki et al., 2000]. To study whether lithium imposes any influence on the proliferation of hepatocellular carcinoma cells, the effect of lithium on the growth of 7721 cells was examined by MTT assay. As shown in Figure 1A, the proliferation of 7721 cells was inhibited by LiCl in a dose-dependent manner. In contrast, the same doses of NaCl or KCl did not exhibit significant inhibition, indicating specificity for the lithium and ruling out a counter-anion or a salt effect.

To further characterize the inhibitory effect of lithium on cell proliferation, we monitored the cell cycle profiles of 7721 cells by flow cytometry. As shown in Figure 1B, a 36 h exposure of 7721 cells to LiCl (0–20 mM) resulted in a dose-dependent increase of G2/M phase cells which was accompanied by a decrease in G1 phase cells. The greatest effect was observed at 20 mM LiCl, with 53% occurrence of cells in the G2/M phase compared to 10% of the control. At a 30 mM LiCl concentration, the G2/M arrested cells decreased, but a significant increase in cells with sub-G1 phase arose, indicating cell death induction. However, we failed to detect any evidences of apoptosis (data not shown), and the death of cells was possibly attributed to the cytotoxic effect of lithium.

The increase of tetraploid cells can be the result of block in G2 phase or M phase. To analyze in more detail, we examined whether lithium could inhibit cells from entering mitosis. As can be seen in Figure 1C, nocodazole served as a positive control and induced an obvious rise of mitotic cells (66%) compared with the control (10%). In contrast, there were no statistical differences in mitotic proportions between lithium-treated groups and the control. We also observed an enlarged, more spread, and flattened appearance in 7721 cells after lithium treatment (data not shown), which is consistent with the previous report [Mao

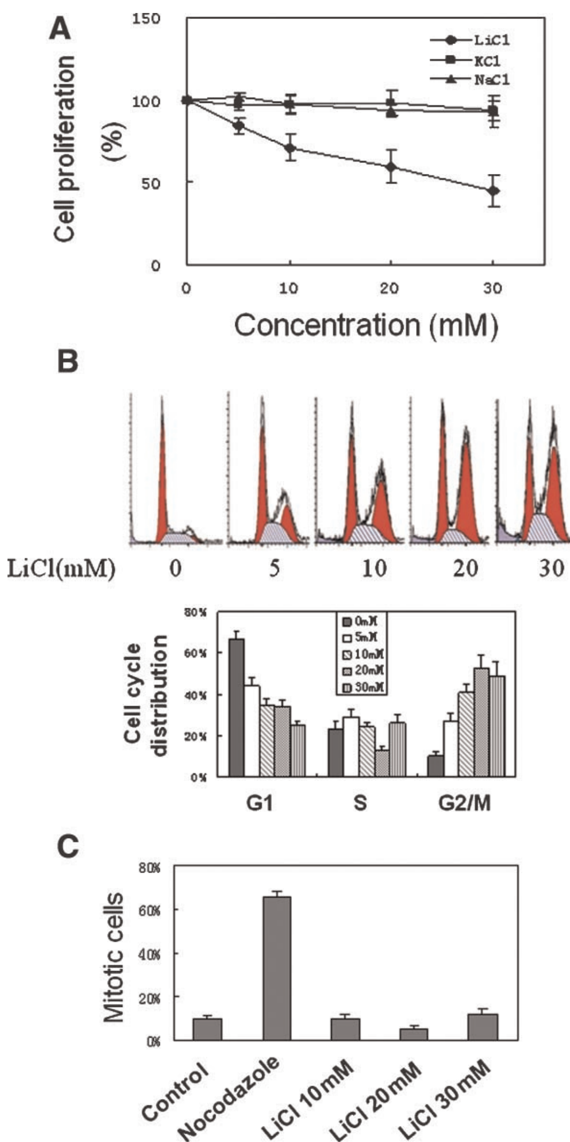


**Fig. 1.** Lithium inhibits SMMC-7721 cells proliferation and induces G2/M cell cycle arrest. **A**: MTT assay. Cells (7721) were seeded in 96-well plates and then were cultured with various concentrations of LiCl, NaCl, or KCl, respectively. After a 72 h treatment, the cells were mixed with MTT for 4 h at 37°C, and MTT activity was measured. Cell proliferation (%) was carried out by comparing samples to the control (100%). **B**: Asynchronously growing 7721 cells were exposed to various concentrations of LiCl. After 36 h, 7721 cells were harvested and fixed in cold 70% ethanol. After staining with propidium iodide, cell cycle profiles were analyzed by flow cytometry. **C**: Cells (7721) were treated with 2.5 μg/ml nocodazole or various concentrations of LiCl for 24 h, and then the cells were stained with DAPI. The mitotic cells were determined by scoring for cells with condensed chromosomes, using immunofluorescence microscopy. All experiments were performed at least three times and the data are expressed as mean ± SD. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

et al., 2001]. This indicates that cell cycle arrest occurs in the G2 phase.

Taken together, our study's results demonstrate that lithium inhibits 7721 cells proliferation by preventing G2-M transition.

