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Identification of a novel cyclin required for the intrinsic apoptosis pathway in lymphoid cells

MB Roig[1,4,5], R Roset[1,5], L Ortet[1], NA Balsiger[1], A Anfosso[1], L Cabellos[1], M Garrido[1], F Alameda[2], HJM Brady[3] and G Gil-Gómez*[,1]

We have identified an early step common to pathways activated by different forms of intrinsic apoptosis stimuli. It requires *de novo* synthesis of a novel cyclin, cyclin O, that forms active complexes primarily with Cdk2 upon apoptosis induction in lymphoid cells. Cyclin O expression precedes glucocorticoid and γ-radiation-induced apoptosis *in vivo* in mouse thymus and spleen, and its overexpression induces caspase-dependent apoptosis in cultured cells. Knocking down the endogenous expression of cyclin O by shRNA leads to the inhibition of glucocorticoid and DNA damage-induced apoptosis due to a failure in the activation of apical caspases while leaving CD95 death receptor-mediated apoptosis intact. Our data demonstrate that apoptosis induction in lymphoid cells is one of the physiological roles of cyclin O and it does not act by perturbing a normal cellular process such as the cell cycle, the DNA damage checkpoints or transcriptional response to glucocorticoids.
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The role of cyclins and Cdks in cell cycle regulation is clear, based on compelling data from genetic and biochemical approaches.[1] However, their role in apoptosis is not so well defined, due to the lack of clear genetic evidence.

Thymocytes are a good model to study the connection between apoptosis and cell cycle, as about 90% of the cells of the normal thymus are quiescent[2] and do not progress through the cell cycle *in vitro*. As a consequence, any activation of cell cycle-related kinases is not necessarily related to cell cycle progression. Activation of Cdk2 has been shown to be obligatory for the apoptosis of quiescent cells such as thymocytes[3] as well as endothelial cells dying by trophic factor withdrawal.[4] This activation of Cdk2 is specific to apoptosis and is not an abortive attempt to re-enter the cell cycle.[3]

We have shown earlier[5] that Cdk2 activation is an early step in cell death that precedes both loss of plasma membrane asymmetry and activation of apical caspases. This implies that Cdk2 activation must be upstream of the translocation of the Bcl2 family members Bax and Bid to the mitochondria and the loss of mitochondrial function that finally results in the release of cytochrome *c* into the cytoplasm.

Activation of Cdk2 during thymocyte apoptosis does not seem to be a consequence of binding to cyclin A or cyclin E, the canonical Cdk2-activating cyclins in its cell cycle regulatory function.[3] We report here the isolation and characterisation of a Cdk2- and Cdk1-activating cyclin specific to apoptosis. This novel cyclin (cyclin O) is able to bind and activate Cdk2 in response to intrinsic apoptotic stimuli such as glucocorticoids or DNA-damaging agents. The *de novo* synthesis of cyclin O precedes apoptosis induction. Down-regulation of the expression of cyclin O abrogates DNA damage- and dexamethasone-induced apoptosis, whereas CD95-induced apoptosis remains intact. This lack of apoptotic response to DNA damage and glucocorticoids is due to a failure to activate a supramitochondrial step leading to the activation of apical caspases and not due to defective signalling to these intrinsic stimuli. Cyclin O is a novel cyclin that can induce apoptosis in lymphoid cells in concert with Cdk2.

Results

Cloning of mouse and human cyclin O. Our earlier results suggested the possibility that an unidentified cyclin-like protein could be responsible for the activation of Cdk2 during the apoptosis of quiescent cells in response to intrinsic, but not extrinsic, stimuli in thymocytes.[3,6] Using an *in silico* analysis of the human genome, we identified several non-characterised cyclins, from which preliminary results encouraged us to further characterise one of them located on human chromosome 5 (on mouse chromosome 13), later designated as cyclin O.[7]

The generic cyclin O locus encodes four transcripts that arise from the use of three alternative promoters (Figure 1a): P1 (cyclin Oα and the alternatively spliced product cyclin Oβ

