

# Subcellular localisation of Cdc25A determines cell fate

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## Abstract

Cell division cycle 25A (Cdc25A) was shown to colocalise both with nuclear and cytoplasmic proteins. Recently, we have demonstrated that overexpressed Cdc25A promoted the survival of rat 423 cells through indirect activation of PKB-protein kinase B. Using a Cdc25A:ER fusion protein, which can be shuttled from the cytoplasm into the nucleus, the present investigation evidences that the antiapoptotic effect of Cdc25A was restricted to its cytoplasmic localisation in rat 423 cells. In contrast, nuclear Cdc25A overexpression caused dephosphorylation and nuclear retention of the proapoptotic transcription factor Forkhead in rhabdomyosarcoma-like 1 (FKHRL1) in human N.1 ovarian carcinoma cells. This resulted in the increased constitutive expression of the FKHRL1 targets Fas ligand and Bim, and promoted apoptosis. Thus, the Cdc25A oncogene, which was found to be frequently overexpressed in certain human cancers, can increase or decrease the susceptibility to apoptosis depending on the cell-type-specific subcellular distribution.

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**Abbreviations:** Akt, PKB-protein kinase B; ASK, apoptosis signal-regulating kinase; ATP, adenosine triphosphate; Bim, proapoptotic member of the Bcl-2 family of proteins; Cdc25A, cell division cycle 25A; Cdk2, cyclin-dependent kinase 2; DAPI, 4'-6-diamino-2-phenylindol; DTT, DL-dithiothreitol; EDTA, ethylenediaminetetraacetic acid; ER, oestrogen receptor; FasL, Fas ligand; FCS, foetal calf serum; FITC, fluorescein isothiocyanate; GST, glutathione-S-transferase; FKHRL1, Forkhead in rhabdomyosarcoma-like 1; HEPES, (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]); HO, Hoechst 33258; 4-OHT,

4-hydroxytamoxifen; IPTG, (isopropyl[ $\beta$ ]-D-thiogalactoside); NLS, nuclear localisation signal; PBS, phosphate-buffered saline; PI, propidium iodide; PMSF, phenylmethylsulphonyl fluoride; PTEN, phosphatase dephosphorylating phosphatidylinositol 3,4,5-trisphosphate (PIP3); SDS, sodium dodecyl sulphate; TNF $\alpha$ , tumour necrosis factor alpha

## Introduction

In a variety of malignant disorders, cell division cycle 25A (Cdc25A) is frequently overexpressed,<sup>1–4</sup> and it has not been determined so far whether the pattern of overexpressed Cdc25A is confined to specific subcellular compartments in the distinct tumour entities. The family of Cdc25 dual-specificity phosphatases controls the cell cycle at different phases.<sup>5–7</sup> Cdc25A was shown to regulate G1–S transition by dephosphorylating threonine 14 and tyrosine 15 of cyclin-dependent kinase 2 (Cdk2),<sup>8–13</sup> and by competition with p21 for cyclinA/Cdk2 and cyclinE/Cdk2 binding sites.<sup>14</sup> Moreover, Cdc25A degradation is part of a DNA-damage checkpoint mechanism.<sup>15</sup> This implicates that Cdc25A activity is focused to the cell nucleus. However, Cdc25A also interacts with Raf1 by virtue of 14.3.3 proteins<sup>16–18</sup> and apoptosis signal-regulating kinase (ASK)1.<sup>19</sup> This, in contrast, points to a cytoplasmic function of Cdc25A. It was demonstrated recently that in rat 423 cells the bulk of Cdc25A was actually localised in the cytoplasm and that apoptosis was inhibited by Cdc25A due to the PKB-protein kinase B (Akt) survival pathway activation.<sup>18</sup> In contrast, endogenous and ectopic Cdc25A is found in the nucleus in N.1 human ovarian carcinoma cells. The question arose as to how far the spatial distribution of Cdc25A is relevant for its antiapoptotic function.

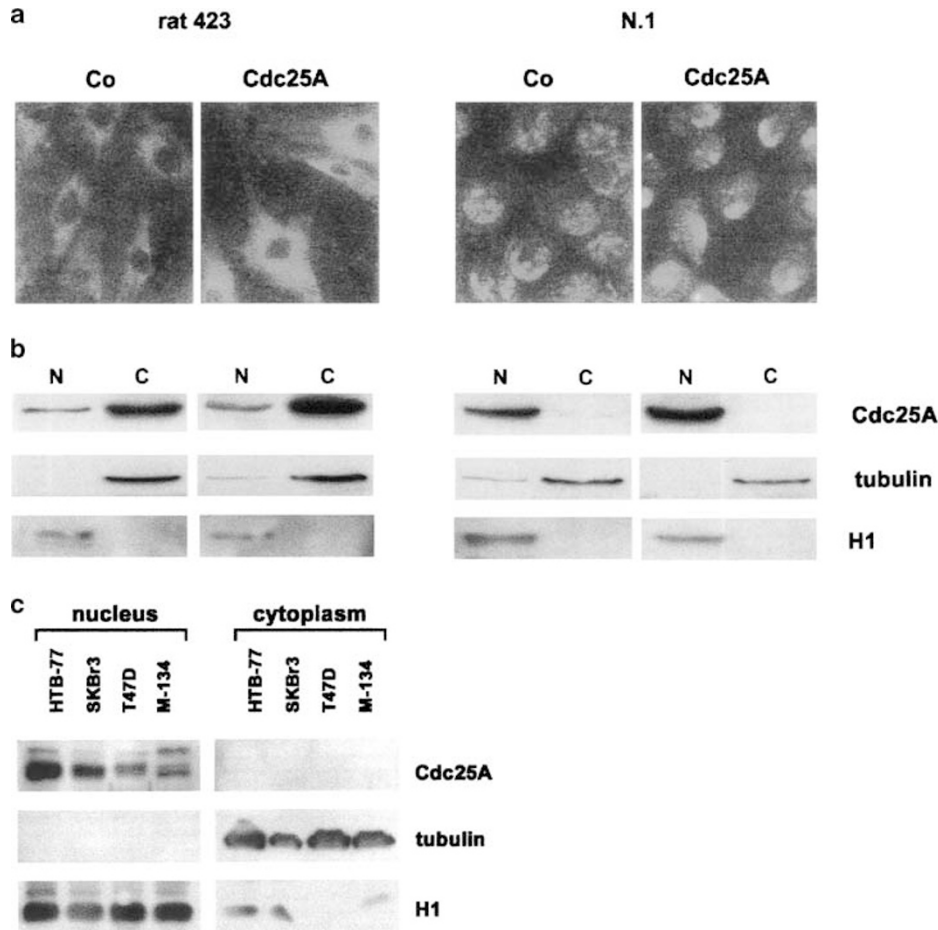
## Results

### Distinct expression of Cdc25A in rat 423 cells and N.1 ovarian carcinoma cells

In earlier studies, it was shown that overexpressed Cdc25A promoted apoptosis in N.1 cells,<sup>20</sup> which in contrast was prevented in rat 423 cells.<sup>18</sup> Immunocytochemistry (Figure 1a) and Western blot analysis of fractionated cell lysates (Figure 1b) revealed that endogenous as well as ectopic Cdc25A was predominantly expressed in the cytoplasm in rat 423 cells, whereas it was restricted to the nuclei in N.1 cells. The analysis of two additional ovarian carcinoma cell lines (HTB-77, M-134) and two breast cancer cell lines (SKBr3 and T47D) revealed that these cell lines also expressed Cdc25A in the nucleus (Figure 1c). Therefore, rat 423 cells were used for further studies regarding the cytoplasmic expression and function of Cdc25A.

