Interference between p53 and cdc25C in cell cycle regulation

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Abstract. The eukaryotic cell cycle is regulated by a network of different protein kinases and phosphatases which are by various mechanisms linked to the growth suppressor p53. Cell cycle regulation is quite similar from yeast to man. Although there is no endogenous p53 in yeast expression of human p53 led to growth arrest of yeast cells which can be suppressed by simultaneous overexpression of cdc25C, a phosphatase regulating entry into mitosis. Herein, we show that over-expression of cdc25C in mammalian cells is insufficient in suppressing a p53 induced growth arrest. We further show that p53 is co-immunoprecipitated with cdc25C and p53 inhibits the cdc25C phosphatase activity in a dose-dependent manner. Thus, our data show that p53 like other binding partners of cdc25C, regulates entry into mitosis by binding to cdc25C.

Introduction

It has been shown that expression of human p53 led to growth arrest of yeast cells, which can be suppressed by simultaneous overexpression of cdc25C (1). In eukaryotes, cell cycle progression is controlled by stoichiometric complexes of cyclin dependent kinases (cdks) and of cyclins. The activity of these cyclin dependent kinases is regulated i) by assembly of the cdks with cyclins, ii) by specific inhibitors, iii) by activating or inactivating upstream kinases, iv) by their subcellular localisation and, v) by phosphatases (reviewed in ref. 2). Among these phosphatases is a family of highly conserved proteins of the cdc25 family namely cdc25A, cdc25B and cdc25C expressed in mammalian cells (3). For each family member, there is a number of known different splice variants (4-6). Each member of the cdc25 family has different functions during the cell cycle by dephosphorylating specific substrates. cdc25A controls progression through the S-phase whereas cdc25B and cdc25C primarily control progression through the G_2-phase and entry into mitosis (7). In late G_2-phase of the cell cycle the cdc25C phosphatase dephosphorylates cdk1 of the cyclin B1/cdk1 complex at both threonine 14 and tyrosine 15 leading to the activation of the cyclin B1/cdk1 complex (8) and to entry into mitosis.

The activity of cdc25C is strictly regulated throughout the cell cycle i.e. the phosphatase activity of cdc25C is low during interphase and enhanced during mitosis (9). Mitotic activation of cdc25C is achieved by phosphorylation in the amino terminus of cdc25C by cdk1 and by polo-like kinases (10). The 14-3-3 protein is a phosphoserine binding protein that negatively regulates cdc25C. During interphase cdc25C is phosphorylated at serine 216 and bound to the 14-3-3 protein whereas during mitosis cdc25C is phosphorylated at serine 214 which prevents phosphorylation at serine 216 and binding of the 14-3-3 protein (11,12). Binding of cdc25C to the 14-3-3 protein leads to a nuclear exclusion of cdc25C and thus to inactivation of cdc25C. In a yeast two-hybrid screen PCNA (proliferating cell nuclear antigen) was found as an interaction partner of cdc25C, this interaction was confirmed to appear *in vitro* and *in vivo* (13). To date, nothing is known about the functional relevance of this interaction.

The growth suppressor protein p53 is a critical mediator of cellular stress response. It preserves genomic integrity by arresting cells in G_1- or G_2-phase of the cell cycle or by inducing apoptosis (14,15). p53 directly binds to cdk1 and down-regulates the cyclin B1/cdk1 activity (16-18). The human cdc25C promoter contains a recognition site for p53 and the expression of cdc25C is down-regulated by p53 (19) indicating that both cdk1 and cdc25C are important components in the G_2 arrest mediated by p53.

Although there is no evidence for a p53 homologue in yeast, human p53 expression in yeast cells leads to a growth arrest indicating that p53 is functional in this heterologous system (20,21). This p53 induced growth arrest was efficiently suppressed by coexpression of human cdc25C indicating a functional interaction of both proteins (1). Later on binding of p53 to cdc25C was at least shown in an *in vitro* binding assay using bacterially expressed and purified proteins (22). In the present study, we analyzed whether overexpression of cdc25C in mammalian cells is sufficient to overcome a p53-induced growth arrest. In contrast to yeast cells we found that cdc25C was unable to suppress a p53-induced growth arrest in mammalian cells. As shown by co-immunoprecipitation experiments p53 bound to cdc25C. This binding of p53 to cdc25C resulted in inhibition of the phosphatase activity indicating also a functional interaction of both proteins in mammalian cells.

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Materials and methods

Cell culture and transfection. The saos2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum (FCS) at 37˚C in a 5% CO_2 atmosphere. For the culture of saosts 138-8 cells (kindly provided by Dr Klaus Roemer, Homburg) the DMEM medium contained 200 μg/ml G418.

For transfection, cells were grown to subconfluence in 10-cm dishes and transfected with either pcDNA3.1, pcDNA3.1-cdc25C_{WT} or pcDNA3.1-cdc25C_{DM} using superfect transfection reagent (Qiagen) according to the manufacturer's instructions. Transfected cells were cultured in medium containing 200 μg/ml G418 and 50 μg/ml hygromycin B. For a G_2/M arrest, cells were shifted to 31˚C for different times. Cells were harvested with a rubber policeman and washed 3 times with phosphate-buffered saline (PBS), pH 7.4. The cell pellet was lysed with a double volume of lysis buffer (100 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.5% NP40). Proteins were extracted for 1 h on ice. Cell debris was eliminated by centrifugation (4˚C, 30 min, 13000 x g). The protein content was determined according to a modified Bradford method with the BioRad reagent dye (BioRad). Protein extracts were used for Western blot analysis, immunoprecipitation and phosphatase activity assays. For the recovery of mitotic cells, cells were treated with 400 μg/ml nocodazol for 24 h. Mitotic cells were collected by mitotic shake off and extracted as described above.