### Lithium Induces G2/M Cell Cycle Arrest in a p53 Independent Pathway

It is well known that the cyclin B/cdc2 complex play a key role at G2/M checkpoint, and we hereby investigated if lithium interfered in the activation of the cyclin B/cdc2 complex. As shown in Figure 2A, an enhancement in phosphorylated cdc2 (Tyr-15) was observed with 7721 cell exposure to lithium for 24 h. This enhancement was dose-dependent and 20 mM LiCl exerted the greater effect. Additionally, an increase of cyclin B1 by lithium was also observed, which might be due to the continuous synthesis of cyclin B1 in G2 phase.

Increased p53 has been reported previously in the lithium-induced G2/M arrest [Mao et al., 2001]. Similarly, we observed an enhanced level of p53 and p21 at low concentrations of lithium (0–5 mM). Conversely, p53 and p21 were down-regulated at relatively high concentrations (10–20 mM). Moreover, the level of cdc2 phosphorylation is not consistent with the change of p53 and p21; it increased in a dose-dependent manner with lithium treatment (Fig. 2A). To further analyze whether p53 is responsible for lithium-induced G2/M phase arrest, we exposed HepG2 cells (wild p53) and Hep3B cells (null p53) to lithium. The results showed that both p-cdc2 (Tyr-15) levels and G2/M phase cells induced by lithium were not significantly different between HepG2 and Hep3B (Fig. 2B,C), indicating that p53 is dispensable for G2/M arrest induced by lithium.

Altogether, our data suggest lithium induces G2/M arrest in hepatocelluar carcinoma cells by phosphorylating cdc2 in a p53 independent pathway.

### Effect of Lithium on Cell Cycle Is Not Concerned With GSK-3 Inhibition or Inositol Depletion, but Is Associated With Chk1 Activity

Lithium has previously been verified as a potent GSK-3βinhibitor. However, in the present study, another more specific GSK-3βinhibitor, SB216763, failed to promote the phosphorylation level of cdc2 or bring about G2/M arrest like lithium, though both enhanced the level of β-catenin, a protein that can be

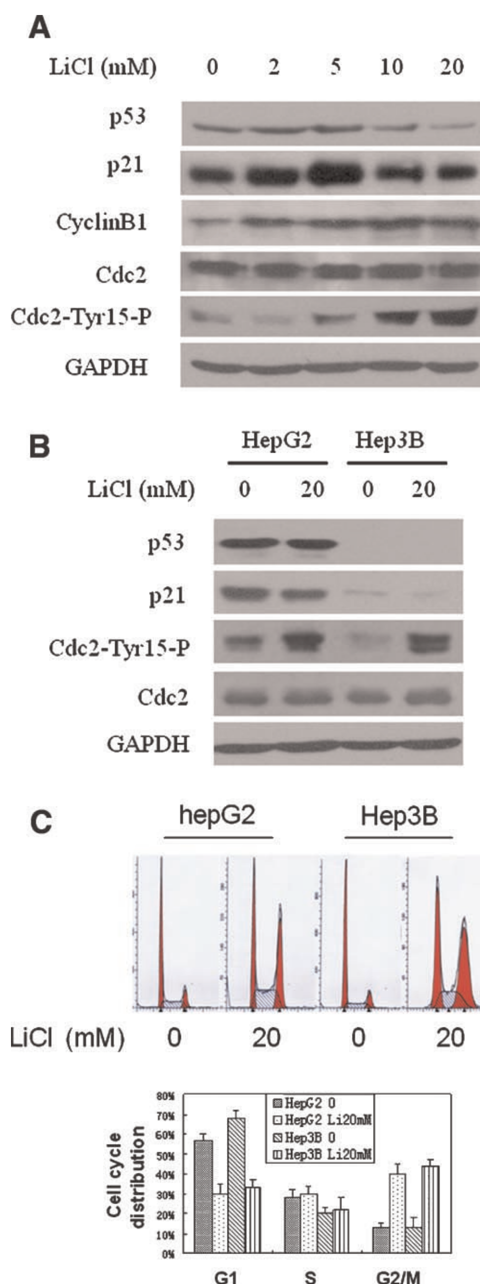


**Fig. 2.** Lithium induces G2/M cell cycle arrest independent of p53. **A**: Cells (7721) were treated with various concentrations of LiCl. **B**: HepG2 cells (wild p53) and Hep3B cells (null p53) were treated with 20 mM LiCl. After a 24 h treatment, the cells were then harvested and prepared for the lysates. Equal amounts (50 μg of protein) of lysates were subjected to 10% SDS–PAGE. After electrotransfer to a PVDF membrane, the proteins were detected by Western blot with corresponding antibodies as described in "Materials and Methods Section." **C**: HepG2 cells and Hep3B cells were treated with 20 mM LiCl. After a 36 h treatment, the cells were harvested and analyzed by flow cytometry. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

phosphorylated and down-regulated by GSK-3 [Hedgepeth et al., 1997] (Fig. 3A,B). Lithium is known also to inhibit IMPase and thereby deplete the endogenous inositol in cells, whereas the addition of inositol, the limiting factor in the inositol pathway turnover, did not weaken the ability of lithium to induce cdc2 phosphorylation or G2/M arrest (Fig. 3A,B). A critical kinase in the regulation of G2/M checkpoint, Chk1, was shown to participate in the lithium action. A potent Chk1 inhibitor, SB218078, largely abrogated the lithium effect both in cdc2 phosphorylation and G2/M arrest (Fig. 3A,B).