### Cdc25A causes dephosphorylation and activation of FKHRL1

In a search for nuclear proteins that elevate the susceptibility to apoptosis when dephosphorylated (e.g. by Cdc25A), we



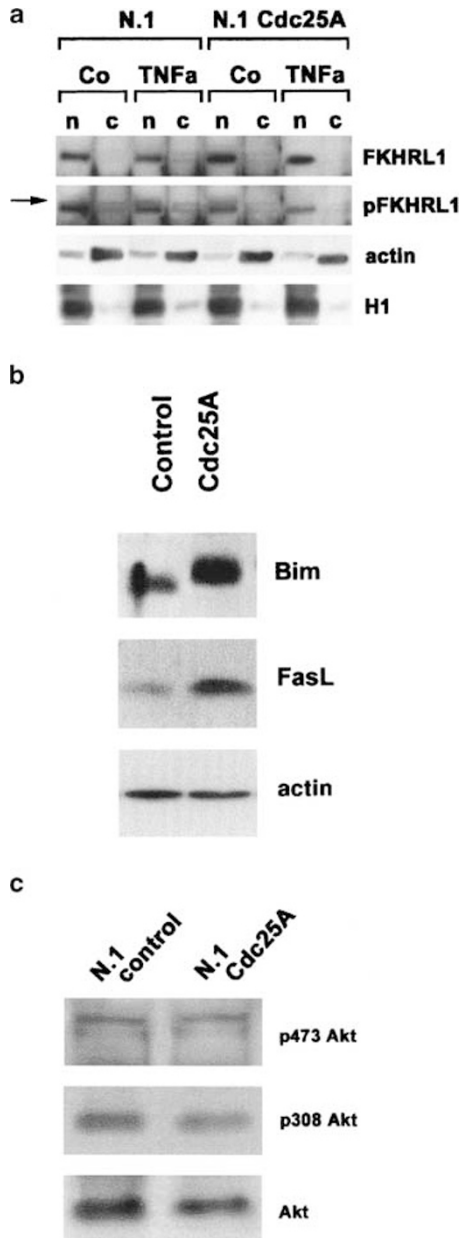
**Figure 1** Detection of subcellular localisation of endogenous and ectopic Cdc25A in rat 423 and human N.1 ovarian carcinoma cells. (a) To detect Cdc25A overexpression by indirect immunofluorescence, the cell clones were grown on siliconised glass slides, then the cells were fixed and incubated with anti-Cdc25A antibody (SC-97), as described in 'Materials and Methods'. Only the nuclei of N.1 cells are stained, but they are larger than the nuclei of rat 423 cells. The intensively stained agglomerations in the Cdc25A-overexpressing N.1 cells are also strictly nuclear. For control, slides were exposed to the second antibody only (not shown). (b) Nuclear (N) and cytoplasmic (C) fractions were prepared from N.1 and rat 423 clones and analysed by Western blotting as described in 'Materials and Methods'. Rat 423 and N.1 cells were either transfected with Cdc25A cDNA (Cdc25A) or with an empty vector (Co). The purity of the subcellular fractions was controlled by analysing the expression of tubulin and histone H1. (c) Detection of subcellular localisation of Cdc25A in human ovarian and breast carcinoma cell lines. Nuclear and cytoplasmic extracts of HTB-77 and M-134 cells (ovarian carcinoma) and of T47D and SKBr3 cells (breast carcinoma) were analysed by Western blotting with monoclonal anti-Cdc25A antibody (Abcam, ab2357). The purity of the subcellular fractions was controlled by analysing the expression of tubulin and histone H1

analysed the expression and the phosphorylation status of the Forkhead transcription factor family members FKHR, Forkhead in rhabdomyosarcoma-like 1 (FKHRL1) and AFX (FOXO4) in Cdc25A-overexpressing N.1 cells, and in N.1-control cells. Tumour necrosis factor alpha (TNF $\alpha$ ) induced the apoptosis of N.1 cells only under serum-deprived culture conditions. Therefore, all the analyses were performed with serum-starved cells. FKHR could not be detected in N.1 clones and AFX did not exhibit a distinct phosphorylation pattern in the cell clones analysed (data not shown). In N.1 cells, FKHRL1 accumulated in the nucleus under serum-starved conditions, which was also shown by others in different cell systems,<sup>21,22</sup> and nuclear expression of FKHRL1 was further increased in Cdc25A-overexpressing cells (Figure 2a, upper panel, compare lanes 1 and 5). It was demonstrated that 14.3.3 proteins bind to phosphorylated FKHRL1 (pFKHRL1) and cause its relocation from the nucleus into the cytoplasm<sup>23,24</sup> where it becomes rapidly

degraded. Nevertheless, pFKHRL1 was still detected as double bands by anti-pFKHRL1 antibody in the nuclei of N.1-control cells and N.1-Cdc25A cells under serum-deprived conditions. However, in N.1-Cdc25A cells, the phosphorylation level of FKHRL1 was 2.5-fold reduced compared to N.1-control cells. This was calculated on the basis of densitometry measurements of pFKHRL1 bands (lane 1 *versus* lane 5, second panel), which were set in relation to total FKHRL1 protein expression levels (lane 1 *versus* lane 5, upper panel), and further standardised by histone H1 expression levels (fourth panel).

Taken together, this implicated that Cdc25A mediated the dephosphorylation of FKHRL1 and caused its nuclear retention.

The slower migrating band of the pFKHRL1 double bands was not detected by the anti-pFKHRL1 antibody in Cdc25A-overexpressing N.1 cells after treatment with TNF $\alpha$  (indicated by an arrow, Figure 2a, second panel, compare lanes 3 and 7).



