

Inhibition of cell proliferation by lithium is associated with interference in cdc2 activation

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Abstract Lithium can interfere with embryonal development in a variety of organisms. We investigated the effect of lithium on the proliferation of early embryonal cells. [³H]Thymidine incorporation of non-committed mouse P19 embryonal carcinoma cells was inhibited by lithium treatment. Similar effects were seen in a variety of other cells. This growth inhibition occurred in the G₂ phase, since cells accumulated with a 4N DNA content, but the appearance of mitotic cells was blocked. Lithium could also prevent the activation of cdc2, thereby inhibiting cyclin B/cdc2 kinase activity. These data indicate that lithium might disturb embryonal development through interference in embryonal cell cycle regulation.

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Key words: Cell cycle; Checkpoint; Cyclin; G₂; Lithium

1. Introduction

Early embryonal stem cells undergo rapid cycles of cell division, but the cell cycle lengthens as cells become more committed. This lengthening is due to the introduction of cell cycle control mechanisms, causing the cells to pause between the alternating S and M phases, in G₁ or G₂ [1]. During G₂, initiation of mitosis is prevented by inhibitory phosphorylation of the mitotic cyclin B/cdc2 complexes. This inhibitory phosphorylation takes place on the Thr-14 and Tyr-15 residues of cdc2 and is mediated by Wee1 and related kinases [2–5]. At the end of the G₂ phase, the phosphatase Cdc25C dephosphorylates these inhibitory sites, triggering the activation of cdc2 [6,7].

Lithium is used as a treatment for bipolar psychiatric disorders [8]. In addition, it has been demonstrated that lithium can stimulate proliferation of some cells [9] and has teratogenic effects [10,11], when used at concentrations applied in human therapy. For example, in *Xenopus* embryos, lithium works as a modifier of mesoderm induction that can induce duplication of the dorsal axis [10]. In *Dictyostelium*, lithium influences cell fate determination by blocking spore cell and promoting stalk cell development [11].

In this paper, we demonstrate that lithium inhibits the proliferation of mouse embryonal cells, as well as in a variety of

other cell lines. The effect of lithium is exerted in the G₂ phase and is associated with an inhibition of cyclin B-associated kinase activity, due to retention of phosphorylation on the Tyr-15 regulatory site of cdc2.

2. Materials and methods

2.1. Cell culture, antibodies and reagents

U₂OS (ATCC), A14 [12] and Rat-1 (ATCC) cells were routinely grown in Dulbecco's modified Eagle's medium (DMEM, Gibco). P19 cells (ATCC) were also cultured in DMEM, on gelatin-coated dishes. SK-N-MC (ATCC) cells were cultured in a 1:1 mixture of DMEM and Ham's F12 (DF12 medium, Gibco). The culture media were supplemented with 10% fetal calf serum (FCS, Integro), 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. For differentiation, P19 cells were grown in aggregates on bacteriological petri dishes in the presence of retinoic acid (RA) 10⁻⁶ M for 5 days (P19^{AG}+RA) [13]. Cells were arrested at the metaphase/anaphase transition of the M phase by treating the cells with nocodazole (2.5 µg/ml; Sigma) for 16 h [14]. To arrest the cells at the G₁/S transition, cells were treated with thymidine (2.5 mM; Sigma) for 24 h. Cells were released from a thymidine block by washing twice with pre-warmed PBS, followed by washing twice with pre-warmed culture medium for 15 min at 37°C.

Protein A/G plus agarose, and the mouse monoclonal antibodies against cyclin B1 (GNS1) and cdc2 (clone 17) were purchased from Santa Cruz. Polyclonal phosphospecific cdc2-Tyr-15 antibody was obtained from New England Biolabs. Histone H1 was obtained from Boehringer Mannheim. Propidium iodide was purchased from Sigma.

2.2. [³H]Thymidine incorporation assay and cell cycle analysis

[³H]Thymidine incorporation was performed as described [15]. For DNA profiles, the cells were grown with or without LiCl (10 mM) for 48 h, after which cells were harvested and fixed overnight in 70% ethanol at 4°C. After washing away the ethanol, the cells were stained with propidium iodide in a solution containing 10 µg/ml propidium iodide and 10 µg/ml DNase-free RNase. The stained cells were analyzed on a fluorescence activated cell sorter (FACS) using Lysis II software flow cytometry analysis (Becton Dickinson).