[1]Apoptosis Signalling Group, Cancer Research Programme, Institut Municipal d'Investigació Mèdica, Parc de Recerca Biomèdica de Barcelona, Dr. Aiguader, 88. 08003 Barcelona, Spain; [2]Department of Pathology, Hospital Del Mar, Universitat Autonoma de Barcelona, 08003 Barcelona, Spain and [3]Molecular Haematology and Cancer Biology Unit, Institute of Child Health and Great Ormond Street Hospital for Children, University College London, London WC1E 6BT, UK
*Corresponding author: G Gil-Gómez, Institut Municipal d'Investigació Mèdica (IMIM), Apoptosis Signalling Group, Dr. Aiguader, 88, Barcelona, 8003, Spain.
Tel: +34 93 316 0432; Fax: +34 93 316 0410; E-mail: ggil@imim.es
[4]Present address: Department of Biochemistry, Oxford, OX1 3QU, UK
[5]These two authors contributed equally to this work
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Abbreviations: ELISA, enzyme-linked immunosorbent assay; HPRT, hypoxanthine-guanine phosphoribosyltransferase; mcyclin O, mouse cyclin O; PARP, poly (ADP-ribose) polymerase; RT-PCR, reverse transcription polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; S.E.M., standard error of the mean; shRNA, short hairpin RNA; SPF, specific pathogen free
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both expressed at least at the mRNA level in human and mouse cells), P2 (cyclin Oγ, expressed only in human cells) and P3 (cyclin Oδ, expressed only in mouse cells). About two-thirds of the cyclin Oα (hereforth cyclin O) sequence encompasses a highly conserved cyclin box, sharing about 28% of identity with human cyclins A2 and B1. Neither cyclin Oγ nor cyclin Oδ is protein coding and their function is unknown.

Expression, regulation and subcellular localisation of mouse cyclin O. The expressions of the different transcripts coming from *mcyclin O* gene were analysed by quantitative RT-PCR in mRNA coming from thymus and splenocytes. Expression of cyclin Oα, -β and -δ was detected in both samples at low levels (Figure 1b).

The cyclin O protein is detected by immunohistochemistry as a cytosolic protein (see below). The use of cytosol-enriched samples from cell lines such as colon adenocarcinoma cell line HT-29 and immortalised mouse embryonic fibroblasts allowed us to detect cyclin O by western blotting. Cyclin O migrates as a diffuse group of bands of around 40 kDa in SDS-PAGE gels, suggesting post-translational modifications (Figure 1c), and exclusively in cytosol-enriched extracts (SN).

Whole-body irradiation of mice with 10 Gy of γ-radiation from a ^{137}Cs source results in a time-dependent induction of mouse cyclin O (mcyclin O) mRNA expression (Figure 1d and e). To determine if mcyclin O mRNA expression is regulated by Bcl2 family members, as is Cdk2 kinase activity in irradiated thymocytes,[3] we measured the kinetics of induction of its mRNA after γ-irradiation in mice transgenic for Bax or Bcl2 and compared it with non-transgenic littermates. The induction of mcyclin O mRNA after γ-radiation treatment was independent of both Bax and Bcl2, but they can modulate its levels at late time points: Bax transgenic mice show significantly elevated levels of expression of mcyclin O mRNA with respect to non-transgenic controls, whereas in Bcl2 transgenic thymocytes, its expression lags behind significantly (Figure 1d and e).

Cyclin O activation correlates with apoptosis induction *in vivo*. We analysed the expression of mcyclin O and active caspase-3 as an apoptosis marker in the thymus of mice treated with 10Gy of γ-radiation 2, 3, 6 and 8 h after the treatment. Both cyclin O and active caspase-3 staining are negative at time 0 (Figure 2a). Radiation treatment leads to a time-dependent increase in the expression of cyclin O in the cytoplasm of the thymocytes located mainly in the cortex of the thymus, in parallel with an increased number of apoptotic cells positive for active caspase-3 and showing pyknotic nuclei in the haematoxylin–eosin staining. To demonstrate that thymocytes that upregulate cyclin O are committed to apoptosis, we performed double-immunofluorescence staining for cyclin O and active caspase-3 (Figure 2b). Two hours after total body radiation, double-positive cells start to appear in the cortex of the thymus. The number of cells staining simultaneously for cyclin O and active caspase-3 increases with time, although the kinetics is slower in the case of dexamethasone-treated mice, so the time course was extended from 4 to 14 h (Figure 2b, lower panel).

Similarly, both total body irradiation and dexamethasone treatment induced cyclin O expression and caspase-3 activation in the spleen of the mice (Supplementary Figure 1). Again, both proteins colocalised in the cytosol of apoptotic cells. However, apoptotic cells do not accumulate, most likely due to the presence of high amounts of phagocytic cells in the spleen.