Immunofluorescence. For immunofluorescence, cells were grown on coverslips in 10-cm petri dishes up to 50-70% confluence. We used the mouse monoclonal anti-α tubulin antibody (Sigma), the mouse monoclonal anti-p53 antibody PAb421, the rabbit polyclonal anti-cdc25C antibody C-20 (Santa Cruz Biotechnology) or the mouse anti-cdc25C antibody C2-2 (BD, Biosciences, Pharmingen). Cells were rinsed with PBS and fixed in 3.7% formaldehyde in PBS for 10 min at room temperature. Cells were washed 3x10 min with PBS and permeabilised with 0.5% Triton X-100 for 10 min. Cells were washed again 3x10 min with PBS and incubated in PBS + 10% bovine serum albumin (BSA) at room temperature for 10 min to block non-specific protein binding. Cells were incubated with the primary antibody for 1 h at room temperature or at 37˚C for 30 min. After 3 washes with PBS containing 0.1% Tween-20 at room temperature, cells were incubated with an appropriate FITC- or TRITC-conjugated anti-mouse or anti-rabbit antibody (#488 or #595, Molecular Probes) at room temperature for at least 30 min. Cells were washed again under the same conditions. For DAPI staining, cells were incubated with 50 μl DAPI (4', 6-Diamidino-2-phenylindol, 0.1 μg/μl) per coverslip at 37˚C for 15 min, and then washed twice with PBS and once with water. The coverslips were mounted on a drop of mounting medium and analyzed under a fluorescence microscope.

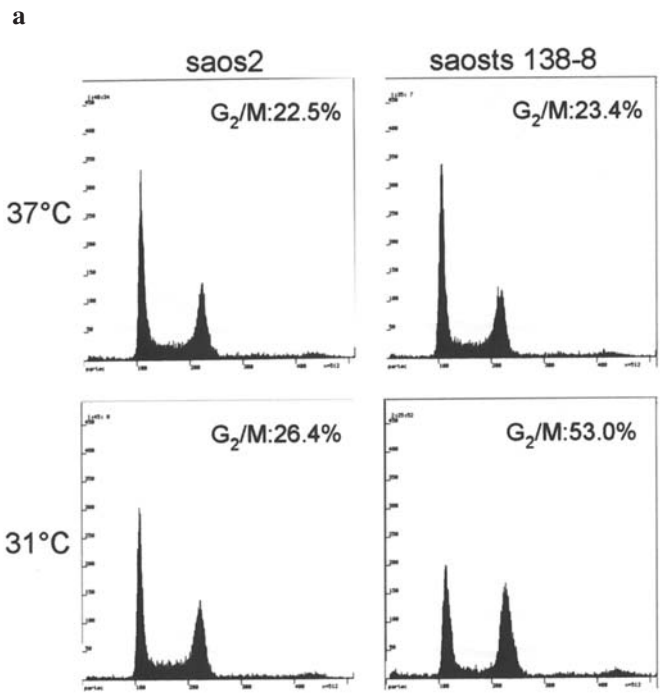
Determination of the mitotic index. The mitotic index was determined as described (23). Briefly, cells were grown to 50-70% confluence on Cellocate coverslips with 55-μm grid size (Eppendorf, Hamburg, Germany) in 6-cm petri dishes. Coverslips were transferred to fresh medium, containing 25 mM hepes buffer (pH 7.8), shortly before injection. After injection, coverslips were placed into fresh medium containing 10% FCS. Cellocate coverslips were pre-coated with poly-L-lysine (Sigma) as described by the manufacturer. For microinjection experiments, we used rhodamine-conjugated bovine brain tubulin (10 mg/ml, tubulin-rhodamine, Molecular Probes). Tubulin-rhodamine, dissolved in dialysis buffer or the dialysis buffer (20 mM Tris-HCl, pH 8.0, 100 mM KCl, 5 mM $MgCl_2$, 0.1% (v/v) Tween-20) alone were centrifuged for 30 min at 90000 x g at 4˚C. The supernatants were mixed and used for the experiments. After microinjection, cells were incubated at 37˚C or 31˚C for different times. They were washed with PBS and fixed for 10 min at room temperature in 3.7% formaldehyde. After washing twice with PBS, cells were briefly rinsed with water to remove salts, and finally coverslips were mounted with a drop of mounting medium and the cells analyzed under a fluorescence microscope.

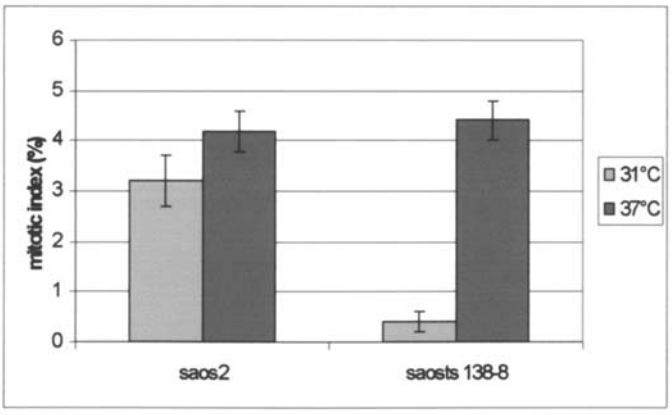
Cytofluorimetry. For cytofluorimetric analysis cells were washed twice with cold PBS and harvested by trypsinizing. Cells were spun down (400 x g, 4˚C, 10 min) and resuspended in 200 μl PBS. Cells were fixed by adding 2 ml ice-cold 70% ethanol and left for at least 30 min at -20˚C. Cells were harvested by centrifugation and resuspended in 800 μl PBS. RNase (100 μl: 1 mg/ml) and 100 μl propidium iodide (400 μg/ml) were added and cells were incubated for 30 min at 37˚C and then sonified for 30 sec at 4˚C. Cell cycle analysis was performed using a PAS III cytofluorimeter (Partec).