To determine the percentage of mitotic cells, cells were harvested, fixed in 70% ethanol and cytospins were prepared. DNA was stained using 4',6-diamidino-2-phenylindole (DAPI, 20 µg/ml) [16]. The percentage of mitotic figures was scored using fluorescence microscopy (≥ 200 cells per time point).

2.3. Western blotting, immunoprecipitation and in vitro kinase reactions

For Western blotting, cells were lysed directly in Laemmli sample buffer without β-mercaptoethanol or bromophenol blue and the samples were boiled for 5 min. Protein concentrations were determined using the Lowry protein assay [17]. Subsequently, β-mercaptoethanol and bromophenol blue were added. Proteins were separated on a polyacrylamide (PAA) gel and blotted to a nitrocellulose membrane. Proteins were detected with antibody incubation (mouse monoclonal anti-cdc2, recognizing human, mouse and *Xenopus* cdc2, non-cross-reactive with other cdk's; rabbit polyclonal phosphospecific anti-cdc2-Tyr-15) overnight at 4°C. This was followed by the secondary antibody for 1 h at room temperature. The blots were developed using enhanced chemiluminescence (ECL).

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Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; ECL, enhanced chemiluminescence; FACS, fluorescence activated cell sorter; GSK-3, glycogen synthase kinase 3; IMPase, inositol monophosphatase; PAA, polyacrylamide; RA, retinoic acid

For immunoprecipitation, the cells were washed with ice-cold PBS and lysed in NETN (400 mM NaCl, 20 mM Tris pH 8.0, 1 mM EDTA, 0.5% NP-40 supplemented with 10 mM β -glycerophosphate, 10 mM NaF, 1 mM Na_2VO_3 , 1 mM phenylmethylsulfonyl fluoride, 10 $\mu\text{g}/\text{ml}$ aprotinin, 10 $\mu\text{g}/\text{ml}$ leupeptin and 10 $\mu\text{g}/\text{ml}$ trypsin inhibitor) for 30 min at 4°C. Lysates were centrifuged at 15000 rpm at 4°C. Cyclin B1 was immunoprecipitated with 0.3 μg of anti-cyclin B1 antibody and protein A/G plus agarose for 4 h to overnight at 4°C. Subsequently, immunoprecipitates were washed three times with lysis buffer. For cdc2 immunoblotting, precipitates were washed once again with lysis buffer, after which sample buffer was added. Then the samples were boiled for 5 min, the proteins were separated on a 12% PAA gel and cdc2 was detected by Western blotting. In vitro kinase reactions were performed as described [15].

3. Results

3.1. Lithium inhibits proliferation of P19 cells

To study if lithium can affect the proliferation of early embryonal cells, we used mouse P19 embryonal carcinoma cells as a model for embryonal cells [18]. We found that proliferation was dramatically inhibited, as determined by [^3H]thymidine incorporation (Fig. 1A). To determine whether this effect was restricted to non-committed early embryonal cells, we analyzed the effect of lithium on proliferation in differentiated P19 cells. To this end, P19 cells were cultured in aggregates in the presence of RA for 5 days, inducing mesodermal and neuronal differentiation [13]. Treating such differentiated P19 cells for 24 h or 48 h with LiCl resulted in a decrease of [^3H]thymidine incorporation of $\sim 30\%$ and $\sim 45\%$, respectively (Fig. 1A). Although the effect of lithium is not as dramatic in the differentiated P19 cells compared to the undifferentiated cells, we do not think this is due to a difference in sensitivity to lithium but rather to the slower proliferation rate of the differentiated cells.

In addition, we examined the dose dependence of growth inhibition by lithium. As shown in Fig. 1B, proliferation is already inhibited at a concentration of 0.5 mM LiCl.

To determine in which phase of the cell cycle lithium exerts its growth-inhibitory effect, we analyzed cell cycle profiles of asynchronously growing P19 cells, which were treated with LiCl for 48 h. Treatment with LiCl resulted in an increase in the number of cells with a 4N DNA content from 26% in untreated cells to 47% in LiCl-treated cells (Fig. 1C). Also differentiated P19 cells showed an accumulation of cells in the G_2/M phases upon LiCl treatment (data not shown).