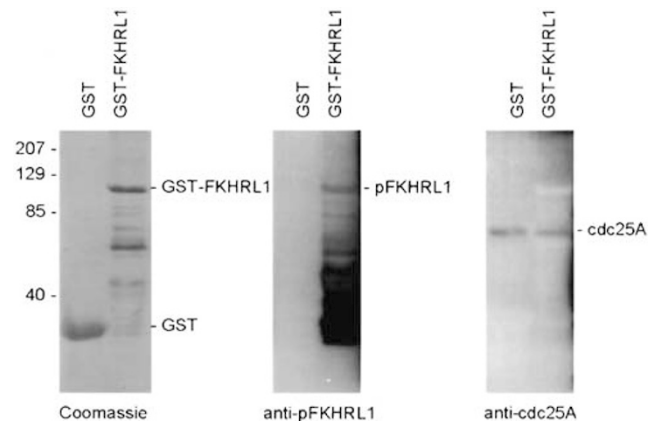
**Figure 2** (a) Expression and phosphorylation of FKHL1 in distinct N.1 clones following TNF $\alpha$  treatment. Control cells (N.1) and Cdc25A-overexpressing cells (N.1-Cdc25A) were grown to 50% confluence, then the serum was withdrawn for 24 h and cells were treated for another 8 h with TNF $\alpha$  (40 ng/ml). Nuclear (N) and cytosolic (C) fractions were prepared and analysed by Western blotting using anti-FKHL1 (upper panel) and anti-phospho<sub>T32</sub> FKHL1 (pFKHL1; second panel) antibodies. The arrow indicates a higher phosphorylated form of FKHL1, which migrates slower. The purity of the subcellular fractions and equal sample loading was controlled by analysing the expression of beta-actin and histone H1 (third and fourth panels). (b) Expression of Bim, FasL and beta-actin in N.1-pwz clones. Total protein of N.1-Cdc25A (Cdc25A) and N.1-control cells was isolated and separated on SDS-PAGE, transferred onto PVDF membranes and immunoblotted with anti-Bim, anti-FasL and anti-beta-actin antibodies. (c) Phosphorylation of Akt in N.1 clones. Total protein of N.1-Cdc25A (Cdc25A) and N.1-control cells was isolated and separated on SDS-polyacrylamide gels, transferred onto PVDF membranes and immunoblotted with an antibody directed against phospho-serine 473 of Akt (p473Akt) and phospho-threonine 308 of Akt (p308Akt). For control reasons, the total Akt expression (Akt) was also investigated

This substantiated the former observation that nuclear over-expression of the phosphatase Cdc25A promoted the dephosphorylation of FKHL1.

Only the nuclear localisation of the FKHL1 transcription factor enables the transactivation of its target genes such as Bim and fasL<sup>23,25,26</sup>. Western blot analyses of Bim,<sup>26,27</sup> and FasL<sup>23,28</sup> evidenced increased expression in N.1-Cdc25A cells compared to N.1 controls, whereas the expression of actin, which is not controlled by FKHL1, remained unchanged (Figure 2b).

It was shown that cytoplasmic Cdc25A indirectly activated Akt by inducing phosphorylation of Akt-threonine 473 presumably by involvement of Raf1.<sup>18</sup> Akt is a kinase regulating FKHL1 phosphorylation, and Akt activity itself is regulated by phosphorylation. Therefore, the phosphorylation levels of Akt at serine 473 and at threonine 308 were investigated. Figure 2c demonstrates that neither of the two sites of Akt became differently phosphorylated in Cdc25A-overexpressing or N.1-control cells, which made an Akt-mediated alteration of FKHL1 phosphorylation in Cdc25A-overexpressing cells unlikely.

Next, we investigated whether Cdc25A interacted directly with FKHL1. A glutathione-S-transferase fusion construct (GST-FKHL1), which was expressed in *Escherichia coli*, was bound to glutathione-sepharose and incubated with native N.1 cell lysate (containing a phosphatase inhibitor) and adenosine triphosphate (ATP) to phosphorylate GST-FKHL1. However, the specific binding of Cdc25A (which was contained in native N.1 cell lysates as an endogenous protein) to FKHL1 could not be detected. The faint Cdc25A bands showing up in the presence of GST-FKHL1 and GST alone (control) were nonspecific contaminations, which remained trapped by sepharose (Figure 3). Even the addition of 150–800 ng of recombinant active Cdc25A to phosphory-



**Figure 3** Interaction of Cdc25A with FKHL1. GST-FKHL1 and GST (for control reasons) was bound to glutathione-sepharose and incubated with native N.1 cell protein lysate and 1 mM ATP at room temperature for 20 min to phosphorylate GST-FKHL1 (pGST-FKHL1) and to promote the binding of Cdc25A to the potential substrate. Then, the incubation was terminated, GST-sepharose and GST-FKHL1-sepharose were washed and centrifuged, resuspended in PAGE sample buffer, boiled and subjected to electrophoretic separation and Western blot analysis. The expression of GST and GST-FKHL1 (Coomassie blue staining; left panel), the phosphorylation level of GST-FKHL1 (middle panel) and the presence of Cdc25A (right panel) were analysed by Western blotting

lated GST-FKHRL1 did not show a specific interaction (data not shown). Furthermore, IP-Western assays, which were performed with native N.1 cell lysates, could not demonstrate a direct interaction of Cdc25A with FKHRL1 (data not shown). Therefore, the dephosphorylation of FKHRL1 in the presence of overexpressed Cdc25A was not mediated by a direct mechanism.

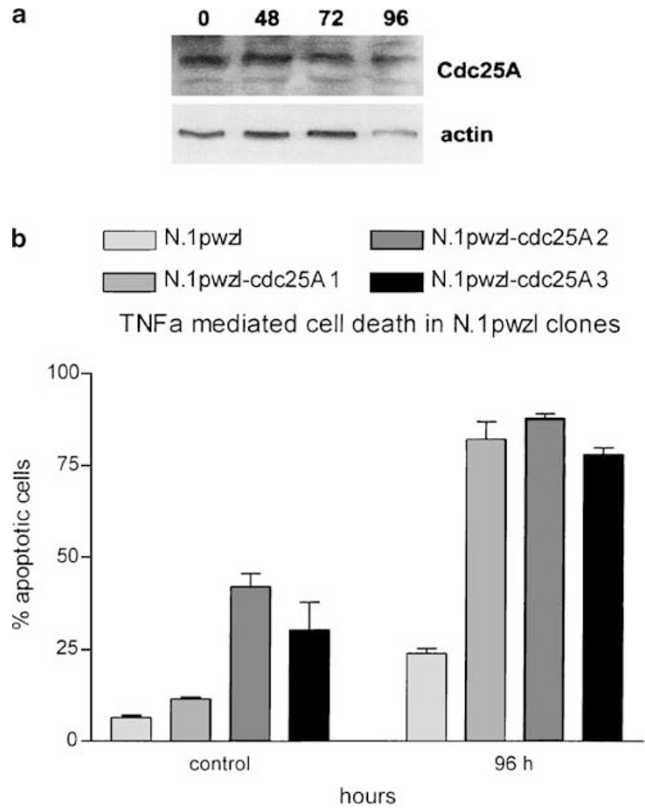
### Cdc25A overexpression supports TNF $\alpha$ -induced apoptosis of N.1 cells