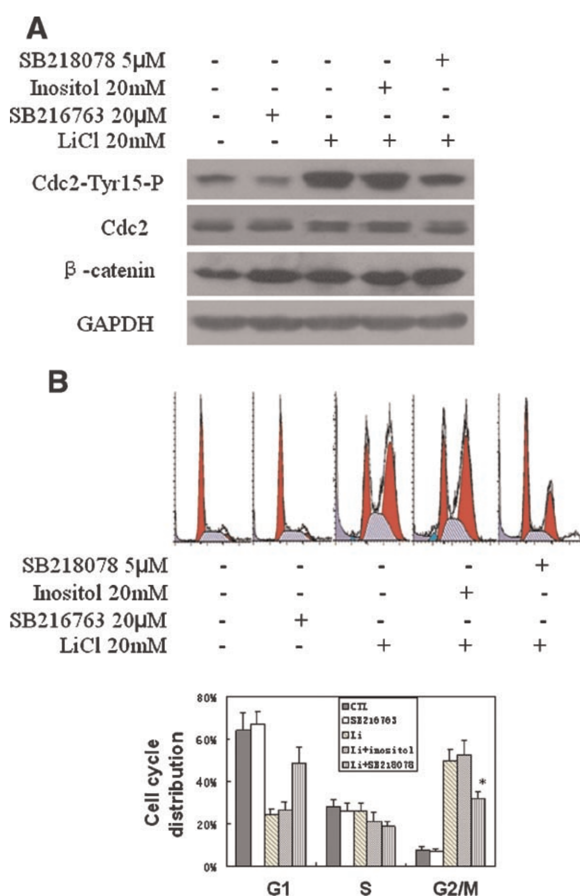


**Fig. 3.** Effect of lithium on cell cycle is partly reversed by Chk1 inhibitor, not concerned with GSK-3 inhibition or inositol depletion. Cells (7721) were treated with SB216763 (a specific GSK-3 inhibitor), LiCl, or LiCl in the presence of inositol or SB218078 (a potent Chk1 inhibitor) for 36 h. **A**: After treatment, cells lysates were prepared and corresponding proteins were detected with relevant antibodies by Western blot. **B**: Cells were harvested and fixed in cold 70% ethanol, and then cell cycle profiles were analyzed by flow cytometry. Each point represents the mean ± SD of at least three independent experiments. *$P < 0.05$ as compared with LiCl group. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Together, the inhibition of GSK-3 and the depletion of the inositol pathway were excluded, and Chk1 was suggested to be associated with the cell cycle arrest by lithium.

### Lithium Induces cdc2 Phosphorylation Through Chk1 Independent of Phosphorylation of Chk1 on Ser-317 and Ser-345

The reversible effect of SB218078 on lithium-induced G2/M arrest indicates that lithium can promote the activity of Chk1. Chk1 is activated by phosphorylation on its specific residue(s); thus, we next examined whether lithium can affect the phosphorylation status of Chk1. As shown in Figure 4A, hydroxyurea and etoposide, two different types of DNA damage agents, led to a distinct phosphorylation of Chk1 (Ser-345,Ser-317) and Chk2 (Thr-68). However, lithium hardly induced any phosphorylation on Chk1 or Chk2. Despite this, the phosphoryltion of cdc2 (Tyr-15) was increased by lithium, hydroxyurea, and etoposide.

We next investigated the effects of SB218078 and caffeine on the lithium- or hydroxyurea-induced phosphorylation of cdc2. As shown in Figure 4B, the Chk1 inhibitor SB218078 abrogated the phosphorylation of cdc2 induced by lithium and hydroxyurea, while it exhibited a stronger attenuation on cdc2 phosphorylation by lithium than that by hydroxyurea. On the other hand, caffeine, a well-known ATM/ATR inhibitor [Sarkaria et al., 1999], abrogated effectively the phosphorylation of Chk1 and cdc2 by hydroxyurea, while it hardly had any influence on lithium-induced phosphorylation of cdc2. This indicates that hydroxyurea and lithium may cause cdc2 phosphorylation through different pathways.

To summarize these results, the phosphorylation of cdc2 by lithium involves the role of Chk1, and it is interesting to note that lithium might promote Chk1 activity independent of phosphorylation on its Ser-317 and Ser-345.

### Chk1 siRNA and Kinase-Dead Chk1 Abrogates G2/M Arrest by Lithium

To further verify the role of Chk1 in lithium-induced G2/M arrest, Chk1 siRNA and kinase-dead Chk1 (D130A) were adopted in the following examination. As can be seen in Figure 5A, the down-regulation of Chk1 by Chk1 siRNA partially blocked the increase of cdc2 phosphorylation by lithium. In addition, the over-expression of kinase-dead Chk1 caused a similar