In vitro phosphatase assay in total cell extract. The phosphatase assay was performed as described (24). Briefly, 300 μg proteins from a total cell extract were dissolved in 140 ml phosphatase assay buffer [30 mM Tris-HCl, pH 8.5, 75 mM NaCl, 0.67 mM EDTA, 0.033% (w/v) BSA, 1 mM DTT] to a total volume of 150 μl and incubated with 20 μg fluorescein diphosphate (FDP) for 15 min at room temperature. The fluorescence emission was detected by a 'fluorescence reader' (GENios Spectra Fluor Plus, Tecan) at 535 nm.

Immunoprecipitation. Cells were harvested and resuspended in extraction buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5% Triton-X-100 and protease inhibitor Complete™ (Roche Diagnostics) and lysed by sonification. The lysate was cleared by centrifugation (13000 x g, 4˚C, 25 min) and then incubated with protein A-sepharose (Amersham Bioscience) for 60 min at 4˚C. This pre-adsorption step was repeated at least once and proteins binding unspecifically to protein A-sepharose were used as control. Protein A-sepharose (80 μl) was incubated with antibody C-20 for 90 min at 4˚C. Then, the cell extract was incubated with the protein A-sepharose bound antibody at 4˚C overnight. Finally, the protein A-sepharose was washed 3 times with extraction buffer and bound proteins were eluted with sample buffer and analyzed on a 10% SDS-polyacrylamide gel as described previously (25).

Western blotting. For Western blot analysis proteins were transferred to a PVDF membrane by tank blotting with 20 mM Tris-HCl, pH 8.7, 150 mM glycine as transfer buffer. Membranes were blocked with PBS containing 0.1% Tween-20





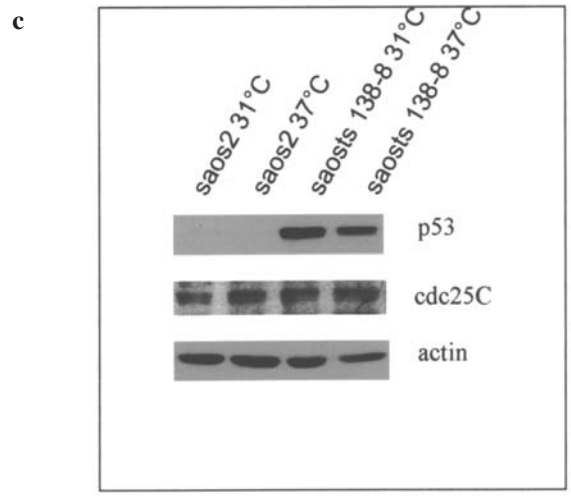


Figure 1. Characterization of the cell system. (a) Cytofluorimetry. saos2 and also saosts 138-8 cells were incubated at 37˚C and then either left at 37˚C or shifted to 31˚C for 24 h. Cells were analyzed by cytofluorimetry. The inserts show the percentage of cells in G_2/M phase. (b) Mitotic index. saos2 and saosts 138-8 cells were grown on coverslips at 37˚C and then incubated further at 37˚C or shifted to 31˚C for 24 h. Cells were fixed with formaldehyde and permeabilised with Triton-X 100 and then analyzed with the mouse monoclonal anti-α tubulin antibody (Sigma). FITC-conjugated anti-mouse antibodies were used as secondary antibodies. One thousand cells were counted and the mitotic index was determined. (c) Western blot analysis. saos2 and saosts 138-8 cells grown at 37˚C were incubated further at 37˚C or shifted to 31˚C for 24 h. Cell extracts (100 μg) were separated through an SDS-polyacrylamide gel and transferred onto a PVDF-membrane. cdc25C and p53 were detected with specific antibodies and visualized by the Lumilight system of Roche. Loading of the lanes was demonstrated by incubation of the membrane with the α-actin antibody I-19.

and 5% dry milk for 1 h at room temperature. The membrane was incubated with the primary antibody (PAb421 for p53, C-20 or C2-2 for cdc25C, I-19 for actin) usually in a dilution of 1:1000 in PBS-Tween-20 with 1% dry milk for another hour. After incubation with the primary antibody blot membranes were washed with PBS-Tween-20 three times before incubating with the peroxidase-coupled secondary antibody in a dilution of 1:30000 in PBS-Tween-20 with 1% dry milk. Signals were detected with the Lumilight system of Roche Diagnostics (Mannheim, Germany).

FDP phosphatase assay. The activity of immunopurified cdc25C was also measured with fluorescein diphosphate as a substrate essentially as described (26). Immunopurified cdc25C was washed 3 times with washing buffer [30 mM Tris-HCl, pH 7.5, 75 mM NaCl, 0.67 mM EDTA and 1 mM dithiothreitol (DTT)]. Then, cdc25C at the protein A-sepharose beads was resuspended in 140 μl reaction buffer (washing buffer containing 20 μM FDP) and transferred to a 96-well microtitre plate. Fluorescence emission from the product fluorescein monophosphate was measured with a multiwell plate reader at 32˚C.