Increased FKHRL1 activity was expected to enforce an apoptotic response due to the transcription of FasL and Bim.<sup>29</sup> Therefore, apoptosis was measured in Cdc25A-overexpressing N.1 cells. To obtain comparable results, Cdc25A was introduced into N.1-pwz1 cells by retroviral transduction (see 'Materials and Methods'). In N.1 cells, it was demonstrated that the induction of apoptosis by TNF $\alpha$  and benzamide riboside repressed endogenous Cdc25A.<sup>30,31</sup> Hence, we analysed the expression of Cdc25A in the presence of TNF $\alpha$  by Western blotting to confirm that ectopic Cdc25A remained expressed (Figure 4a). This was essential for the performance of subsequent experiments. To determine cell death rates, N.1-Cdc25A and N.1-control cells, were exposed to TNF $\alpha$  for 96 h (Figure 4b). On average, TNF $\alpha$  induced 23.8% cell death in control cells and 82.7% cell death in Cdc25A-overexpressing clone pools after 96 h (three independently pooled clones, triplicate experiments). Thus, in analogy to Myc-induced apoptosis,<sup>32</sup> there was also a Cdc25A-dependent increase in cell death rates. Also, in a particular subclone of Rat1 fibroblasts<sup>33</sup> and in vascular smooth muscle cells,<sup>34</sup> Cdc25A promoted apoptosis.

Cdc25A is known to activate Cdk2 and the improper activity of cell cycle protagonists (i.e. Cdk2) may be a potent apoptotic trigger. Therefore, the effect of Cdk2 overexpression on the induction of apoptosis was investigated. However, N.1 cells, in which cdk2 cDNA was stably introduced (Figure 5a), did not show significant differences upon TNF $\alpha$ -induced apoptosis when compared to controls containing an empty vector (Figure 5b). Also, rat 423 cells did not exhibit increased apoptosis rates when Cdk2 was overexpressed (data not shown).

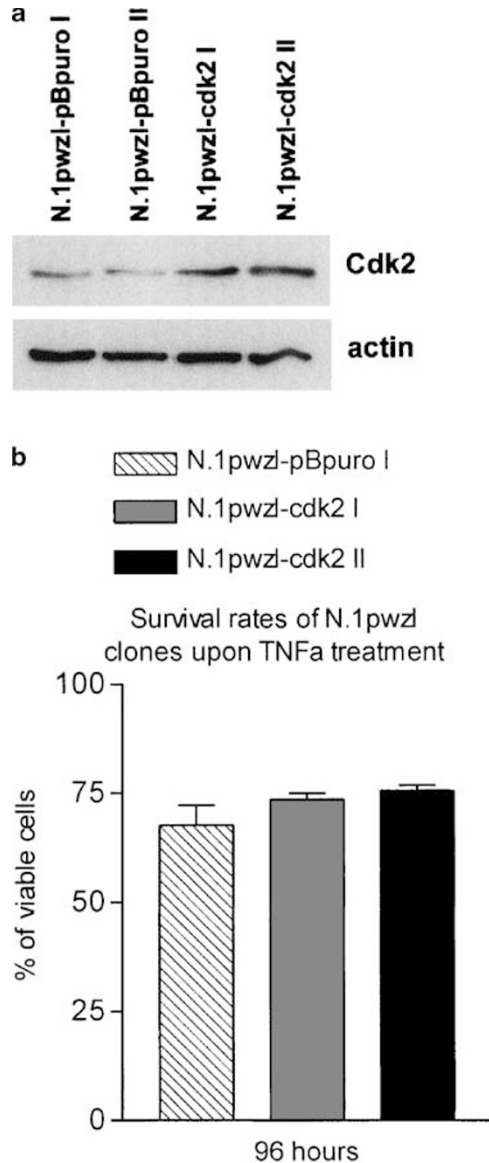
### Cytoplasmic expression of Cdc25A:ER promotes survival

To study the effect of cytoplasmic Cdc25A in a conditional model, the cDNA of Cdc25A was fused to a Tamoxifen (4-hydroxytamoxifen, 4-OHT)-responsive mutant of the oestrogen receptor (ER) resulting in a Cdc25A:ER construct. In the absence of 4-OHT, Cdc25A:ER localises in the cytoplasm. Therefore, a cell line was required, in which the nuclei were constitutively Cdc25A depleted (i.e. rat 423 cells), otherwise the nuclear expression of endogenous Cdc25A and cytoplasmic expression of ectopic Cdc25A:ER would have biased the readout of the survival assays. Since N.1 cells and the cell lines HTB-77, M-134, SKBr3 and T47D expressed endogenous Cdc25A in the nucleus, we had to use rat 423 cells, in



**Figure 4** (a) Sustained expression of ectopic Cdc25A upon treatment with TNF $\alpha$ . N.1-Cdc25A cells were grown to 50% confluence, then the serum was withdrawn for 24 h and 40 ng/ml TNF $\alpha$  was added for the times (in hours) indicated. Thereafter, total protein was isolated and Cdc25A expression was investigated by Western blot analysis. To control sample loading, the expression of beta-actin was analysed. (b) Cell death rates upon TNF $\alpha$ -treatment of independent N.1 clones expressing ectopic Cdc25A. Independently pooled N.1-pwz1 clones were grown in 10% FCS, then the serum was withdrawn for 24 h and thereafter clones were treated with 40 ng/ml TNF $\alpha$  for 96 h. Cells floating in the supernatant were pooled with cells, which were trypsinised from the culture dish, stained with HO/PI and apoptotic cells were identified and counted under UV light in conjunction with a DAPI filter using an inverse microscope. Values represent averages from triplicate experiments with three independent pooled clones (1, 2 and 3). Error bars indicate S.E.M. Significance (s; *t*-test): Control: N.1 versus Cdc25A 1,  $P=0.041$  (non-s); N.1 versus Cdc25A 2,  $P=0.0006$  (s); N.1 versus Cdc25A 3,  $P=0.0357$  (non-s); 96 h TNF $\alpha$ : N.1 versus Cdc25A 1,  $P=0.0003$  (s); N.1 versus Cdc25A 2,  $P=0.0001$  (s); N.1 versus Cdc25A 3,  $P=0.0001$  (s)

which the nuclei were devoid of endogenous Cdc25A, and therefore the Cdc25A:ER fusion construct was stably transfected into this cell line. The ectopic expression of Cdc25A:ER was analysed by Western blotting with anti-ER and anti-Cdc25A antibodies (Figure 6a). To confirm the conditional, 4-OHT-mediated translocation of Cdc25A:ER from the cytoplasm into the nuclei, cells were grown in 10% foetal calf serum (FCS), treated with 4-OHT for 2, 4 and 8 h, and then cytoplasmic and nuclear extracts were prepared and analysed by Western blotting. In response to 4-OHT, cytoplasmic Cdc25A:ER levels decreased time dependently and were redistributed into the nuclei, because the nuclear Cdc25A:ER level increased after 2 h of 4-OHT treatment. Unexpectedly, after 8 h of 4-OHT treatment, the nuclear expression levels of Cdc25A:ER decreased (Figure 6b).