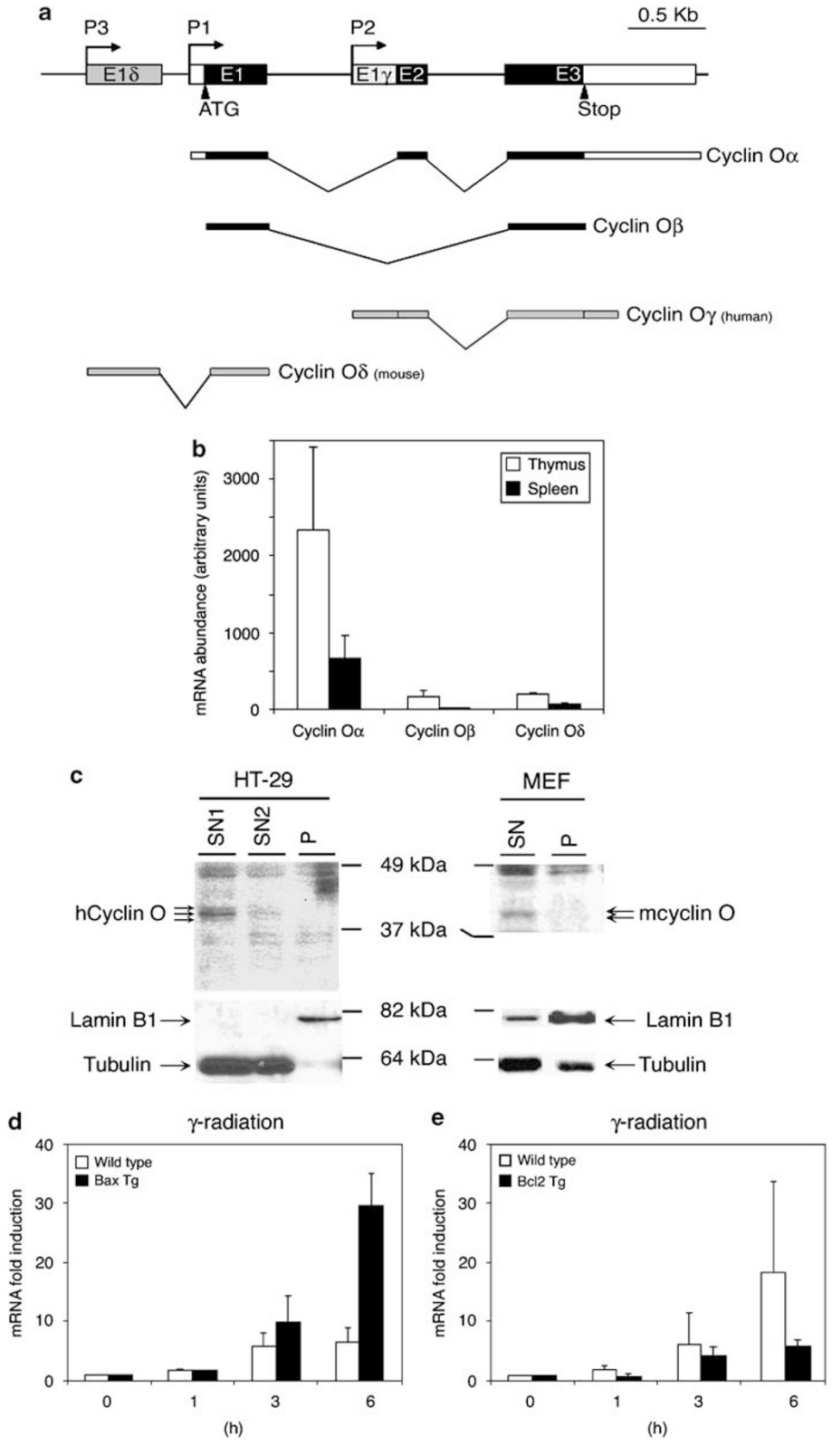
These data suggest that cyclin O expression precedes or is concomitant with apoptosis induction.