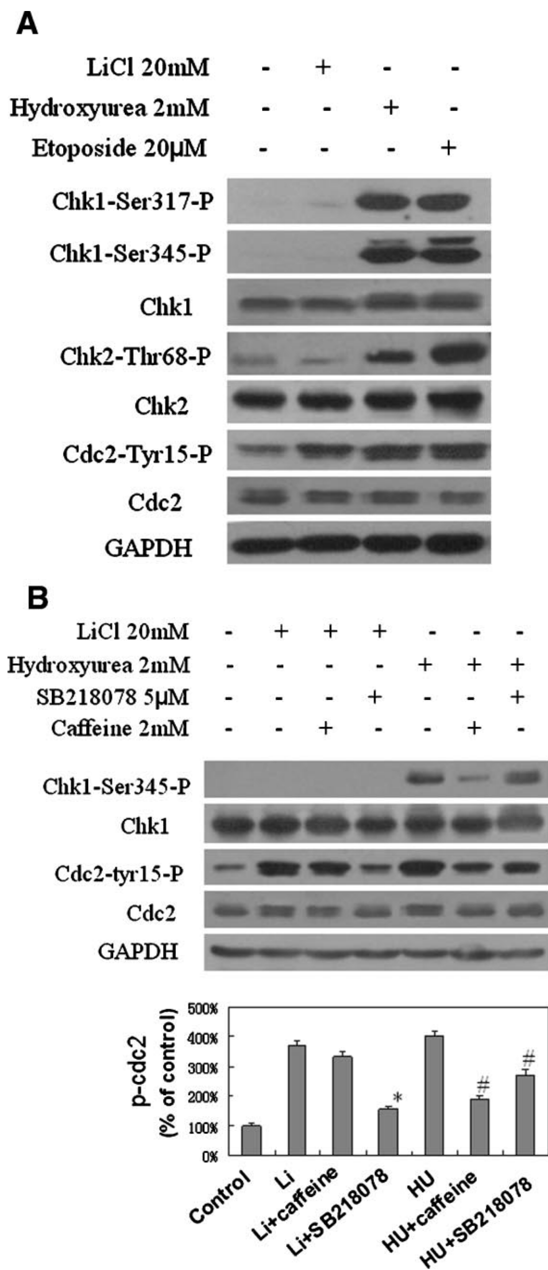


**Fig. 4.** Lithium induces cdc2 (Tyr-15) phosphorylation through Chk1 without phosphorylation of Chk1 on Ser-317 and Ser-345. **A**: Cells (7721) were treated with 20 mM LiCl, 2 mM hydroxyurea (HU), and 50 μM etoposide, then the cells were lysed after 24 h treatment and were processed for Western blot analysis. **B**: Cells (7721) were pretreated with caffeine or SB218078 for 1 h, and then were treated with 20 mM LiCl or 2 mM HU. After a 24 h treatment, cell lysates were prepared for Western blot described in ''Materials and Methods Section.'' Each point represents the mean ± SD of at least three independent experiments. *$P < 0.05$ as compared with LiCl group. $^{\#}P < 0.05$ as compared with HU group.

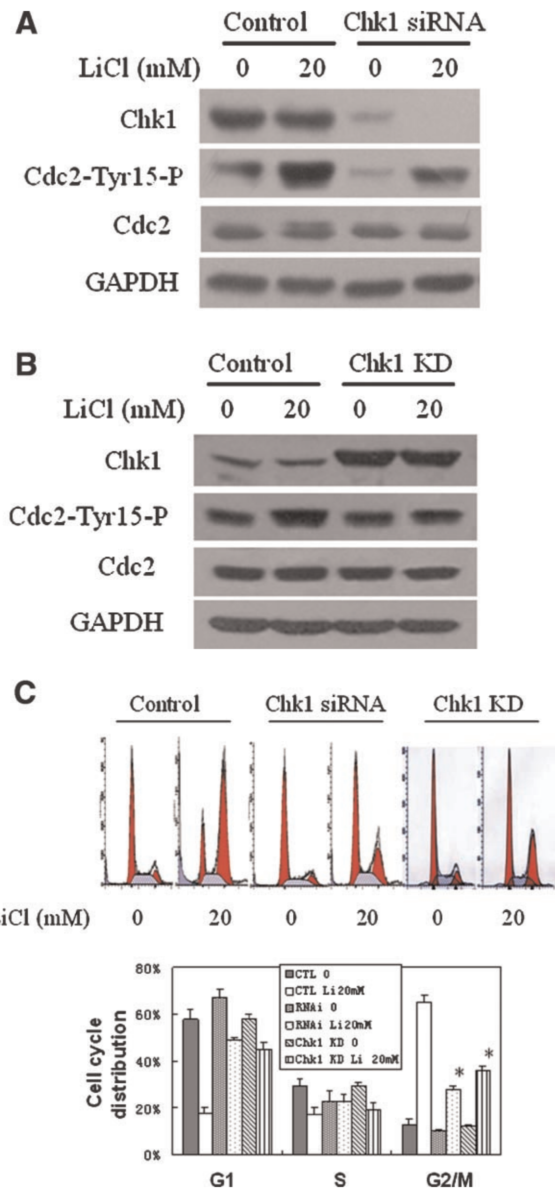


**Fig. 5.** Chk1 siRNA and kinase-dead Chk1 abrogates G2/M arrest by Lithium. **A**: Cells (7721) were transfected with Chk1 siRNA or control siRNA. **B**: Cells (7721) were transfected with pcDNA3.1-Chk1 (D130A) plasmids or a control vector. After 24 h, the cells were treated with 20 mM LiCl for additional 24 h. The cell lysates were then prepared for Western blot analysis. **C**: Cells (7721) were transfected with Chk1 siRNA or pcDNA3.1-Chk1 (D130A) plasmids. After 24 h, the cells were treated with 20 mM LiCl for 36 h and then were harvested and fixed in 70% ethanol. Cell cycle profiles were analyzed by flow cytometry. Each point represents the mean ± SD of at least three independent experiments. *$P < 0.05$ as compared with control group. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

effect, which inhibited the lithium-induced cdc2 phosphorylation (Fig. 5B). Cell cycle analysis by flow cytometry also showed that both silence of Chk1 and over-expression of kinase dead Chk1 led to a weaker G2/M arrest than that of control after lithium treatment (Fig. 5C). These results suggest that Chk1 at least is partially involved in G2/M arrest induced by lithium.