**Figure 5** Effect of Cdk2 overexpression in N.1 ovarian carcinoma cells on survival. (a) Expression of Cdk2 was analysed in N.1-pwzl cells, in which cdk2 cDNA (in vector pBpuro) was stably introduced (cdk2 I and cdk2 II are two independently pooled clones). For control reasons, an empty vector was introduced (pBpuro I and pBpuro II are two independently pooled clones). (b) After withdrawal of serum for 24 h, the independent clones were exposed to 40 ng/ml TNF $\alpha$  for 96 h. Then, adherent cells were trypsinised and counted. The marginal differences in the survival rates of control and Cdk2-overexpressing N.1-pwzl clones were not significant (*t*-test). Error bars indicate S.E.M.

After the induction of apoptosis by serum withdrawal, the survival rates of rat 423 control and Cdc25A:ER-expressing cells were determined. In previous investigations, it was shown that the ectopic overexpression of cytoplasmic Cdc25A prevented apoptosis of rat 423 cells,<sup>18</sup> and here we demonstrate that Cdc25A:ER also accumulated in the cytoplasm in the absence of 4-OHT and rendered rat 423 cells refractory to apoptosis (Figure 6c). In the presence of 4-OHT (added 2 h after serum withdrawal), the antiapoptotic effect of Cdc25A:ER was reverted. Hence, Cdc25A prevented

apoptosis only when it was localised in the cytoplasm. The proapoptotic effect of nuclear Cdc25A:ER (upon 4-OHT-mediated relocation) was weak, because Cdc25A:ER became rapidly degraded in the nuclei of 423 cells, but it was significant (see Figure 6c).

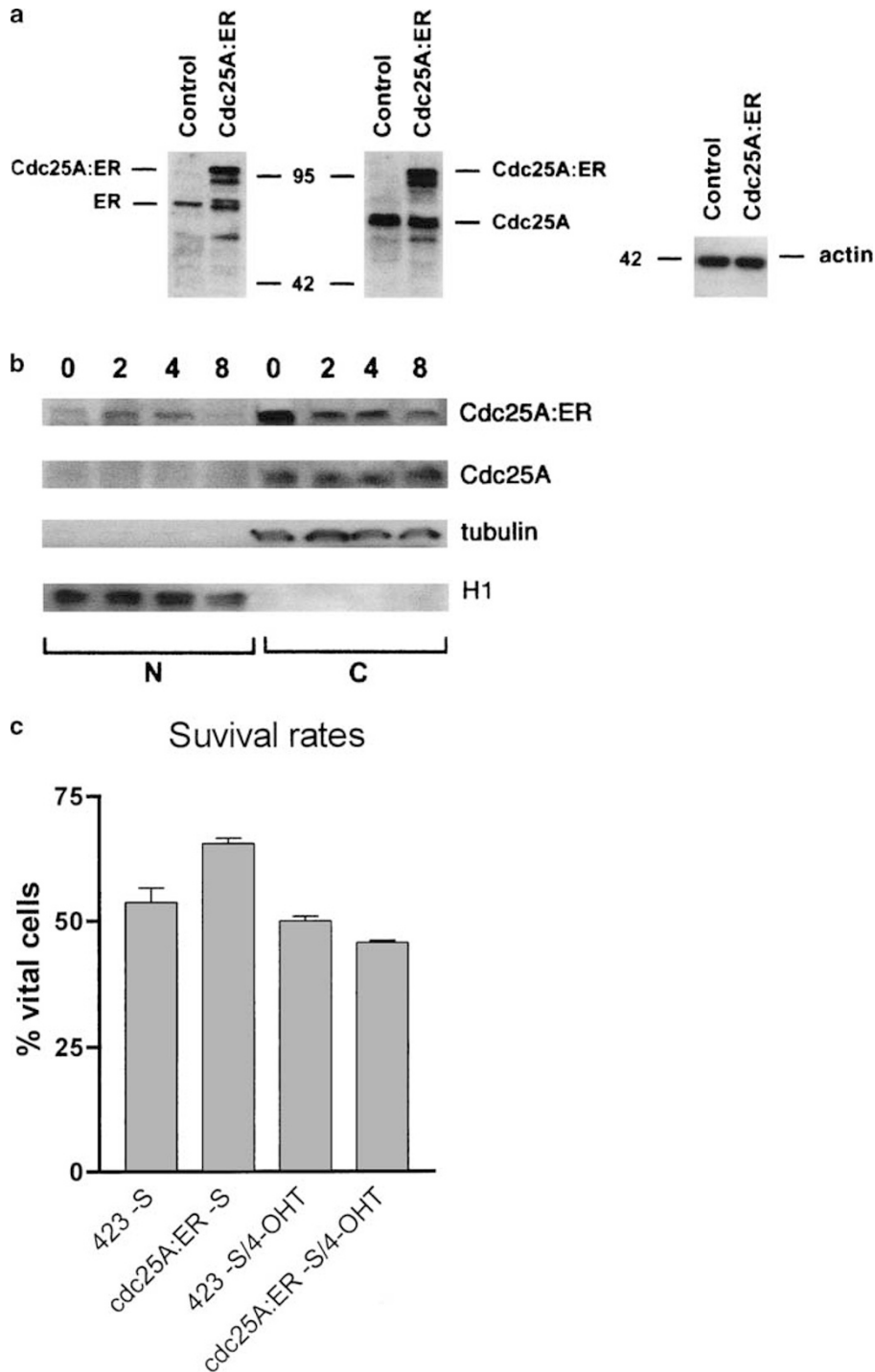
## Discussion

Former work regarding the function of Cdc25A raised indirect evidence for its ubiquitous distribution within cells. On the one hand, Cdc25A was described as a key regulator of G1-S transition by dephosphorylating Cdk2<sup>8</sup> and this implicated that Cdc25A is active in the nucleus. On the other, Cdc25A also interacts with Raf1 by virtue of 14.3.3 proteins,<sup>16,17</sup> implicating a cytoplasmic activity of Cdc25A.