Cyclin O binds and activates preferentially Cdk2. Maltose-binding protein (MBP)-cyclin O can bind stoichiometrically and activate the histone H1 kinase activity of glutathione-*S*-transferase (GST)-Cdk2 (Supplementary Figure 2a), but not the activity of the inactive mutant D145N (Cdk2 DN), which can bind cyclins but lacks kinase activity.[8] To study the substrate preferences of cyclin O-containing complexes, HA-tagged cyclin O was immunoprecipitated from whole-cell extracts of transiently transfected HEK293 cells and a kinase assay of the immunoprecipitates was carried out using either purified histone H1 or GST-RNA polymerase II C-terminal domain (GST-CTD) as the exogenous substrate. The cyclin O immunocomplexes efficiently phosphorylate histone H1 but not GST-CTD. Conversely, immunoprecipitation of Cdk7, a *bona fide* RNA polymerase II kinase, leads to very efficient phosphorylation of GST-CTD but not of histone H1 (Supplementary Figure 2b).

To find out which Cdk is the preferred partner of cyclin O, we took into account the fact that active Cdk2 complexes, immunoprecipitated from apoptotic thymocytes, are inhibited by the ATP analogue roscovitine.[3] Cyclin O complexes immunoprecipitated from transfected HEK293 cells were as sensitive to roscovitine *in vitro* as Cdk1 or Cdk2 complexes (Supplementary Figure 2c). Of all the Cdks tested, only Cdk1, Cdk2, Cdk5, Cdk7 and Cdk9 are known to be efficiently inhibited *in vitro* by roscovitine;[9] so they were the best candidates to be cyclin O partners. Given the fact that the immunoprecipitated cyclin O complexes poorly phosphorylate GST-CTD, this excludes Cdk7 and Cdk9, as they are efficient CTD kinases.[10] In addition, pull-down experiments show that they fail to interact significantly with cyclin O *in vitro* (data not shown).

Cdk1 and Cdk2 were efficiently co-immunoprecipitated *in vivo* from transiently transfected HEK293 cells, whereas no interaction can be detected with Cdk4, a roscovitine-insensitive Cdk[9] (Figure 3a). To detect the interaction of endogenous cyclin O with Cdk2 and Cdk1, whole-cell extracts from fibroblasts derived from either wild-type or Cdk2 knockout mice[11] were immunoprecipitated with the anti-mcyclin O C2 antibody. The immunoprecipitates were then analysed by western blotting against Cdk1 and Cdk2 (Figure 3b). The membranes were stripped and probed with the anti-cyclin O antibody as a control. Endogenous cyclin O could be co-immunoprecipitated with Cdk2 only from extracts from wild-type mouse fibroblasts, whereas the complexes with Cdk1 could be recovered from extracts of either wild-type or Cdk2 knockout mouse fibroblasts. Therefore, we can conclude that endogenous cyclin O forms stable complexes *in vivo* with Cdk1 and Cdk2.

To investigate if cyclin O–Cdk1 and cyclin O–Cdk2 complexes are active, we incubated the MBP-mcyclin O fusion protein bound to amylose beads with whole-cell extracts made from wild-type mouse fibroblasts (Figure 3c, lower panel) and from Cdk2 KO fibroblasts (upper panel). Following incubation, the beads were extensively washed,



and the MBP-cyclin O fusion protein together with the associated proteins was eluted in native conditions. Aliquots of the eluate were immunoprecipitated using normal rabbit IgGs or antibodies against Cdk1 or Cdk2, and a kinase assay was performed in the immunoprecipitates using histone H1 as the exogenous substrate. Only the antibodies against Cdk2 immunoprecipitated significant amounts of kinase activity from wild-type fibroblasts. However, if Cdk2 is not present (Cdk2 KO fibroblasts), then the kinase activity is recovered in the anti-Cdk1 immunoprecipitates.

This experiment strongly suggests that, at least *in vitro*, Cdk2 is the preferred binding partner for cyclin O, which Cdk1 can substitute for Cdk2, and that cyclin O binds and activates Cdk1 and Cdk2.

Cyclin O overexpression induces apoptosis. We looked for direct evidence that cyclin O induces apoptosis by overexpressing it in cell lines both stably and transiently. The poor transfectability of the lymphoid cell lines precluded most of the experiments directed to demonstrate a pro-apoptotic role for cyclin O expression. We, therefore, used the highly transfectable cell lines HEK293 and U2OS to characterise the effect.

U2OS cells were transfected with an mcyclin Oα expression vector including neomycin resistance gene and then selected in G418 for 2 weeks. Colonies were fixed, stained with Coomassie Blue and counted. Around fivefold more colonies were consistently obtained in the empty vector-transfected cells *versus* the cyclin O-transfected cells (Figure 4a).