### Lithium Induces Phosphorylation of cdc25C Through Chk1

In eukaryotic cells, the phosphorylation of cdc2 (Tyr-15) is regulated by Wee1 kinase and cdc25 family phosphatases [Gautier et al., 1991; Watanabe et al., 1995; Liu et al., 1997]. Cdc25C is an important substrate for Chk1 and can be phosphorylated at Ser-216 by active Chk1. We investigated next whether any change occurred in Wee1 or cdc25C after lithium treatment. As shown in Figure 6A, no obvious change happened to the total Wee1, while total cdc25C showed a time-dependent decrease after lithium treatment. The level of phosphorylated cdc25C (Ser-216) was elevated by lithium initially (0–12 h), then it declined with the decrease of total cdc25C (Fig. 6A). We speculate that the decrease of cdc25C conceals the elevation of cdc25C phosphorylation in long-term treatment. The decreased cdc25C is not expected in transcriptive inhibition because we did not find the decrease of cdc25C mRNA level by lithium (0–20 mM, data not shown). The increase of its degradation is a possible explanation because the phosphorylation of cdc25C on Ser-216 has been shown to promote its ubiquitination and proteasomal degradations [Eymin et al., 2006]. The increase of phosphorylated cdc25C can be relieved by SB218078 and Chk1 siRNA (Fig. 6C), indicating that the phosphorylation is carried out by Chk1. Lithium treatment can also induce a decrease of nuclear cdc25C (Fig. 6B), which is accorded with the nuclear export of cdc25C after phosphorylation on its Ser-216 residue [Peng et al., 1997].

To determine whether the protein kinase activity of Chk1 was regulated by lithium, immune complex kinase assays were performed in vitro (Fig. 6D). As a positive control, the protein kinase of Chk1 isolated from HU-treated cells was approximately twofold higher than that from untreated cells. Interestingly, Chk1 isolated from lithium-treated cells was also observed to have a higher kinase activity

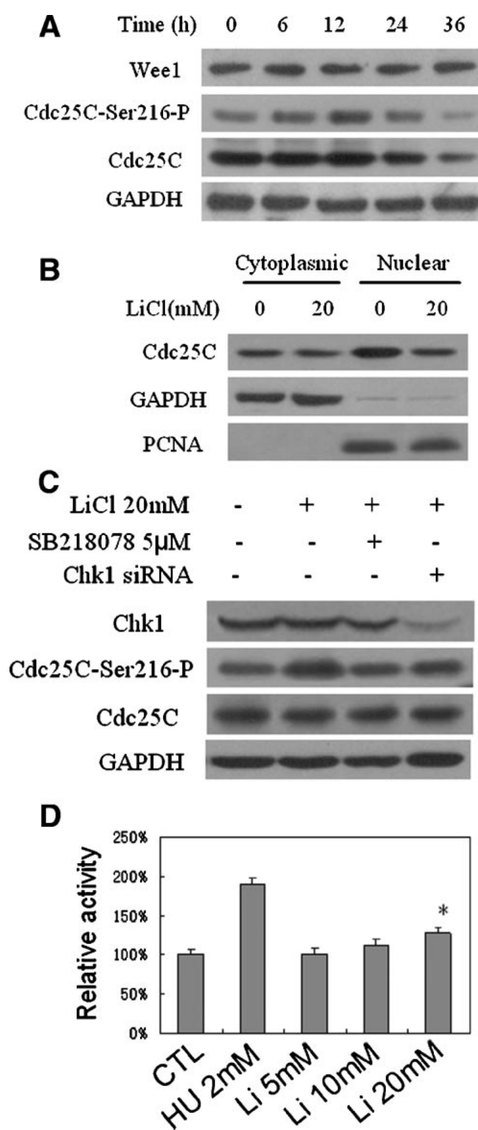


**Fig. 6.** Lithium induces phosphorylation of cdc25C (Ser-216) through Chk1. **A**: Cells (7721) were incubated for indicated time with 20 mM LiCl, and then were lysed for Western blot with corresponding antibodies. **B**: Cells (7721) were left untreated or treated with 20 mM LiCl for 12 h. After the treatment, nuclear extracts were prepared, and cdc25C was detected by Western blot. GAPDH and PCNA were used as cytoplamic and nuclear controls, respectively. **C**: Cells (7721) were pretreated with SB218078 for 2 h or Chk siRNA for 24 h and then were treated with 20 mM LiCl for additional 12 h. After treatment, cell lysates were prepared for Western blot analysis. **D**: Cells (7721) were untreated or treated with 2 mM HU or 5–20 mM LiCl for 12 h. Clarified cellular lysates were incubated with Chk1 antibodies. After precipitation with protein A/G agarose, Chk1 kinase assay was performed in vitro in the presence of 5 μg of CHKtide Substrate Peptide. Each point represents the mean ± SD of at least three independent experiments. $^{*}P < 0.05$ as compared with control group.

than that of control. The increase of Chk1 activity by lithium showed a dose-dependent manner. At a 20 mM lithium concentration, the kinase activity of Chk1 was about 1.3-fold greater than that of control.