In rat 423 cells, the bulk of anti-Cdc25A immunostaining was in fact localised in the cytoplasm.<sup>18</sup> This was confirmed by immunoblotting rat 423 nuclear and cytoplasmic protein extracts (Figures 1a and b). In rat 423 cells, cytoplasmic Cdc25A was shown to activate the Akt-survival pathway indirectly, thereby preventing apoptosis.<sup>18</sup> Another report demonstrated that Cdc25A inhibited cytoplasmic ASK1 independent of its phosphatase activity, thereby blocking cell death triggered by oxidative stress.<sup>19</sup> Hence, we fused the cDNA of Cdc25A to that of ER, which allows for Tamoxifen (4-OHT)-mediated shuttle of the Cdc25A:ER protein from the cytoplasm into the nucleus. The results from this investigation suggested that Cdc25A protected from apoptosis only when it was localised in the cytoplasm, because upon 4-OHT treatment, the cytoplasmic Cdc25A:ER level diminished and increased in the nuclei of rat 423 cells within 2 h, followed by degradation within 8 h. Hence, the loss of antiapoptotic function of Cdc25A:ER correlated with the decreased cytoplasmic expression upon exposure to 4-OHT. The rapid nuclear degradation of imported Cdc25A:ER (mediated by 4-OHT) may explain why the proapoptotic effect of (short-lived) nuclear Cdc25A:ER was weak (but significant).

Upon treatment with 4-OHT, the decay of nuclear Cdc25A:ER might have been triggered by the ER portion and ubiquitination of ER might have also tagged Cdc25A to proteasomal degradation. The stability of ER was shown to depend on the type of ligand bound to ER and on the cellular context.<sup>35</sup> Alternatively, Cdc25A itself might have been the prime target for proteolytic degradation as soon as it was relocated to the nucleus of rat 423 cells. Since the bulk of endogenous Cdc25A was found in the cytoplasm, but not in the nuclei of rat 423 cells, this favours the possibility of nucleus-specific proteolysis of Cdc25A in this cell line and makes rat 423 cells a valuable tool to study spatial functions of Cdc25A. Furthermore, Cdc25A contains both a nuclear localisation signal and a nuclear export signal, and it is possible that either of the two mechanisms might be dysregulated, thereby causing a decrease in nuclear Cdc25A levels. Future work in our lab will focus on the subcellular expression of Cdc25A in distinct cell types and on physiological relocation mechanisms.

As pointed out in this investigation, Cdc25A accumulated *a priori* in the nuclei of N.1 human ovarian carcinoma



**Figure 6** (a) Ectopic Cdc25A:ER expression in rat 423 cells. Total protein of rat 423-Cdc25A:ER cells (clone B/N2; Cdc25A:ER) and of rat 423 (Control) cells was isolated and separated on SDS-PAGE, transferred onto a PVDF membrane and immunoblotted with anti-ER  $\alpha$  (left side panel) and anti-Cdc25A (SC-7157; right side panel) antibodies. Equal loading was confirmed by analysing beta-actin expression. (b) 4-OHT-mediated subcellular shuttle of Cdc25A:ER. Cdc25A:ER-expressing rat 423 cells were treated with 4-OHT (750 nM) for 0, 2, 4 and 8 h. Then nuclear (N) and cytoplasmic (C) fractions were isolated and analysed by Western blotting with an anti-Cdc25A (SC-7389) antibody. The upper panel shows ectopic Cdc25A:ER-expression and the second panel shows endogenous Cdc25A-expression. The purity of the subcellular fractions was controlled by analysing the expression of tubulin and histon H1 (third and fourth panels). (c) Survival rates of Cdc25A:ER-expressing rat 423 cells upon serum deprivation and 4-OHT treatment. Cells were grown in 10% FCS to confluence, then the serum was withdrawn. After 2 h, 4-OHT (500 nM) was added. After 24 h, viable cells that remained attached to the culture plate were trypsinised and counted. Values show the average of triplicate experiments in percent. Error bars indicate S.E.M. Significance (s; *t*-test): 423 *versus* Cdc25A:ER,  $P=0.0014$  (s); 423 *versus* 423 + 4-OHT,  $P=0.2308$  (non-s); 423 *versus* Cdc25A:ER + 4-OHT,  $P=0.0005$  (s); 423 + 4-OHT *versus* Cdc25A:ER + 4-OHT,  $P=0.0001$  (s)

cells. Therefore, a survival-promoting effect of Cdc25A seemed unlikely. Notably, ectopic Cdc25A overexpression resulted in increased spontaneous apoptosis rates in pooled N.1 clones.

The ectopic overexpression of Cdc25A was ~2-fold in N.1 cells and that of Cdc25A:ER ~3-fold in rat 423 cells. Also in earlier investigations,<sup>18</sup> we did not observe that ectopic Cdc25A was expressed beyond two- to three-fold over endogenous expression. We assume that excessive (nuclear) expression has adverse effects for these cells, and therefore such clones, which vastly overexpress Cdc25A, will vanish by negative selection. Surprisingly, even in rat 423 cells, in which Cdc25A was shown to predominate in the cytoplasm and protects from apoptosis, only a two- to three-fold overexpression was observed in a panel of 12 independent Cdc25A overexpressing clones. This suggests that in various cell types, only a limited level of Cdc25A expression is tolerated and further implicates that Cdc25A seems to be a fine-tuned key regulator (perhaps even rate limiting) for different cellular processes. This assumption is supported by the fact that even moderate overexpression of Cdc25A caused dramatic effects that are beyond a direct correlation with respect to the expression level. Interestingly, the constitutive Cdc25A-expression levels of HTB-77 *versus* M-134 ovarian carcinoma cells differed by a factor of ~10. It can be speculated that HTB-77 possess an appropriate (distorted) genetic background, which allows for this high Cdc25A expression without adverse effects.

This investigation furthermore demonstrates that at least phospho-threonine 32 of FKHRL1, which targets FKHRL1 for 14.3.3-mediated cytoplasmic export, became dephosphorylated upon nuclear Cdc25A overexpression in N.1 cells. FKHRL1 is a transcription factor<sup>23,28,36,37</sup> and to be active its nuclear abundance is mandatory. Also phosphorylation at serine 253 defines cytoplasmic localisation by disrupting a nuclear localisation signal (NLS) on FKHRL1.<sup>36</sup> Both phosphorylation sites may cooperate in determining the subcellular distribution of FKHRL1. The regulatory functions of phosphorylated serine 315 and of other phosphosites are currently unknown. Dephosphorylation of FKHRL1 is necessary for its nuclear retention and in fact the level of nuclear FKHRL1 was elevated in Cdc25A-overexpressing N.1 cells. This may have caused the activation of FasL<sup>28,23</sup> and Bim transcription,<sup>27,26</sup> and the enhanced apoptotic response in Cdc25A-overexpressing N.1 cells.