HEK293 cells were transiently transfected by the calcium phosphate method with empty expression plasmid or a mcyclin O expression plasmid (CycO, Figure 4b). Cells floating in the medium or adhered to the plate were quantitated 72 h after transfection by a flow cytometry-based total cell count. The number of adherent cells is about the same in both conditions, whereas the number of floating cells is much higher in cultures transfected with mcyclin O expression plasmid (Figure 4b). It is likely that the cells that die and detach from the plate are replaced following division of the remaining cells on the plate. Analysis of the DNA content shows that the adherent cells contain a significantly higher number of cells with subdiploid DNA content in the culture transfected with the mcyclin O expression vector (Figure 4c). In the floating cells, a high percentage of sub-G1 nuclei were detected. No obvious differences were observed in the cell cycle distribution of live cells regardless of the expression of cyclin O, suggesting a lack of effect on the cell cycle.

To confirm that cyclin O overexpression induces apoptosis and its specificity *versus* other cyclins also reported to induce apoptosis, HEK293 cells transiently transfected with cyclins O, A2 and D1 were analysed for activation of caspase-3 by western blotting. Only cyclin O overexpression led to the activation of this executor caspase (Figure 4d).

These data demonstrate that mcyclin Oα overexpression can induce caspase-dependent apoptosis in cell lines of non-lymphoid origin.

Cyclin O expression is necessary for apoptosis induced by intrinsic stimuli. Thymocytes are quiescent, short-lived cells in culture that cannot be transfected. The WEHI7.2 cell line is derived from a mouse T-cell lymphoma that is sensitive to glucocorticoids and DNA-damaging agents as well as to extrinsic stimuli such as CD95 agonistic antibodies.[12] This cell line expresses cyclin O at very low levels in basal conditions but dramatically upregulates its expression in a time-dependent manner in response to DNA damage or the glucocorticoid dexamethasone similarly to its regulation in thymocytes (Supplementary Figure 3).

To determine whether cyclin O induction is necessary for apoptosis to take place in WEHI7.2 cells, we downregulated its expression level by shRNA. To avoid off-target effects, we designed two different shRNA constructs directed against exon 3 of the gene and obtained stable clones expressing each one. As a negative control, we transfected the empty pSuperpuro vector[13] and the same vector containing an shRNA construct against GFP.

After puromycin selection, we obtained single-cell clones and analysed the effect of the shRNA on apoptotic response. Given the impossibility of measuring directly the levels of cyclin O by western blotting, the effectivity of the shRNA was assessed by two independent methods: checking the disappearance of the immunoreactivity by immunohistochemistry and abrogation of the induction of cyclin O-associated kinase activity of the cyclin O shRNA clones after etoposide treatment (Supplementary Figure 4). Clones containing empty vector ('V' clones) or the GFP shRNA clones ('shG' clones) behaved indistinguishably from the parental cells in response to apoptotic stimuli. Clones containing the different shRNAs directed against exon 3 of the gene were designated '3' clones and '5' clones according to the cyclin O shRNA construct transfected and followed by the number of the clone. To rule

Figure 1 Cyclin O locus and expression. (**a**) The cyclin O locus encodes four transcripts that arise from the use of three alternative promoters: P1 (human/mouse cyclin Oα and the alternatively spliced product human/mouse cyclin Oβ), P2 (human cyclin Oγ) and P3 (mouse cyclin Oδ). Black boxes denote coding regions. White boxes denote 5′ and 3′ non-coding regions. Grey boxes denote non-coding transcripts. (**b**) Expression of cyclin Oα, -β and -δ transcripts was measured by quantitative RT-PCR in normal mouse thymus (white bars) and spleen (black bars) of the same mouse, using HPRT levels as control. RNA levels were measured in triplicate and the mean values ± the S.E.M. are shown. (**c**) Human cyclin O was detected by western blotting in cytosol-enriched extracts (SN1 and SN2) or in nuclear protein-enriched extracts (P) of the human colon adenocarcinoma cell line HT-29 (left panel) using the N1 antibody or in mouse fibroblast extracts (MEFs) using the C2 antibody (right panel). In both cases, SuperSignal West Pico chemiluminescent substrate was used. The membranes were stripped and reprobed with antibodies against Lamin B1 as a nuclear marker and α-tubulin as a cytosolic marker. Mice transgenic for (**d**) Bax and non-transgenic littermates (wild-type) and (**e**) Bcl2 and non-transgenic littermates (wild-type) were irradiated as described in the Materials and Methods section and thymuses isolated and processed for RNA isolation. The levels of mcyclin Oα mRNA were analysed by quantitative RT-PCR. As a loading control, the mRNA levels of HPRT were determined in the same samples. The experiment was repeated twice and the result of a representative measure of the cyclin O performed in triplicate is shown (mean plus S.D.) as fold of induction over the levels of the non-transgenic mice at time 0. Error bars show the calculated error range for two independent experiments