Collectively, these results suggest that cdc25C phosphorylation induced by lithium involves the role of Chk1.

## DISCUSSION

Cell cycle checkpoints play pivotal roles in the regulation of DNA replication and chromosome segregation [Sancar et al., 2004]. It has been well established that cyclinB/cdc2 activation is an indispensable event for the entry into mitosis. Sustained phosphorylation of cdc2 (Thr-14, Tyr-15) by Wee1 and Myt1 inhibits its activity, and the dephosphorylation of these residues by cdc25C activates cdc2 kinase [Gautier et al., 1991; Watanabe et al., 1995]. In response to stalled replication or genotoxic stress, Chk1 is activated by phosphorylation at Ser-317 and Ser-345 through ATM/ATR kinase. Activated Chk1 leads to an inhibitory phosphorylation of cdc25C on Ser-216 and causes G2/M arrest [Ng et al., 2004; Karlsson-Rosenthal and Millar, 2006]. The loss of cell-cycle checkpoints is a hallmark of human cancers. Modifications in cdc2 kinase function are also common in human tumors, and mutations of Chk1 and Chk2 have been observed in many types of human cancers [Bertoni et al., 1999; Liu et al., 2000; Stewart et al., 2003].

Lithium has been shown to inhibit cell proliferation and has the quality to reduce the risk of cancer development [Cohen et al., 1998]. However, the underlying mechanism involved in this is not well explained. In the present study, we demonstrated that lithium inhibited the proliferation of human hepatocellular carcinoma 7721 cells by inducing the phosphorylation of cdc2 (Tyr-15) to create G2/M checkpoint arrest. We revealed that Chk1, a critical kinase in G2/M checkpoint, was involved in the lithium actions. Interestingly, the promotion of Chk1 activity by lithium is independent of phosphorylation on its Ser-317 and Ser-345 and is different from the well-known ATM/ATR pathway.

Lithium-induced G2/M arrest has been reported before. Smits et al. [1999] have observed a lithium-induced G2/M arrest in various carcinoma cell lines, and they have shown that the cyclin B/cdc2 activity was impaired by lithium due to the phosphorylation of cdc2 on Tyr-15, which was also confirmed by the present study. G2/M arrest has also been reported in bovine aortic endothelial cells, where the effect of lithium was shown to involve the stabilization of p53 [Mao et al., 2001]. Although p53 can affect cyclin B/cdc2 activity through inducing the expression of gadd45, 14-3-3, and p21 [Taylor and Stark, 2001], the present study revealed that p53 was down-regulated by lithium, and it did not account for the lithium-induced G2/M arrest. The apparent discrepancy between the two studies may be due to the difference of lithium dose and exposure time. Actually, we also found that lithium (5–30 mM) ultimately down-regulated p53 in long term treatment in spite of initial increase (data not shown), which is consistent with an early study on cerebellar granule cells [Chen and Chuang, 1999]. To date, two well-documented pharmacological targets of lithium are GSK-3 and IMPase [Hallcher and Sherman, 1980; Klein and Melton, 1996; Ryves and Harwood, 2001], both of which can be effectually inhibited by lithium. However, SB216763, a specific GSK-3 inhibitor, failed to mimic a similar G2/M arrest and cdc2 phosphorylation, and the addition of inositol was unable to attenuate these effects of lithium too. Therefore, GSK-3 inhibition and inositol depletion do not seem to account for our results.

In this study, we initially revealed that Chk1 is associated with lithium-induced G2/M arrest, for the effect of lithium could be largely abrogated by SB218078, a potent Chk1 inhibitor. Though lithium failed to induce phosphorylation on Chk1 (Ser-317 or Ser-345), we still obtained some evidences to support the functional role of Chk1 in the lithium-induced G2/M arrest. First, the down-regulation of Chk1 by Chk1 siRNA and the over-expression of kinase dead Chk1 relieved the G2/M arrest and cdc2 phosphorylation by lithium, which is similar to the effect of Chk1 inhibitor. Second, lithium caused an elevated phosphorylation of cdc25C on Ser-216 and an attenuation of nuclear cdc25C. Cdc25C is a well-known substrate of Chk1. Phosphoryated cdc25C at Ser-216 by active Chk1 creates a 14-3-3 binding site and promotes nuclear export of cdc25C [Peng et al., 1997]. The decrease of nuclear cdc25C leads to the phosphorylation of cdc2 on Tyr-15 and Thr-14, which ultimately causes G2/M arrest.

In the present study, the phosphorylation of cdc25C (Ser-216) could also be attenuated by both SB218078 and Chk1 siRNA, which support the role of Chk1 in the lithium action. Finally, in vitro kinase assay showed that lithium treatment can promote Chk1 kinase activity though it is relatively weaker than hydroxyurea. Based on these evidences, it seems reasonable to conclude that Chk1 is at least partly responsible for lithium-induced G2/M arrest. p38 MAP kinase is also reported to phosphorylate cdc25C on Ser-216 and causes G2/M arrest [Bulavin et al., 2001]. We did observe an enhanced phosphorylation of p38 MAP kinase (Thr-180/Tyr-182) in this study, but it was not expected to account for lithium-mediated cell cycle arrest because SB203580 (a p38 MAP kinase inhibitor) failed to abrogate either the phosphoryation of cdc2 or G2/M arrest by lithium (data not shown).