The dephosphorylation of FKHRL1 requires a direct interaction with phosphatase(s). However, neither *in vitro* binding studies with a GST-FKHRL1 fusion protein nor pull-down assays from native cell extracts (data not shown) confirmed direct binding of Cdc25A to FKHRL1. Hence, Cdc25A seems unlikely to dephosphorylate FKHRL1 itself, but to either induce another phosphatase or to inactivate a kinase that regulates FKHRL1 phosphorylation. It was reported that FKHRL1 becomes directly phosphorylated by kinases such as Akt,<sup>23,28,38,39</sup> whereas PTEN mediates indirect dephosphorylation of FKHRL1.<sup>40</sup>

In conclusion, this study demonstrates that the nuclear overexpression of Cdc25A indirectly mediated the dephosphorylation of threonine 32 of FKHRL1 and promoted

apoptosis, whereas the cytoplasmic overexpression of Cdc25A was apoptosis protective.

Since numerous human cancers overexpress Cdc25A,<sup>1–4</sup> the determination of the spatial expression may be relevant to evaluate its impact on prognosis and therapy.

## Materials and Methods

### Materials

Human cdc25A cDNA was a kind gift from Dr. David Beach (Cold Spring Harbour Laboratories, NY, USA), cdk2 cDNA was a gift from Dr. Hannes Hofmann (IMP, Vienna), the pBabe-puro (pBpuro) vector was a gift from Dr. Trevor Littlewood (ICRF, London, UK), the GST-FKHRL1 fusion construct was a gift from Dr. Anne Brunet (Harvard, Boston, USA) and the pwz1 plasmid was a gift from Dr. Manuel Serrano (National Center of Biotechnology, Madrid, Spain). The Phoenix ecotropic packaging cell line was a gift from Dr. Gary Nolan, (Department of Molecular Pharmacology, Stanford University School of Medicine). The cell lines HTB-77 and M-134 were a gift from Dr. Thomas Grunt and T47D cells were a gift from Dr. Sabine Wohlfart (both University of Vienna, Vienna).

The recombinant active CDC25A enzyme (10 U/ $\mu$ l; 61 U/ $\mu$ g) was obtained from Upstate. Anti-Cdc25A (Abcam ab2357) 1:250, anti-Cdc25A (Santa Cruz, sc-97) 1:10 000, anti-Cdc25A (sc-7157) 1:5000, anti-Cdc25A (sc-7389) 1:200, anti-Cdk2 (sc-163) 1:10 000, anti-histon H1 (sc-8030) 1:20 000, anti-tubulin (sc-8035) 1:20 000, anti-ER $\alpha$  (sc-542) 1:500, anti-Akt (Cell Signaling #9272) 1:1000, anti-P-Akt (Thr 308; Cell Signaling #9275) 1:1000, anti-phospho (Thr32) FKHRL1 (Cell Signaling #9464) 1:1000, anti-phospho-AFX (Cell Signaling #9471) 1:1000, anti-FKHRL1 (Upstate #06-951) 1:500, anti-FasL (BD Transduction Lab. 610410) 1:1000, anti-Bim (Pro Sci. Inc. 2065) 1:1000 and anti-beta-actin (Sigma, A5441) 1:20 000 were used for Western blotting in the indicated dilutions.

### Cell culture

Ovarian carcinoma cell lines HTB-77 and M-134 were grown in alpha-MEM, the breast cancer cell line SKBr3 was grown in McCoys medium and the breast cancer cell line T47D and rat 423 cells were grown in DMEM, all containing 10% FCS and 1% penicillin–streptomycin. Rat 423-Cdc25A:ER cells were grown in DMEM containing 10% FCS, 1% penicillin–streptomycin and 2  $\mu$ g/ml puromycin.

The monoclonal human ovarian adenocarcinoma N.1 cell line is a derivative of the heterogeneous HOC-7 cell line that was cultured in MEM supplemented with 10% FCS (GIBCO, Paisley, UK), penicillin–streptomycin (100 U/ml–100  $\mu$ g/ml, Gibco-BRL) (medium A), at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>.

N.1-pwz1 cells were grown in MEM containing 200  $\mu$ g/ml G-418 sulphate.

N.1-pwz1-Cdc25A and N.1-pwz1-Cdk2 cells (both in pBpuro), and N.1-pwz1-pBpuro cells (control) were grown in a-MEM with the addition of 0.4  $\mu$ g/ml puromycin and 200  $\mu$ g/ml G-418 sulphate.

### Subcloning procedures

The cdc25a or egfp (for control reasons) cDNA was subcloned into the *Xho*I and *Not*I site of the pCI<sup>neo</sup> mammalian expression vector, which contains a CMV promoter (Promega, Mannheim, Germany) or blunt end ligated into the *Sna*BI site of pBpuro.

**Cdc25A:ER construction:** The *cdc25a* cDNA was released from the pBluescript vector with *Bss*HI and *Bsr*GI. In all, 5' overhangs were filled with Klenow and ligated into the Klenow-filled *Bam*HI site of Myc:ER that is inserted in the pBpuro vector (*Bam*HI releases myc from this construct, which was a kind gift of Trevor Littlewood<sup>41</sup>). The correct reading frame was checked by DNA sequencing. C-terminal loss of six amino acids does not infringe the active centre of the Cdc25A phosphatase domain.

### Transfection of cells

Rat 423 cells were plated into 60 mm dishes and grown for 2 days to 40–50% confluence. Lipofection of cells with Cdc25A:ER cDNA (in pBpuro) was performed utilising the Transfectam reagent (Promega). The cells were washed once with prewarmed DMEM without FCS. A total of 5  $\mu$ g DNA (1  $\mu$ g/ $\mu$ l) and 10  $\mu$ l Transfectam were separately pipetted into 0.75 ml DMEM each and then both mixtures were combined. Transfectam–DNA complexes were allowed to form for 10 min and were then applied onto rat 423 cells for 3 h in a humidified incubator. The transfection was stopped by adding 4 ml DMEM containing 10% FCS. The next day, the cells were split into new dishes with a fresh medium and 48 h after transfection, selection drug 2  $\mu$ g/ml was added and single clones were isolated with cloning cylinders and were propagated separately. Where indicated, pooled clones were propagated.

### Generation of ecotropic mouse retrovirus transducible N.1 human ovarian carcinoma cells

N.1 cells were stably pretransfected with mouse retrovirus receptor (pwzI, G-418 selection) and then various single clones were infected with EGFP. Those clones, which expressed functional pwzI (identified by green fluorescence when inspected under UV light in conjunction with a fluorescein isothiocyanate (FITC) filter), were pooled and used for control reasons. The parental clones, which were identified by this assay (N.1-pwzI) to express the functional retrovirus receptor, were also pooled and this pooled clone was subsequently infected with ectopic mouse retrovirus containing Cdc25A. This procedure generated five independent Cdc25A pools from two independently packaged retrovirus stocks. Cdc25A overexpression was tested by Northern and Western blot analyses.