Results

In *S. pombe* cells human p53 can induce growth arrest which is efficiently suppressed by co-expression of human cdc25C (1). In order to analyze whether cdc25C can also suppress a p53 induced growth arrest in mammalian cells we used saosts 138-8 cells which are saos2 cells stably transfected with a temperature sensitive $p53_{Ala138Val}$. To verify the cell system saosts 138-8 cells were cultured at 37˚C and either kept at 37˚C or shifted to 31˚C for 25 h and then cells were analyzed for their cell cycle distribution by cytofluorimetry. As a control the same experiment was repeated for the parental saos2 cells. As shown in Fig. 1a, saos2 cells showed a normal cell cycle profile at both temperatures with most of the cells in G_1-phase of the cell cycle. A similar cell cycle profile was obtained for saosts 138-8 cells at 37˚C indicating that the cells grew normally at this temperature. However, saosts 138-8 cells shifted to 31˚C for 48 h showed a decreased level of cells in G_1-phase and an elevated level of cells in G_2/M-phase of the cell cycle. Fig. 1a shows the percentage of cells in G_2/M-phase under the different conditions. From this figure it is clear that saosts 138-8 cells at 31˚C accumulated in the G_2/M-phase of the cell cycle. To confirm that saosts 138-8 cells indeed arrested in G_2-phase of the cell cycle we determined the mitotic index of cells

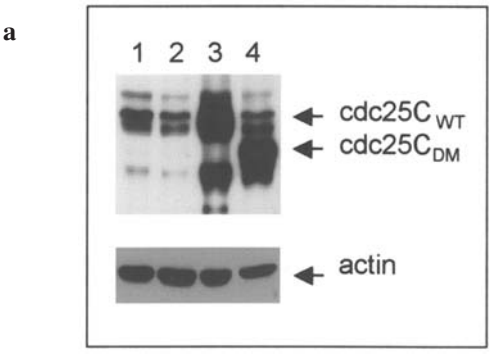
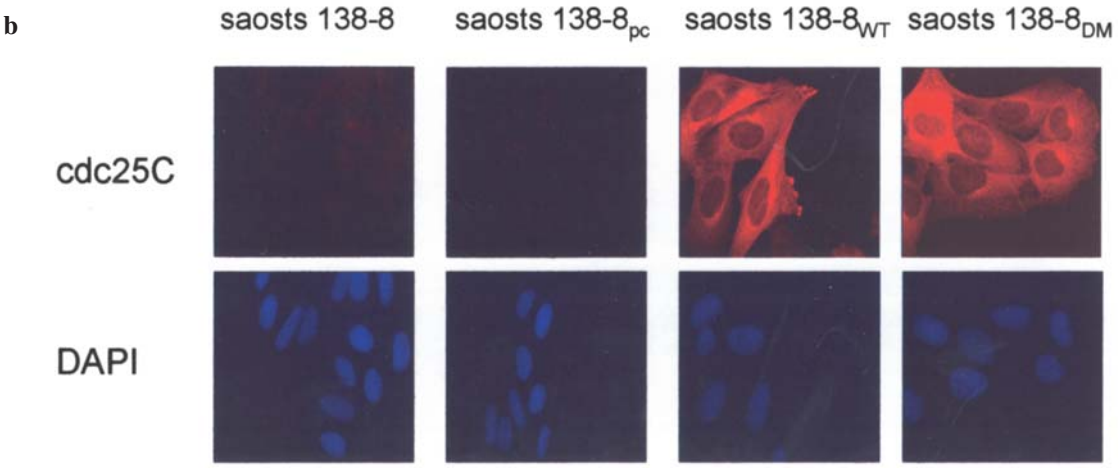


Figure 2. Detection of cdc25C. (a) By Western blot analysis. saosts 138-8 (lane 1), saosts 138-8_{PC} (lane 2), saosts 138-8_{WT} (lane 3) and saosts 138-8_{DM} (lane 4) cells were grown at 37˚C. Total protein (100 μg) from cell extracts were separated by SDS-polyacrylamide gel electrophoresis and then transferred onto a PVDF-membrane. cdc25C was detected with antibody C2-2 and the Lumilight system of Roche. Loading of the lanes was checked by analysis of α-actin. (b) By immunofluorescence analysis. saosts 138-8, saosts 138-8_{pc}, saosts 138-8_{WT} and saosts 138-8_{DM} cells were grown on coverslips at 37˚C. The cells were fixed with 3.7% formaldehyde and permeabilised with 0.5% Triton-X 100. Cells were analyzed with the rabbit polyclonal anti-cdc25C antibody C-20. FITC-conjugated anti-rabbit antibodies were used as secondary antibodies. Nuclear staining was performed with DAPI. Magnification: x400.



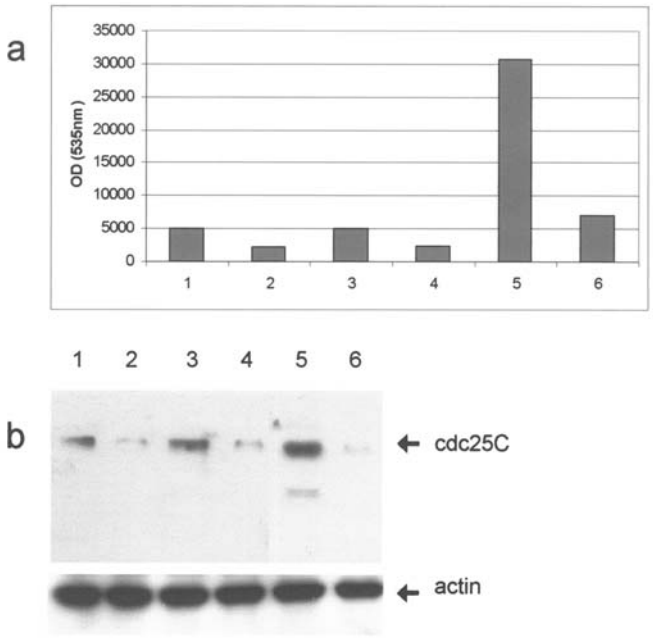


Figure 3. Phosphatase assay in cell extracts before and after depletion of cdc25C. (a) saosts 138-8_{pc} (1, 2), saosts 138-8_{WT} (3,4) and saosts 138-8_{DM} (5,6) cells were grown at 37˚C. Cells were treated with nocodazol (400 ng/ml) for 24 h and mitotic cells were separated by mitotic shake off. The cell extracts were left untreated (1, 3, 5) or depleted for cdc25C with antibody C-20 (2, 4, 6) and then incubated with fluorescein diphosphate (FDP): The fluorescence emission was measured by a 'fluorescence reader' (GENios Spectra Fluor Plus, Tecan) at 535 nm. (b) The same amount of cell extract was analyzed on an SDS polyacrylamide gel. Proteins were transferred to a PVDF-membrane, which was incubated with an antibody directed against cdc25C. To show equal loading of the lanes we used antibody I-19 against actin. Proteins were visualized by the Lumilight system of Roche.