3.2. Lithium induces growth inhibition in several cell lines

To analyze if the effect of lithium on G_2/M progression is more general, we investigated its effect on the proliferation of a diverse range of cell lines. The effect of lithium on DNA synthesis in U₂OS cells (human osteosarcoma), SK-N-MC cells (human neuroepithelioma), Rat-1 cells (rat fibroblast) and NIH 3T3-derived A14 cells (mouse fibroblast) was determined by [^3H]thymidine incorporation in the absence or presence of LiCl. Treatment of U₂OS, SK-N-MC and A14 cells with LiCl resulted in a decrease of [^3H]thymidine incorporation from $\sim 45\%$ in SK-N-MC cells to $\sim 60\%$ in U₂OS cells and up to $\sim 75\%$ in A14 cells (Fig. 2A). [^3H]Thymidine incorporation in Rat-1 cells was not influenced by LiCl treatment (Fig. 2A).

Also in these cells the growth inhibition occurred in G_2/M . Treating U₂OS cells with LiCl for 24 h resulted in an increase

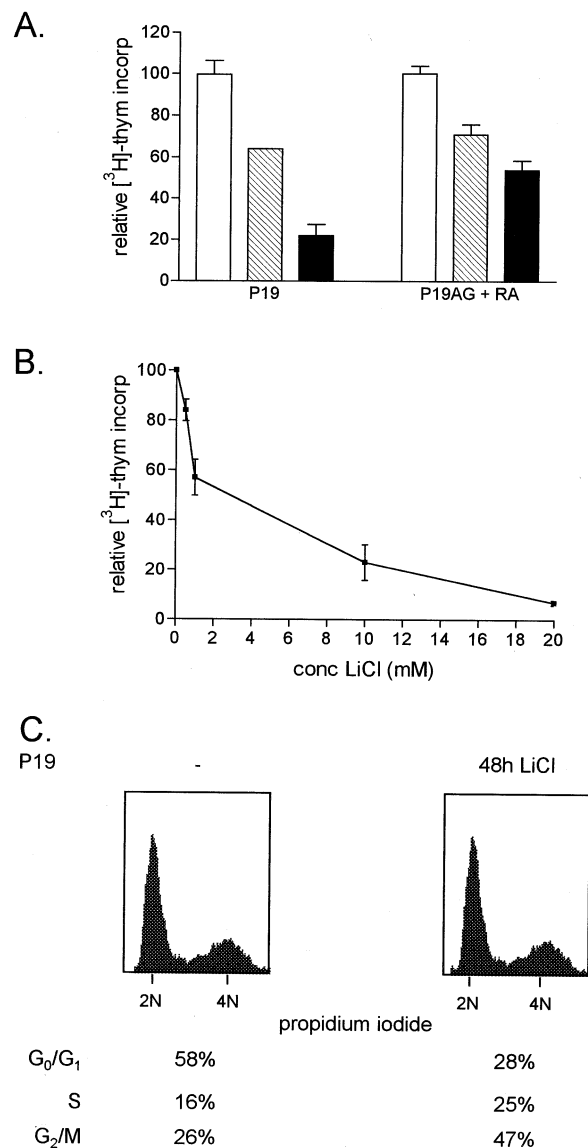


Fig. 1. Lithium inhibits proliferation of P19 cells by a block in G_2/M . A: Asynchronously growing P19 cells or differentiated P19 cells (P19^{AG}+RA) were seeded in 24-well dishes at 40000 cells per well. After 24 h, the cells were left untreated (white bars) or treated with LiCl (10 mM) for 24 (gray bars) or 48 h (black bars). [^3H]Thymidine incorporation was determined in triplicate and is represented as percentage incorporation of untreated cells (actual, P19: 14×10^5 cpm/well; P19^{AG}+RA: 0.7×10^5 cpm/well). Error bars represent the S.E.M. B: Asynchronously growing P19 cells were seeded in 24-well dishes at 10000 cells per well. After 24 h, the cells were treated with different concentrations of LiCl for 24 h. [^3H]Thymidine incorporation was determined as described in A. C: Asynchronously growing P19 cells were left untreated or treated with LiCl (10 mM). After 48 h, the cells were harvested and cell cycle profiles were obtained by staining with propidium iodide.

in the 4N population from 20% to 32% (data not shown), whereas treatment for 48 h resulted in an increase up to 49% (Fig. 2B). Treatment of SK-N-MC cells and A14 cells with LiCl also resulted in an increase in the amount of cells in the G_2/M phase (data not shown), indicating that lithium has a similar effect on a diverse range of cell lines.