### Production of retrovirus titre

Phoenix cells were grown in 100 mm dishes in DMEM, supplemented with 10% FCS and 50  $\mu$ g/ml hygromycin B. The transfection procedure (Transfectam) was upscaled for 100 mm dishes and performed, as described in the Promega protocol. The cells were transfected at 80% confluence with 12.5  $\mu$ g plasmid DNA (concentration 1  $\mu$ g/ $\mu$ l) in 37.5  $\mu$ l Transfectam (1 : 3 ratio w/vol). After 1 h of incubation, the medium was replaced by 10 ml fresh medium with 10% FCS. After 24 h, the medium was replaced with 4 ml of complete medium (10% FCS). At 48 h after transfection, the retrovirus containing cell culture supernatant was filtered (0.22  $\mu$ m filter), aliquoted, shock-frozen in liquid nitrogen and stored at  $-80^{\circ}$ C.

### Infection of cells

N.1-pwzI cells were grown in six-well plates to 50% confluence. A measure of 500  $\mu$ l virus suspension was mixed with 500  $\mu$ l MEM, containing 10% FCS. Then Polybrene (Sigma) was added to a final concentration of

4  $\mu$ g/ml. After an infection period of 3 h in the cell culture incubator, 2 ml of MEM with 10% FCS was added. After 24 h, cells were transferred into larger dishes to allow cell growth. At 48 h after infection, a selective medium (1  $\mu$ g/ml puromycin, Sigma) was added to kill noninfected cells. After 10–12 days of selection, the puromycin concentration was reduced to 0.4  $\mu$ g/ml.

Several independent Cdc25A and control pools of infected N.1-pwzI cells were used for further experiments.

### Immunocytochemistry

Cell clones were grown on siliconised glass slides. Then, cells were washed twice with phosphate-buffered saline (PBS) (pH 7.2), air-dried, fixed with ice-cold acetic acid : ethanol (1 : 3) for 10 min and permeabilised with Triton X-100 (0.5% in PBS) for 3 min at room temperature. Then slides were washed three times with washing buffer (0.5 M Tris-HCl, pH 7.5) to remove Triton X-100. Anti-Cdc25A rabbit polyclonal antibody was diluted in 0.5 M Tris buffer, 1% BSA pH 7.5 (anti-Cdc25A antibody dilution was 1 : 50 of a 100  $\mu$ g/ml stock) and incubated in a humid chamber for 1 h at room temperature. After washing, the slides were incubated with FITC-conjugated second antibody (goat anti-rabbit IgG, dilution 1 : 30; Dako) for 30 min. Finally, slides were covered with a mounting medium (Geltol mounting medium for fluorescence, Lipshaw) and a coverslip. For control, slides were exposed to the second antibody only.

### Nucleus–cytoplasm fractionation

Cells were washed in PBS and lysed in buffer A (10 mM, (HEPES), 10 mM KCl, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mM EGTA, 1 mM DL-dithiothreitol (DTT), 1.5% NP-40, PIM, phenylmethylsulphonyl fluoride (PMSF)), on ice for 10 min and centrifuged at 12 000 rpm. Supernatants were collected as cytoplasmic extracts.

The pellets were washed, resuspended in buffer B (20 mM HEPES, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.05% NP-40, PIM, PMSF) and agitated at  $4^{\circ}$ C for 20 min. After centrifugation at 12 000 rpm, the supernatants were collected as nuclear extracts. The extracts were analysed by Western blotting.

### Western blotting

Experiments were terminated by washing attached cells twice with ice-cold PBS (pH 7.2) and harvesting the monolayer with 200  $\mu$ l sodium dodecyl sulphate (SDS) sample buffer, or with the buffers indicated. The viscous samples were sonicated and equal amounts of total protein were separated by polyacrylamide gel electrophoresis (PAGE). Subsequent to the electrophoretic separation, the proteins were electroblotted onto PVDF-membranes (Hybond, Amersham) overnight at  $4^{\circ}$ C. To confirm equal sample loading, membranes were stained with Ponceau S. After washing with PBS or TBS, membranes were blocked for 1 h (blocking solution: 5% nonfat dry milk in PBS containing 0.5% Tween-20 or in TBS containing 0.1% Tween-20) and washed  $3 \times$  with PBS/Tween-20 (PBST) or TBS/Tween-20 (TBST). Then, membranes were incubated with the first antibody (in blocking solution) by gently rocking at  $4^{\circ}$ C, overnight. Thereafter, the membranes were washed in blocking solution ( $1 \times$ ) and in PBS/TBST ( $2 \times$ ) and further incubated with the second antibody (peroxidase-conjugated goat anti-rabbit IgG or anti-mouse IgG, dilution 1 : 2000–1 : 5000 in PBST or TBST; Calbiochem) for 1–2 h. Chemoluminescence was developed by the ECL detection kit (Amersham, UK) and then membranes were exposed to Kodak X-OMAT UV films.



## Cell death experiments and analysis

Apoptotic cell death was induced by stimulation with TNF $\alpha$ . To determine the number of surviving cells, the individual N.1 clones were grown in six-well plates until 20% confluence. After 24 h of serum withdrawal (0% FCS), TNF $\alpha$  was added for the times indicated. The surviving cells were trypsinised and pooled with supernatant cells and stained with Hoechst 33258/propidium iodide (HO/PI; Hoechst: final concentration 5  $\mu$ g/ml and PI: final concentration 2  $\mu$ g/ml) to monitor nuclear chromatin condensation, using a 4'-6-diamino-2-phenylindol (DAPI) filter in connection with a Zeiss Axiovert microscope.<sup>42–44</sup>

Apoptosis of rat 423 cells, which was induced by serum withdrawal ( $\pm$ 500 nM 4-OHT), was measured as described.<sup>18</sup>

## Expression, *in vitro* phosphorylation of GST-FKHRL1 and conjugation to glutathione-sepharose

The GST (in vector pGEX) and GST-FKHRL1 (aa 1–525) fusion protein were produced in *E. coli* strain BL21. Protein expression from pGEX plasmids was induced in liquid cultures by the addition of 1 mM isopropyl [ $\beta$ ]-D-thiogalactoside (IPTG) for 1 h. Cells were sonicated in PBS containing 0.1% Triton X-100 to obtain protein lysates. For purification and conjugation and immobilisation, the lysates containing GST (control) and GST-FKHRL1 were incubated with glutathione-sepharose for 1 h at 40°C with gentle agitation. After several washing steps, the immobilised GST and GST-FKHRL1 (conjugated to glutathione-sepharose) was incubated with native N.1 cell lysate and 1 mM ATP to allow/enhance the phosphorylation of GST-FKHRL1. Protein expression and phosphorylation status was analysed by Coomassie blue staining and Western blotting.

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