cultured at 37˚C or shifted to 31˚C (23). As shown in Fig. 1b, the mitotic index of saos2 cells is similar at both temperatures whereas the mitotic index of saosts 138-8 cells at 31˚C is drastically reduced when compared to the same cells at 37˚C and compared to saos2 cells at both temperatures. Thus, using different methods we could demonstrate that saosts 138-8 cells showed a p53-dependent growth arrest in the G_2-phase of the cell cycle. In order to analyze whether the temperature shift or the presence of wild-type p53 might have an influence on the expression of cdc25C we used the same conditions for cell cultivation as described above. Cells were extracted and the cell extracts were analyzed on an SDS polyacrylamide gel followed by a Western blot analysis for p53 and cdc25C using appropriate antibodies. The PVDF-membrane was also incubated with an antibody against actin to demonstrate loading of the individual lanes. As shown in Fig. 1c and as expected, p53 is only expressed in saosts 138-8 cells and not in the parental saos2 cells. We found no alteration in the expression of cdc25C in $p53^{-/-}$ and $p53^{+/+}$ cells at either temperature indicating that p53 has no influence on the expression of cdc25C. Thus, neither the temperature shift nor the presence of wild-type p53 had an influence on cdc25C expression.

Having shown that saosts 138-8 cells is a suitable cellular system for the analysis of a suppression of a p53-induced growth by cdc25C we stably transfected saosts 138-8 cells either with an empty vector for control or with $cdc25C_{WT}$. A splice variant of cdc25C ($cdc25C_{DM}$) was discovered (6) which lacks two regions in the N-terminus including 3 out of 5 cdk1 phosphorylation sites. The splice variant $cdc25C_{DM}$ was also stably transfected into saosts 138-8 cells. The corresponding

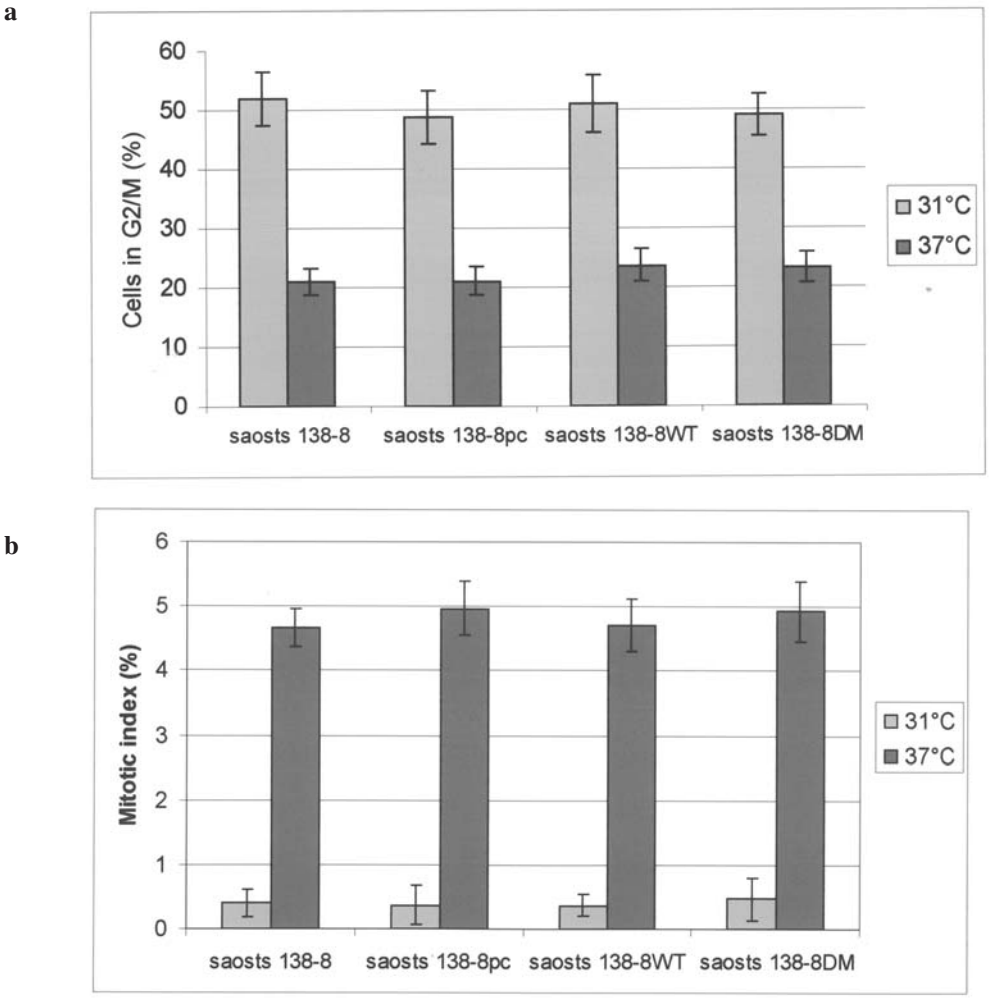


Figure 4. G_2/M arrest of saosts 138-8, saosts 138-8pc, saosts 138-8_{WT} and saosts 138-8_{DM} cells at 37˚C and after shift to 31˚C. (a) Cytofluorimetry. Cells were analyzed by cytofluorimetry and the percentage of cells in the G_2/M phase of the cell cycle was determined. (b) Mitotic index. Cells were incubated for 2 days at 37˚C or 31˚C (cells arrested in G_2/M). After microinjection of tubulin-rhodamine cells were incubated for 1 h at 37˚C or 31˚C in a 5% CO_2 atmosphere. They were washed with PBS and fixed for 10 min at room temperature in 3.7% formaldehyde. The coverslips were mounted on a drop of mounting medium and the cells were analyzed under a fluorescence microscope. The mitotic index of microinjected cells was determined by counting the spindles.