3.3. Lithium inhibits G_2 phase progression, not mitosis

To determine whether lithium-treated cells are blocked in

the G₂ phase or in M phase, we tested whether lithium could inhibit cells from entering mitosis. To this end, U₂OS cells were treated with nocodazole, to arrest them at the metaphase/anaphase transition of the M phase, in the presence or absence of LiCl. At several time points, cells were harvested and the percentage of mitotic cells was determined by immunofluorescence staining with the DNA-interchelating dye DAPI. In the samples treated with nocodazole, the first mitotic cells appeared within 6 h (Fig. 3). The percentage of mitotic cells increased with time and after 16 h a large percentage of the cells (~56%) was in the M phase. In contrast, cells treated with nocodazole and LiCl clearly showed a delay in entering mitosis. After 16 h, we could only detect ~12% mitotic cells.

In addition, U₂OS cells were synchronized at the G₁/S transition using thymidine. When the cells were released in the presence of LiCl the appearance of mitotic cells was delayed compared to the untreated cells (data not shown), again indicating that the block occurs prior to initiation of mitosis.

3.4. Lithium prevents the activation of *cdc2*

For entering M phase, kinase activity of the mitotic cyclin

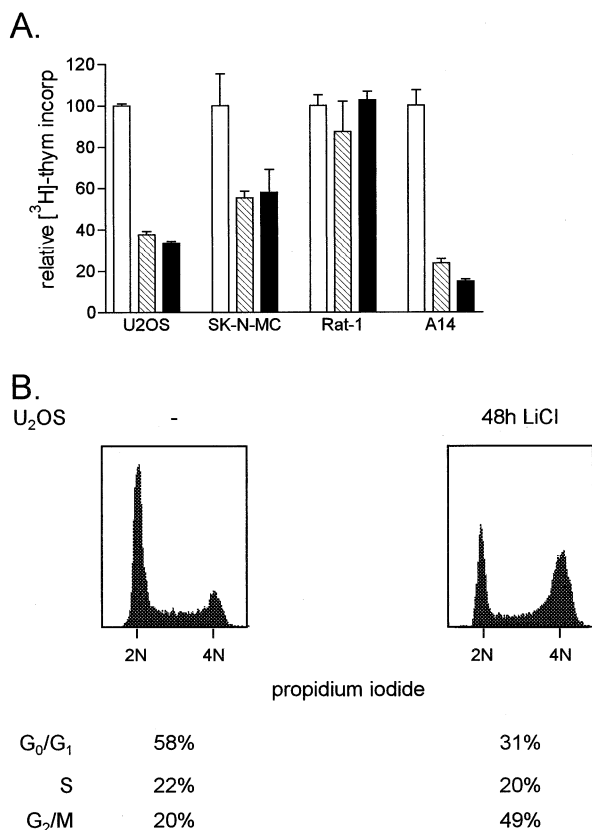


Fig. 2. Lithium inhibits proliferation of A14, U₂OS and SK-N-MC cells, but not Rat-1 cells. A: U₂OS, SK-N-MC, Rat-1 and A14 cells were seeded in 24-well dishes at 40000 cells per well. After 24 h, the cells were left untreated (white bars) or treated with LiCl (10 mM) for 24 (gray bars) or 48 h (black bars). [³H]Thymidine incorporation was determined in triplicate and is represented as percentage incorporation of untreated cells (actual, 1.2×10^5 to 4.2×10^5 cpm/well). Error bars represent the S.E.M. B: U₂OS cells were left untreated or treated with LiCl (10 mM) for 48 h. Then, the cells were harvested and cells cycle profiles were obtained by staining with propidium iodide.