Interestingly, the increase of Chk1 activity by lithium seems to be independent of the phosphorylation on its Ser-317 and Ser-345. It may be different from DNA damage agents, which cause phosphorylation of Chk1 on Ser-317 and Ser-345 through ATM/ATR kinase. Our study's results support this hypothesis by showing that caffeine, a known ATM/ATR inhibitor [Sarkaria et al., 1999], effectively abrogated the hydroxyurea-induced cdc2 phosphorylation, but failed to relieve the same effect by lithium. Correspondingly, SB218078, a potent Chk1 inhibitor, could relieve the cdc2 phosphorylation by both lithium and hydroxyurea. How lithium promotes Chk1 activity still remains unclear. It is possible that lithium causes phosphorylation on other residue(s) of Chk1, but we failed to detect obvious electrophoretic mobility change of Chk1 after lithium treatment (Figs. 4A, 5A,B, 6C). Therefore, further works are certainly needed to clarify the mechanism of lithium action on Chk1.

The present study shows that Chk1 is involved in lithium-induced G2/M arrest. Chk1 is an evolutionarily conserved protein kinase. It plays a key role in G2/M arrest induced by DNA stress, and its mutation has been reported in many human cancers [Bertoni et al., 1999]. Liu et al. [2000] have shown that Chk1 gene deficiency results in a severe proliferation defect, and Chk1 heterozygosity modestly enhances the tumorigenesis phenotype of WNT-1 transgenic mice. Chk1 is hereby proposed to be a novel class of essential tumor suppressor. Considering the preventive quality of lithium in human cancer development [Cohen et al., 1998], it will be interesting and significant to reveal the mechanism of lithium on Chk1 and cell cycle.

## ACKNOWLEDGMENTS

We thank Drs. Kum Kum Khanna, David Gillespie, and Kun Li for their generous gifts of reagents. This work was supported in part by grants from the National Natural Science Foundation of China.

## REFERENCES

Bartek J, Lukas J. 2003. Chk1 and Chk2 kinases in checkpoint control and cancer. Cancer Cell 3:421–429.

Bertoni F, Codegoni AM, Furlan D, Tibiletti MG, Capella C, Broggini M. 1999. CHK1 frameshift mutations in genetically unstable colorectal and endometrial cancers. Genes Chromosomes Cancer 26:176–180.

Boggs DR, Joyce RA. 1983. The hematopoietic effects of lithium. Semin Hematol 20:129–138.

Bulavin DV, Higashimoto Y, Popoff IJ, Gaarde WA, Basrur V, Potapova O, Appella E, Fornace AJ, Jr. 2001. Initiation of a G2/M checkpoint after ultraviolet radiation requires p38 kinase. Nature 411:102–107.

Chen RW, Chuang DM. 1999. Long term lithium treatment suppresses p53 and Bax expression but increases Bcl-2 expression. A prominent role in neuroprotection against excitotoxicity. J Biol Chem 274:6039–6042.

Cohen Y, Chetrit A, Cohen Y, Sirota P, Modan B. 1998. Cancer morbidity in psychiatric patients: Influence of lithium carbonate treatment. Med Oncol 15:32–36.

Diehl JA, Cheng M, Roussel MF, Sherr CJ. 1998. Glycogen synthase kinase-3beta regulates cyclin D1 proteolysis and subcellular localization. Genes Dev 12:3499–3511.

Ding Z, Parchment RE, LoRusso PM, Zhou JY, Li J, Lawrence TS, Sun Y, Wu GS. 2001. The investigational new drug XK469 induces G(2)-M cell cycle arrest by p53-dependent and -independent pathways. Clin Cancer Res 7:3336–3342.

Eymin B, Claverie P, Salon C, Brambilla C, Brambilla E, Gazzeri S. 2006. p14ARF triggers G2 arrest through ERK-mediated Cdc25C phosphorylation, ubiquitination and proteasomal degradation. Cell Cycle 5:759–765.

Gao H, Jin S, Song Y, Fu M, Wang M, Liu Z, Wu M, Zhan Q. 2005. B23 regulates GADD45a nuclear translocation and contributes to GADD45a-induced cell cycle G2-M arrest. J Biol Chem 280:10988–10996.

Gautier J, Solomon MJ, Booher RN, Bazan JF, Kirschner MW. 1991. cdc25 is a specific tyrosine phosphatase that directly activates p34cdc2. Cell 67:197–211.

Hallcher LM, Sherman WR. 1980. The effects of lithium ion and other agents on the activity of myo-inositol-1-phosphatase from bovine brain. J Biol Chem 255:10896–10901.