cell lines were named saosts 138-8_{PC} (vector control), saosts 138-8_{WT} (full-length cdc25C) and saosts 138-8_{DM} (splice variant of cdc25C). In order to show an overexpression of cdc25C_{WT} and cdc25C_{DM} in the transfectants cell extracts from these cell lines and from the parental saosts 138-8 cells were analyzed on an SDS-polyacrylamide gel followed by Western blotting with antibody C2-2 directed against cdc25C. For control the filter was stained with an anti-α-actin antibody as a loading control. Fig. 2a, lanes 1 and 2 show the endogenous cdc25C either from saosts 138-8 cells or from cells transfected with an empty vector. Lane 3 shows cdc25C_{WT} in the stably transformed saosts 138-8_{WT} cells and lane 4 shows the expression of the splice variant from saosts 138-8_{DM} cells. This experiment demonstrates a massive overproduction of cdc25C_{WT} and cdc25C_{DM} in these stably transformed cells.

Early studies on the subcellular localization of cdc25C described cdc25C as a nuclear protein (27,28) whereas more recent studies revealed that cdc25C is located in the cytosol during interphase and translocated into the nucleus just before the entry into mitosis (12,29). Since the Western blot analysis had shown a strong expression of cdc25C we wanted to know its localization within the cell. An immunofluorescence analysis showed no signal for cdc25C in the parental saosts 138-8 or in the cells transfected with the empty vector which is due to the low level of expression of endogenous cdc25C. However, strong staining for cdc25C_{WT} as well as for the splice variant cdc25C_{DM} was observed in saosts 138-8_{WT} or in saosts 138-8_{DM} cells which is in agreement with the results obtained in the Western blot experiment. In both cases cdc25C was almost exclusively found in the cytosol (Fig. 2b).

In order to show that overexpression of cdc25C correlates with an elevated cdc25C activity we analyzed the phosphatase activity of cdc25C. Equal amounts of total protein in cell extracts of saosts 138-8 cells, saosts 138-8_{WT} cells and saosts 138-8_{DM} cells were analyzed for phosphatase activity with fluorescein diphosphate as a substrate (26). In order to concentrate on cdc25C activity we treated cells with nocodazol for 24 h and harvested cells by mitotic shake off. The highest cdc25C activity was observed for cells overexpressing

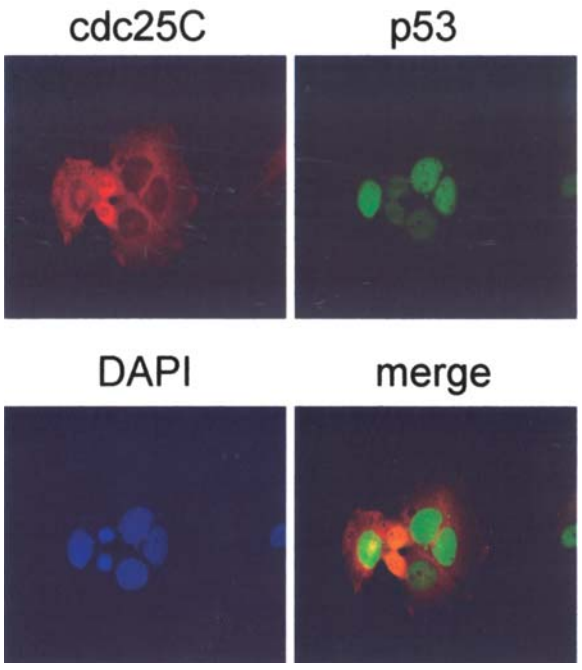


Figure 5. Immunofluorescence analysis of cdc25C and p53 in saosts 138-8_{WT} cells. Cells were grown on coverslips at 37˚C. Cells were fixed with 3.7% formaldehyde and permeabilised with 0.5% Triton-X 100. Cells were analyzed with the mouse monoclonal anti-p53 antibody (PAb421) and the rabbit polyclonal anti-cdc25C antibody C-20 (Santa Cruz). FITC-conjugated anti-mouse and TRITC-conjugated anti-rabbit antibodies were used as secondary antibodies. Nuclear staining was performed with DAPI. Magnification: x400.

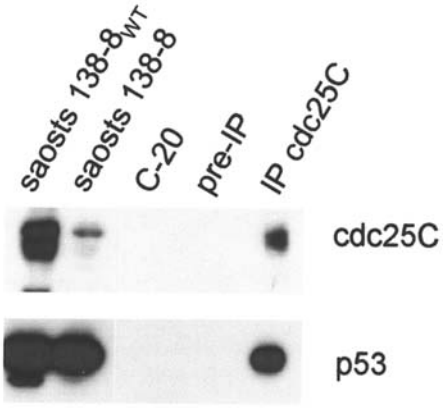


Figure 6. Co-immunoprecipitation of p53 with cdc25C. Protein A-sepharose was pre-incubated with the antibodies (C-20 or PAb421) and washed with PBS. Cell extract (1 mg) was pre-incubated with protein A-sepharose (Amersham-Pharmacia) to remove unspecific-binding proteins. The supernatant was applied to the pre-incubated sepharose-antibody matrix and incubated for 1 h. The supernatant was removed and the antibody matrix was washed 5 times with PBS. The immune complex was subjected to SDS-polyacrylamide gel electrophoresis followed by Western blotting. Proteins were visualized by the Lumilight system of Roche.