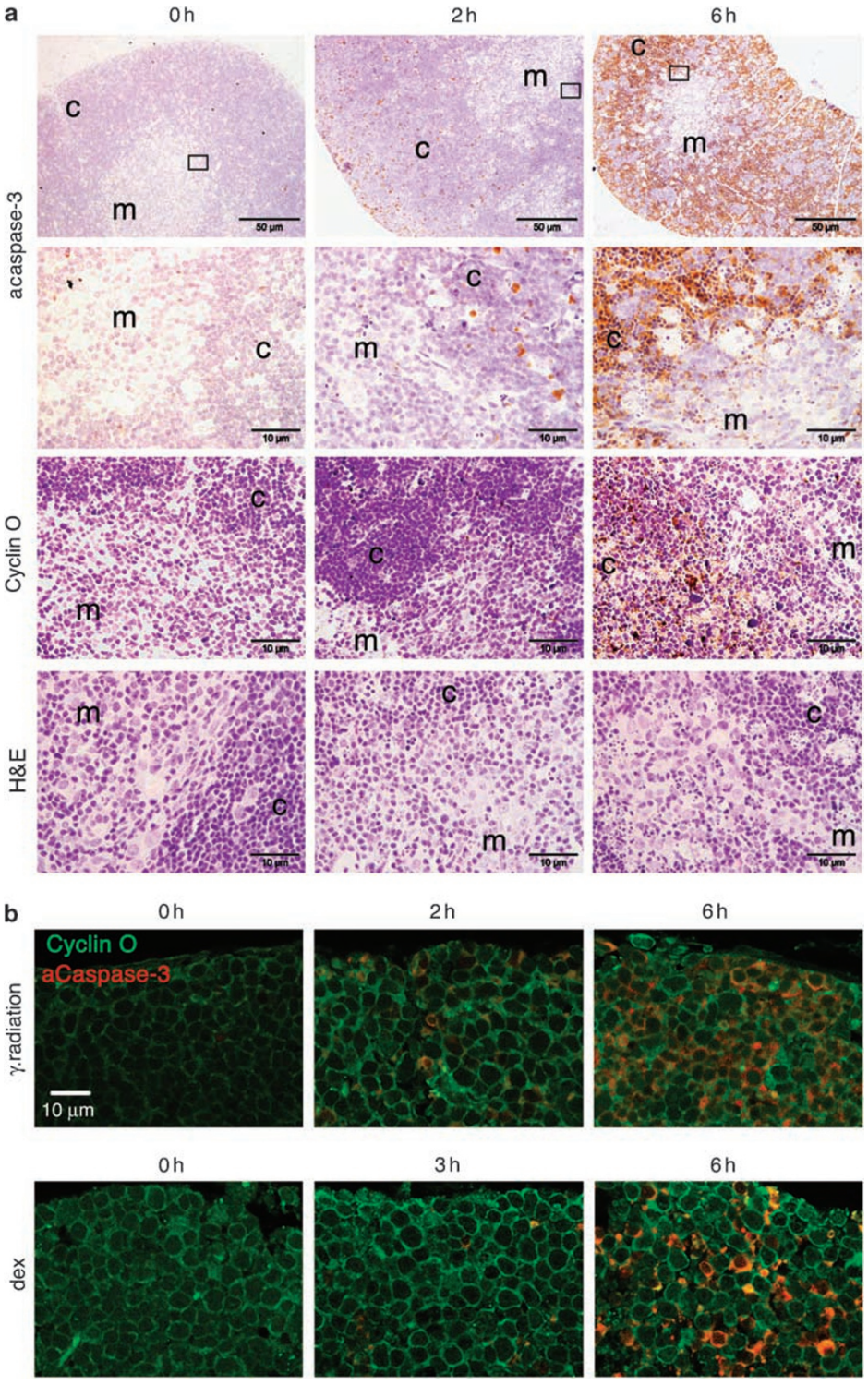


Figure 2 Mouse cyclin O is induced during thymocyte apoptosis *in vivo* and precedes caspase-3 activation. (**a**) Swiss CD-1 mice were irradiated as described in the Materials and Methods section. At the indicated times, mice were killed and the thymuses processed for cyclin O (mcyclin O) or active caspase-3 (acaspase-3) detection by immunohistochemistry. Samples were counterstained with haematoxylin. A section of the same samples was stained with haematoxylin–eosin (H&E) as a control. In the case of active caspase-3, no EnVision™ signal-enhancing system was used. c: cortex; m: medulla. The region magnified in the active caspase-3 staining is indicated by a rectangle. (**b**) Swiss CD-1 mice were irradiated or injected with dexamethasone as described in the Materials and Methods section, the thymuses collected at the indicated times and processed for immunofluorescence detection of cyclin O using the biotinylated N1 antibody revealed by streptavidin-fluorescein and the active caspase-3 using the 5A1 antibody followed by an anti-rabbit antibody labelled with Cy3. Fluorescence was detected using a Leica SP2 confocal microscope

out possible clonal effects, three to five independent clones from each group were characterised for their response to apoptosis, and a representative clone of each group was chosen to show the results of the tests performed. Both shRNA constructs behaved very similarly.

In contrast to the control clones V3 and shG1, clones 3.7 and 5.8 were refractory to etoposide- and dexamethasone-induced apoptosis (Figure 5a and b). To investigate if downregulation of cyclin O expression had any effect on the signalling of the extrinsic stimuli, we studied the response of clone 3.7 to the CD95 agonistic antibody Jo2 in the presence of cycloheximide. The apoptotic response to anti-CD95 remains intact in the presence of downregulated cyclin O expression (Figure 5c).