Hedgepeth CM, Conrad LJ, Zhang J, Huang HC, Lee VM, Klein PS. 1997. Activation of the Wnt signaling pathway:

A molecular mechanism for lithium action. Dev Biol 185:82–91.
Huot J, Nosal G, Radoucu-Thomas S. 1972. Effects of lithium chloride on normal and neoplastic cells in vitro. Experientia 28:456–457.
Kao KR, Elinson RP. 1998. The legacy of lithium effects on development. Biol Cell 90:585–589.
Karlsson-Rosenthal C, Millar JB. 2006. Cdc25: Mechanisms of checkpoint inhibition and recovery. Trends Cell Biol 16:285–292.
Khanna KK. 2000. Cancer risk and the ATM gene: A continuing debate. J Natl Cancer Inst 92:795–802.
Klein PS, Melton DA. 1996. A molecular mechanism for the effect of lithium on development. Proc Natl Acad Sci USA 93:8455–8459.
Kulikov R, Boehme KA, Blattner C. 2005. Glycogen synthase kinase 3-dependent phosphorylation of Mdm2 regulates p53 abundance. Mol Cell Biol 25:7170–7180.
Liu F, Stanton JJ, Wu Z, Piwnica-Worms H. 1997. The human Myt1 kinase preferentially phosphorylates Cdc2 on threonine 14 and localizes to the endoplasmic reticulum and Golgi complex. Mol Cell Biol 17:571–583.
Liu Q, Guntuku S, Cui XS, Matsuoka S, Cortez D, Tamai K, Luo G, Carattini-Rivera S, DeMayo F, Bradley A, Donehower LA, Elledge SJ. 2000. Chk1 is an essential kinase that is regulated by Atr and required for the G(2)/M DNA damage checkpoint. Genes Dev 14:1448–1459.
Mao CD, Hoang P, DiCorleto PE. 2001. Lithium inhibits cell cycle progression and induces stabilization of p53 in bovine aortic endothelial cells. J Biol Chem 276:26180–26188.
Ng CP, Lee HC, Ho CW, Arooz T, Siu WY, Lau A, Poon RY. 2004. Differential mode of regulation of the checkpoint kinases CHK1 and CHK2 by their regulatory domains. J Biol Chem 279:8808–8819.
Nyberg KA, Michelson RJ, Putnam CW, Weinert TA. 2002. Toward maintaining the genome: DNA damage and replication checkpoints. Annu Rev Genet 36:617–656.
Ohteki T, Parsons M, Zakarian A, Jones RG, Nguyen LT, Woodgett JR, Ohashi PS. 2000. Negative regulation of T cell proliferation and interleukin 2 production by the serine threonine kinase GSK-3. J Exp Med 192:99–104.
Peng CY, Graves PR, Thoma RS, Wu Z, Shaw AS, Piwnica-Worms H. 1997. Mitotic and G2 checkpoint control: Regulation of 14- 3-3protein binding by phosphorylation of Cdc25C on serine-216. Science 277:1501–1505.
Phiel CJ, Klein PS. 2001. Molecular targets of lithium action. Annu Rev Pharmacol Toxicol 41:789–813.
Rossig L, Badorff C, Holzmann Y, Zeiher AM, Dimmeler S. 2002. Glycogen synthase kinase-3 couples AKT-dependent signaling to the regulation of p21Cip1 degradation. J Biol Chem 277:9684–9689.
Ryves WJ, Harwood AJ. 2001. Lithium inhibits glycogen synthase kinase-3 by competition for magnesium. Biochem Biophys Res Commun 280:720–725.
Sancar A, Lindsey-Boltz LA, Unsal-Kacmaz K, Linn S. 2004. Molecular mechanisms of mammalian DNA repair and the DNA damage checkpoints. Annu Rev Biochem 73:39–85.
Sarkaria JN, Busby EC, Tibbetts RS, Roos P, Taya Y, Karnitz LM, Abraham RT. 1999. Inhibition of ATM and ATR kinase activities by the radiosensitizing agent, caffeine. Cancer Res 59:4375–4382.
Singh SV, Herman-Antosiewicz A, Singh AV, Lew KL, Srivastava SK, Kamath R, Brown KD, Zhang L, Baskaran R. 2004. Sulforaphane-induced G2/M phase cell cycle arrest involves checkpoint kinase 2-mediated phosphorylation of cell division cycle 25C. J Biol Chem 279:25813–25822.
Sinha D, Wang Z, Ruchalski KL, Levine JS, Krishnan S, Lieberthal W, Schwartz JH, Borkan SC. 2005. Lithium activates the Wnt and phosphatidylinositol 3-kinase Akt signaling pathways to promote cell survival in the absence of soluble survival factors. Am J Physiol Renal Physiol 288:F703–F713.
Smits VA, Essers MA, Loomans DS, Klompmaker R, Rijksen G, Medema RH. 1999. Inhibition of cell proliferation by lithium is associated with interference in cdc2 activation. FEBS Lett 457:23–27.
Stewart ZA, Westfall MD, Pietenpol JA. 2003. Cell-cycle dysregulation and anticancer therapy. Trends Pharmacol Sci 24:135–149.
Taylor WR, Stark GR. 2001. Regulation of the G2/M transition by p53. Oncogene 20:1803–1815.
Watanabe N, Broome M, Hunter T. 1995. Regulation of the human WEE1Hu CDK tyrosine 15-kinase during the cell cycle. EMBO J 14:1878–1891.
Yarden RI, Pardo-Reoyo S, Sgagias M, Cowan KH, Brody LC. 2002. BRCA1 regulates the G2/M checkpoint by activating Chk1 kinase upon DNA damage. Nat Genet 30:285–289.