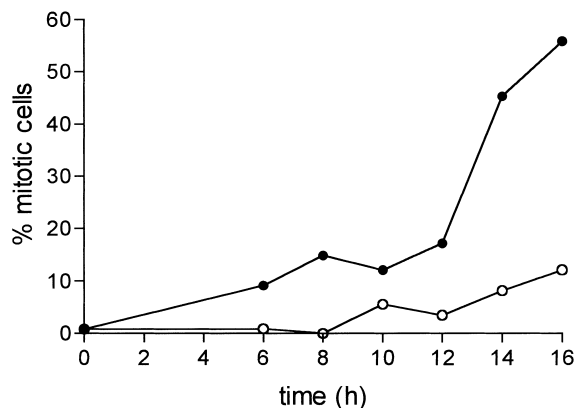


Fig. 3. Lithium inhibits M phase entry. U₂OS cells were treated with nocodazole in the absence (closed circles) or presence (open circles) of LiCl (10 mM). At different time periods, cells were harvested. Then, DNA was stained using DAPI. The percentage of mitotic cells was determined by scoring for cells with condensed chromosomes, using immunofluorescence microscopy.

B/*cdc2* complex is required [3]. We therefore investigated if lithium could interfere with the activation of the cyclin B/*cdc2* kinase. To do so, U₂OS cells were synchronized at the G₁/S transition using thymidine. Next, the cells were released in the presence or absence of LiCl, and nocodazole was added, to ensure that the synchronized cells eventually block at the metaphase-anaphase transition of the M phase. At different time points after the release, we measured cyclin B-associated kinase activity (Fig. 4A). Kinase activity in the untreated samples rose 16 h after the release and increased with time. In contrast, we observed only a minor increase in cyclin B-associated activity in cells released from the thymidine block in the presence of LiCl. This increase occurred at a later time point and the overall kinase activity remained low.

The reduction in cyclin B/*cdc2* kinase activity was not due to inhibition of cyclin B expression, since cyclin B protein levels were not influenced by lithium treatment (data not shown).

We next analyzed the phosphorylation state of *cdc2* after treatment with lithium. Cyclin B immunoprecipitations obtained at the same time points after release from the thymidine block were subjected to immunoblotting for *cdc2*. Dephosphorylation of the Tyr-15 residue of *cdc2* leads to the activation of *cdc2* at the G₂/M transition and results in a mobility shift on PAA gels [19]. In cells blocked at the G₁/S transition using thymidine, only the slower migrating form of *cdc2* could be detected (Fig. 4B). This form represents the inactive form of *cdc2*, phosphorylated on Tyr-15 [19], which we confirmed with a phosphospecific *cdc2*-Tyr-15 antibody (Fig. 4B). Activated *cdc2* was present after a 13 h release in the absence of LiCl and the amount of Tyr-15-dephosphorylated protein increased with time. Releasing the cells in the presence of LiCl showed that cyclin B/*cdc2* complex formation occurred normally, but the Tyr-15 dephosphorylation was inhibited. After 25 h ~80% of the *cdc2* is in its activated, faster migrating form in untreated samples, whereas in the samples treated with LiCl, only ~20% of the *cdc2* is active.

As is shown in Fig. 4C, the inhibition of Tyr-15 dephosphorylation of *cdc2* shows a very similar concentration dependence for LiCl as was observed for the inhibition of [³H]thymidine incorporation (Fig. 1B).