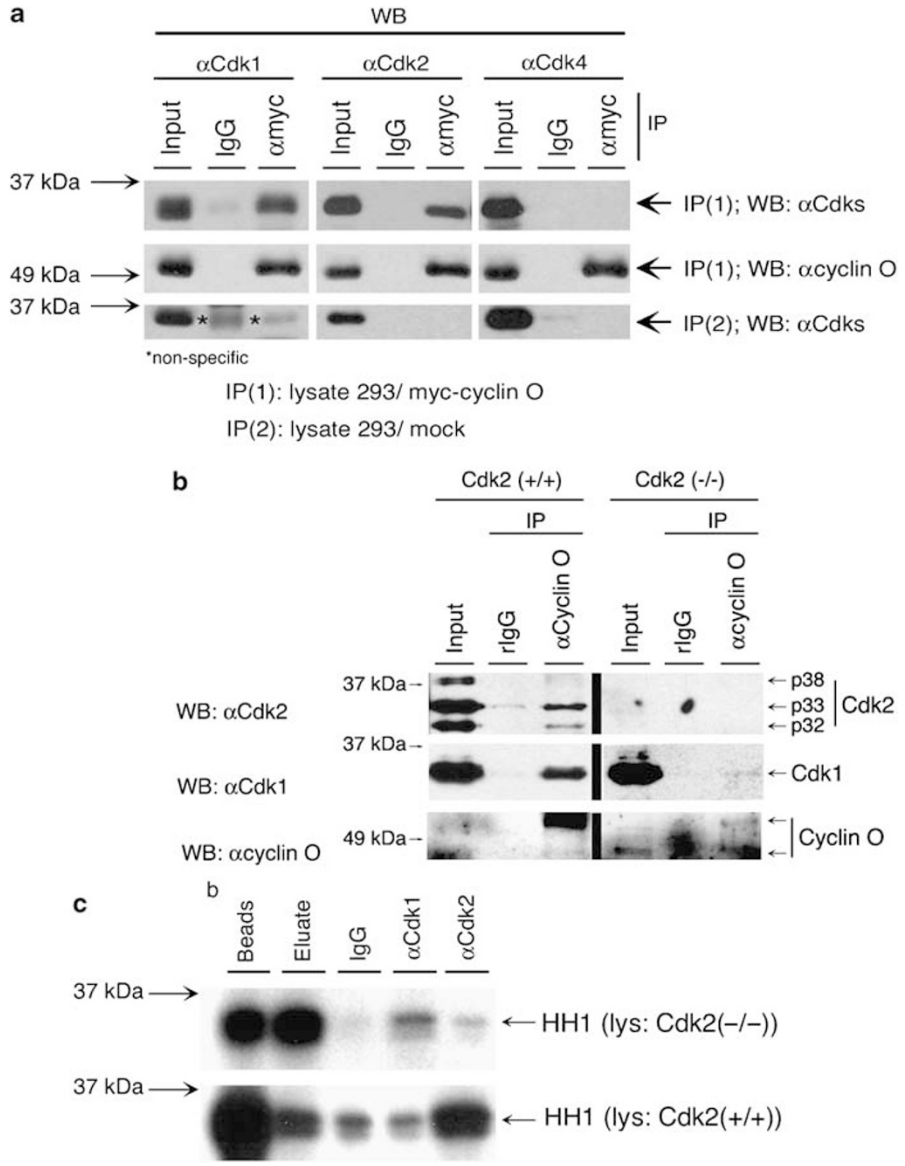


Figure 3 Cyclin O binds preferentially Cdk2. (**a**) Protein extracts of HEK293 cells transiently transfected with an expression plasmid for myc-tagged mcyclin O were immunoprecipitated with a control rabbit IgG (IgG) or with a monoclonal antibody against the myc-tag (9E10) covalently bound to protein-G sepharose beads. After extensive washing, the proteins bound to the beads were first analysed by western blotting with antibodies against Cdk1, Cdk2 and Cdk4 (upper panel) and, after stripping of the membranes, with antibodies against mcyclin O (C2, middle panel). As a control, 2.5% of the input was run in the gel and processed. To run out the possibility that the co-immunoprecipitations were due to the endogenous c-myc protein immunoprecipitated by the 9E10 antibody, cell extracts from mock-transfected HEK293 cells were immunoprecipitated with the 9E10 antibody and probed for the presence of Cdk1, Cdk2 and Cdk4 (lower panel). (**b**) Extracts from Cdk2 wild-type ($+/+$) and Cdk2 knockout ($-/-$) mouse fibroblasts were immunoprecipitated with rabbit immunoglobulins (rIgG), and the anti-cyclin O antibody C2 covalently bound to protein-G sepharose beads. The presence of Cdk1 and Cdk2 in the immunoprecipitates was detected by western blotting. As a control, the membrane was stripped and mcyclin O was detected by western blotting with the antibody C2. (**c**) Recombinant MBP-cyclin O bound to amylose beads was incubated with whole-cell extracts from mouse fibroblasts Cdk2 ($+/+$) or ($-/-$). After washing, the cyclin O complexes were eluted with maltose and immunoprecipitated with normal rabbit IgGs