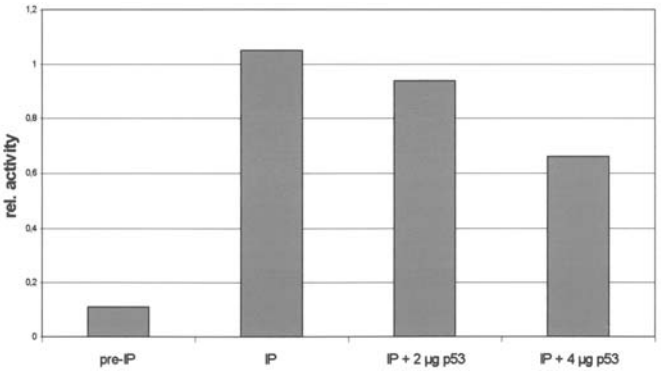


Figure 7. Phosphatase activity of immunopurified cdc25C from saos2 cells in the absence or presence of increasing amounts of p53. saos2 cells were lysed and cdc25C was immunoprecipitated from the cell extract. The immunoprecipitate was divided into three equal aliquots. cdc25C phosphatase activity was measured in the absence (IP) and in the presence of 2 or 4 μg of p53. As a control cdc25C activity was also measured in a pre-immunoprecipitate (pre-IP).

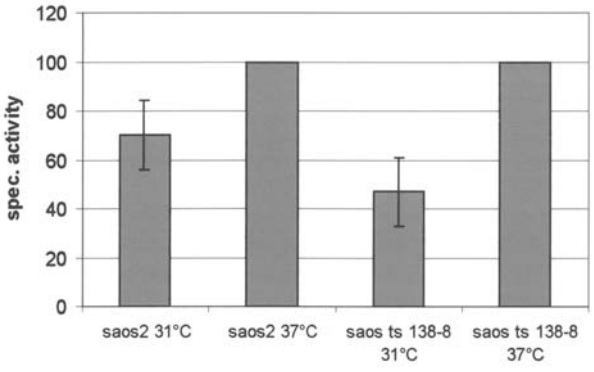


Figure 8. cdc25C phosphatase activity of immunoprecipitated cdc25C from saosts 138-8_{WT} cells and from saos2 cells. Cells were grown at 37˚C and then either further incubated at 37˚C or shifted to 31˚C for 24 h. Cells were lysed and cdc25C immunoprecipitated. Phosphatase activity of immunopurified cdc25C was measured with fluorescein diphosphate as substrate.

cdc25C_{WT} (Fig. 3a). There was a slightly elevated activity for cells expressing cdc25C_{DM} whereas a low activity was found for cell extracts from saosts 138-8 cells or cells transfected with the empty vector. After immunodepletion of cdc25C only a residual activity remained indicating that we indeed measured cdc25C phosphatase activity. Fig. 3b shows the Western blot experiment for cdc25C before and after immunodepletion. After immunodepletion the activity decreased to basal levels corresponding to low level expression of cdc25C. As expected for the splice variant of cdc25C_{DM} we found a considerably lower phosphatase activity due to the missing cdk1 phosphorylation sites. These experiments demonstrated that cdc25C in the transfectants is an active phosphatase and that these cells harbour an elevated phosphatase activity.

Having shown that the transfected cdc25C is functionally active in the next step we wanted to know whether this cdc25C can suppress a p53-induced growth arrest. The parental saosts 138-8 cells and the stably transfected cells were cultured at 37˚C. One half of the cell population was shifted to 31˚C for 24 h. The cell cycle distribution was analyzed by cytofluorimetry. Fig. 4a shows that after a shift to 31˚C all four cell lines accumulated in the G_2/M-phase of the cell cycle regardless of the expression of cdc25C_{WT} or cdc25C_{DM}. In order to analyze whether cell division was indeed inhibited we determined the mitotic index of all four cell lines. As shown

in Fig. 4b, the mitotic index was considerably reduced for the four cell lines upon shift to 31˚C and there was no influence of cdc25C on the cell cycle distribution detectable. Thus, we have shown by two different methods that the overexpressed $cdc25C_{WT}$ and the splice variant $cdc25C_{DM}$ were both unable to suppress a p53 induced growth arrest in these mammalian cells. This observation might be due to different localization of p53 and cdc25C within the cell. Thus, we compared the localization of p53 and of cdc25C in saosts 138-8_{WT} cells by immunofluorescence. In all cells we found p53 exclusively in the nucleus whereas in most of the cells cdc25C was found in the cytoplasm and only in mitotic cells a co-localization of both proteins was found in the nucleus (Fig. 5). According to these results it seems reasonable to assume that cdc25C is unable to overcome a p53 induced growth arrest.

From earlier *in vitro* experiments we know that p53 binds to cdc25C (22). Thus, this interaction might influence the activity of cdc25C directly. Therefore, in the next step we analyzed whether p53 binds to cdc25C also under *in vivo* conditions. saosts 138-8_{WT} cells were extracted and cdc25C was immunoprecipitated with antibody C-20. After intensive washing the immunoprecipitate was loaded onto an SDS-polyacrylamide gel. For control we loaded aliquots of a cell extract from saosts 138-8 cells, from saosts 138-8_{WT} cells, and an antibody control in a non-specific immunoprecipitate on the same gel. Proteins were detected by Western blot analysis with either C-20 for cdc25C or with PAb421 to detect p53. As shown in Fig. 6, we found p53 co-immunoprecipitated with cdc25C (lane IP cdc25C). The experiment was repeated but instead of using C-20 to precipitate cdc25C we used PAb421 for the immunoprecipitation of p53. In this case we found that cdc25C was co-immunoprecipitated with p53 (data not shown). Thus, we have to conclude that p53 binds to cdc25C also under *in vivo* conditions.