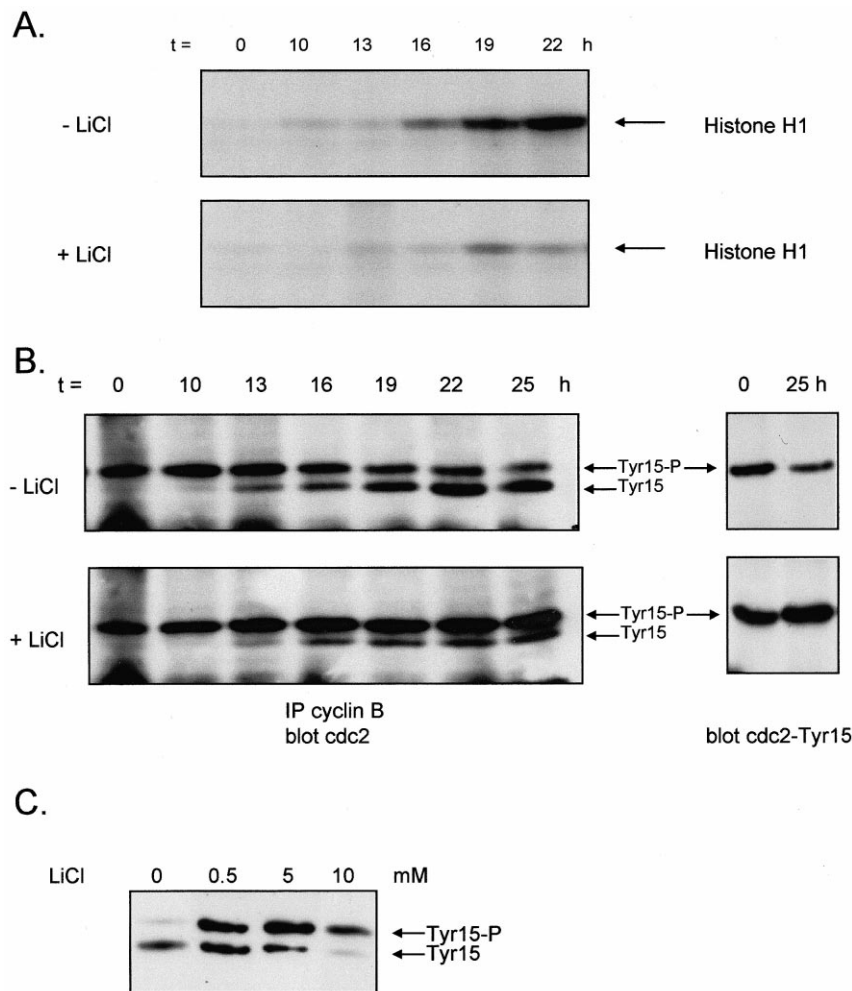


Fig. 4. Lithium inhibits cyclin B/cdc2 activation. A: U₂OS cells were blocked at the G₁/S transition by thymidine treatment for 24 h. Thymidine was washed away and the cells were released in the presence of nocodazole and in the absence or presence of LiCl (10 mM). At different time periods, the cells were lysed. Cyclin B was immunoprecipitated and cyclin B-associated kinase activity was determined using an in vitro kinase reaction with histone H1 as substrate. 5 µg of protein was used in the immunoprecipitation, plateau of the kinase assay at 50 µg. Assays were performed in duplicate and the variation never exceeded 5% (as determined by phosphor imager). B: As A, but after cyclin B immunoprecipitation, cdc2 was detected by immunoblotting. The faster migrating form is the active, Tyr-15-dephosphorylated form of cdc2 (left panel). In total lysates, cdc2-Tyr-15-P was detected by Western blotting with a phosphospecific anti-cdc2-Tyr-15 (right panel). C: Asynchronously growing U₂OS cells were treated with nocodazole in the presence of different concentrations of LiCl. After 16 h, the cells were lysed, cyclin B was precipitated, and cdc2 was detected by immunoblotting. The faster migrating form is the active, Tyr-15-dephosphorylated form of cdc2.

4. Discussion

In this paper, we show that lithium induces a G₂ arrest in a variety of cell lines and inhibits the appearance of cyclin B/cdc2 kinase activity. This inhibition is due to retention of inhibitory phosphorylation of Tyr-15 on cdc2. Dephosphorylation of cdc2-Tyr-15 is a critical step in the activation of the cyclin B/cdc2 complex [6,7]. Our results suggest that lithium might prevent the activation of the mitotic cyclin B/cdc2 complex, through interference with Tyr-15 dephosphorylation.

Two different pathways have been described by which lithium could affect embryonal development. Lithium has been shown to inhibit inositol monophosphatase (IMPase) in vitro [20,21] and lithium can act as an inhibitor of glycogen synthase kinase 3 (GSK-3) and thereby mimic Wingless signaling [22,23]. Whether one of these pathways is involved in the effect of lithium we describe here is currently unknown.

DNA damage will also result in an arrest in G₂ progression and this arrest is caused by inhibition of cdc2 dephosphoryl-

ation [24]. Recent studies showed that the inhibition of cdc2 activation after DNA damage is mediated by the inactivation of Cdc25C [25,26] through phosphorylation-dependent binding to 14-3-3 [27–29]. Present cancer therapies mostly rely on the increased sensitivity of tumor cells to a variety of DNA-damaging agents. Since lithium seems to interfere with the same pathway as these agents, it will be of significant interest to identify the target(s) of lithium and to investigate the relative sensitivity of normal and tumor cells to lithium treatment. In that respect, it is very interesting that lithium has been reported to reduce the incidence of cancer [30].

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References

- [1] Edgar, B.A. and Lehner, C.F. (1996) *Science* 274, 1646–1652.