Next, we wondered whether binding of p53 to cdc25C might have an influence on the phosphatase activity of cdc25C. Therefore, we immunoprecipitated cdc25C from saosts 138-8_{WT} cells with the cdc25C specific antibody C-20. After washing, the immunoprecipitate was incubated with increasing concentrations of p53. Phosphatase activity was measured with fluorescein diphosphate as a substrate. As shown in Fig. 7, increasing concentrations of p53 led to a decrease in the phosphatase activity in a dose-dependent manner. These results show that p53 functions as an inhibitor of cdc25C.

Next, we analyzed the effect of p53 on the phosphatase activity under *in vivo* conditions. For this experiment we used saosts 138-8 and as a control for the effect of the temperature shift we used saos2 cells. Cells were cultured at 37˚C and either left at 37˚C or shifted to 31˚C for 48 h. cdc25C was immunoprecipitated and the phosphatase activity was measured for immunopurified cdc25C (Fig. 8). Compared to cdc25C from saosts 138-8 cells cultured at 37˚C, cdc25C from the same cells shifted to 31˚C the phosphatase activity was reduced to 47% when the activity from 37˚C cells was set to 100%. In the case of saos2 cells i.e. in the absence of p53 the cdc25C activity dropped to 70% upon shift to 31˚C compared to cells kept at 37˚C which shows the temperature effect. Thus, activation of wild-type p53 in saosts138_{wt} at 31˚C resulted in a decrease of the cdc25C activity.

Discussion

In order to proceed through different phases of the cell cycle in a coordinated temporal, spatial and functional fashion the balance of growth and cell division is maintained by different control checkpoints. The underlying checkpoint control mechanisms are conserved from yeast to man (30,31). Major components of this control machinery are stoichiometric complexes of cyclin dependent kinases which are regulated by upstream activators and inhibitors. In yeast cells cdc25 was identified as an inducer of mitotic control (32). In human cells cdc25 proteins are encoded by a multigene family, consisting of cdc25A, cdc25B and cdc25C (33). In addition to these kinases and phosphatases in cell cycle regulation various feed back control mechanisms converge on the growth suppressor protein p53 (14,34). Human p53 seems to function not only in mammalian cells but also in yeast supporting the notion that cell cycle checkpoint control is conserved in eukaryotic cells (1,20).

Therefore, it was not surprising when it was reported that an ectopic expression of p53 in yeast cells induced growth arrest (20,21). Later on, it was reported that this p53 induced growth arrest was efficiently suppressed by coexpression of human cdc25C. Moreover, it was shown that a normal cell cycle checkpoint after UV treatment of yeast cells was restored in p53 and cdc25C co-expressing yeast cells (1). These observations stimulated us to analyze a possible cooperation of p53 and cdc25C in mammalian cells. For this type of analysis we started with saosts 138-8 cells, which express a temperature sensitive p53. At 37˚C these cells express a mutant form of p53 whereas after the shift to 31˚C these cells express wild-type p53 leading to cell cycle arrest in the G_2 phase of the cell cycle. Starting with this cell line we generated stable transformants expressing full length cdc25C. In addition, we stably transfected a splice variant of cdc25C which lacks 90 amino acids in the N-terminus of cdc25C with an insertion of 17 amino acids due to alternative splicing (6). Cdc25C is phosphorylated by cdk1 at least at 5 different sites and this phosphorylation leads to an elevated phosphatase activity (9,35). Since 3 out of 5 sites are missing in the splice variant one might speculate that this variant has an altered activity. It was shown that this variant can complement a cdc25 mutation in yeast, indicating that these phosphorylation sites are not necessary for this activity. Here, it turned out that neither full length cdc25C nor the splice variant cdc25C suppressed the p53 induced growth arrest in mammalian cells. One explanation for the different results in yeast and in mammalian cells might be that in yeast cells mutant p53 was used whereas in the present study we used wild-type p53.

p53 is mainly a nuclear protein whereas cdc25C is located in the cytoplasm throughout the interphase. Thus, one alternative explanation for the different results in yeast and mammals might be an inefficient translocation of cdc25C from the cytoplasm to the nucleus. However, immuno-fluorescence studies performed in the present study as well as microinjection experiments of cdc25C into the nucleus (data not shown) clearly ruled out this possibility. Another possibility is that p53 down-regulated cdc25C in the stable transfectants because it is known that p53 down-regulates cdc25C transcription (19). However, as shown in Fig. 1c we

could not detect a down-regulation of the expression of cdc25C by p53 neither at 37˚C where a mutant form of p53 is expressed, nor at 31˚C where wild-type p53 is expressed. Another possibility might be binding of p53 to the cdc25C protein. We have previously shown that wild-type p53 binds to and inhibits a number of proteins directly which are implicated in cell proliferation such as protein kinase CK2 (36,37), cdk1 (18) and cyclin H of the cyclin H/cdk7/Mat1 complex (38). Furthermore, we showed that p53 binds to cdc25C at least *in vitro* (22). Herein, we showed that p53 was co-immunoprecipitated with cdc25C and moreover p53 inhibited the phosphatase activity of cdc25C in a dose-dependent manner. Thus, this observation is in line with the other binding partners of p53 with regard to the regulation of cell proliferation. Inhibition of the activity of these proteins by direct binding to p53 is more rapid and efficient than suppression of the transcription of the corresponding genes.

PCNA (proliferating cell nuclear antigen) is one of the very few binding partners of cdc25C. This complex formation seems to be involved in the regulation of the onset of mitosis. Cdc25C and PCNA were shown to be transiently co-localized in the nucleus at the beginning of M-phase (13). Similar to p53, it is possible that the cdc25C/PCNA interaction might be a mechanism to prevent the premature onset of mitosis.

We have shown herein that cdc25C overexpression is unable to overcome a p53-induced growth arrest in mammalian cells. We showed that p53 binds to cdc25C and down-regulates its phosphatase activity. In addition to transcriptional repression of cdc25C by p53, binding of p53 to cdc25C seems to be an additional feature and an attractive alternative mechanism for a rapid G_2/M arrest.

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