- [2] Nurse, P. (1990) *Nature* 344, 503–508.
- [3] Dunphy, W.G. (1994) *Trends Cell Biol.* 4, 202–207.
- [4] King, R.W., Jackson, P.K. and Kirschner, M.W. (1994) *Cell* 79, 563–571.
- [5] Mueller, P.R., Coleman, T.R., Kumagai, A. and Dunphy, W.G. (1995) *Science* 270, 86–90.
- [6] Gaultier, J., Solomon, M.J., Booher, R.N., Bazan, J.F. and Kirschner, M.W. (1991) *Cell* 67, 197–211.
- [7] Strausfeld, U., Labbé, J.C., Fesquet, D., Cavadore, J.C., Picard, A., Sadhu, K., Russell, P. and Doreé, M. (1991) *Nature* 351, 242–245.
- [8] Price, L.H. and Heninger, G.R. (1994) *New Engl. J. Med.* 331, 591–598.
- [9] Welshons, W.V., Engler, K.S., Taylor, J.A., Grady, L.H. and Curran, E.M. (1995) *J. Cell Physiol.* 165, 134–144.
- [10] Kao, K.R. and Elinson, R.P. (1989) *Dev. Biol.* 132, 81–90.
- [11] Van Lookeren Campagne, M.M., Wang, M., Spek, W., Peters, D. and Schaap, P. (1988) *Dev. Genet.* 9, 589–596.
- [12] Burgering, B.M.Th., Medema, R.H., Maassen, J.A., van de Wetering, M.L., van der Eb, A.J., McCormick, F. and Bos, J.L. (1991) *EMBO J.* 10, 1101–1109.
- [13] Kranenburg, O., de Groot, R.P., Van der Eb, A. and Zantema, A. (1995) *Oncogene* 10, 87–95.
- [14] Pomies, P. and Block, M.R. (1992) *Biochem. Biophys. Res. Commun.* 189, 1429–1436.
- [15] Medema, R.H., Klompemaker, R., Smits, V.A.J. and Rijksen, G. (1998) *Oncogene* 16, 431–441.
- [16] Lee, L.S. and Garnett, H.M. (1993) *J. Biochem. Biophys. Methods* 26, 249–260.
- [17] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.L. (1951) *J. Biol. Chem.* 193, 267–275.
- [18] McBurney, M.W. and Rogers, B.J. (1982) *Dev. Biol.* 89, 503–508.
- [19] Nishijima, H., Nishitani, H., Seki, T. and Nishimoto, T. (1997) *J. Cell Biol.* 138, 1105–1116.
- [20] Hallcher, L.M. and Sherman, W.R. (1980) *J. Biol. Chem.* 255, 10896–10901.
- [21] Berridge, M.J., Downes, C.P. and Hanley, M.R. (1989) *Cell* 59, 411–419.
- [22] Stambolic, V., Ruel, L. and Woodgett, J.R. (1996) *Curr. Biol.* 6, 1664–1668.
- [23] Hedgepeth, C.M., Conrad, L.J., Zhang, J., Huang, H.C., Lee, V.M.Y. and Klein, P.S. (1997) *Dev. Biol.* 185, 82–91.
- [24] Kharbanda, S., Saleem, A., Datta, R., Yuan, Z.M., Weichselbaum, R. and Kufe, D. (1994) *Cancer Res.* 54, 1412–1414.
- [25] Herzinger, T., Funk, J.O., Hillmer, K., Eick, D., Wolf, D.A. and Kind, P. (1995) *Oncogene* 11, 2151–2156.
- [26] Poon, R.Y.C., Jiang, W., Toyoshima, H. and Hunter, T. (1996) *J. Biol. Chem.* 271, 13283–13291.
- [27] Furnari, B., Rhind, N. and Russell, P. (1997) *Science* 277, 1495–1497.
- [28] Peng, C.Y., Graves, P.R., Thoma, R.S., Wu, Z., Shaw, A.S. and Piwnica-Worms, H. (1997) *Science* 277, 1501–1505.
- [29] Sanchez, Y., Wong, C., Thoma, R.S., Richman, R., Wu, Z., Piwnica-Worms, H. and Elledge, S.J. (1997) *Science* 277, 1497–1501.
- [30] Cohen, Y., Chetrit, A., Cohen, Y., Sirota, P. and Modan, B. (1998) *Med. Oncol.* 15, 32–36.