or antibodies against Cdk1 or Cdk2. The immunoprecipitates, an aliquot of the beads before elution (beads) and 5 μl of the eluate were processed for histone H1 kinase assay. All the experiments were performed at least three times and a representative result is shown

The failure of the cells with downregulated cyclin O to undergo DNA damage-induced apoptosis could be due to a defect in the signal transduction pathway that detects DNA lesions. However, the DNA-damage response parameters analysed are not significantly changed as a result of the downregulation of cyclin O (Supplementary Figure 5a). In agreement with this, the DNA profile of the cells 9 h after treatment with γ-radiation shows a profile similar to the shG1 clone cells, indicating a G1, intra-S and G2 arrest, most likely as a consequence of checkpoint activation (Supplementary Figure 5b).

A characteristic response of lymphoid cells to glucocorticoids is a cell cycle arrest upon treatment previous to apoptosis induction, reflecting the antiproliferative action of these hormones.[14] Dexamethasone treatment of clones shG1 and 3.7 leads to a time-dependent arrest, mainly in G0/G1, that is almost complete in clone 3.7 after 48 h, whereas in clone shG1 appears the sub-G1 apoptotic DNA peak superimposed to the cell cycle arrest (Supplementary Figure 5c). Glucocorticoid-dependent transcription also seems to be unaffected, as the kinetics of induction of the pro-apoptotic BH3-only Bcl-2 family member BimEL, a known glucocorticoid receptor target,[15] is unaltered (Supplementary Figure 5d).

In an attempt to identify the reason why the shRNA clones show defective apoptosis, we measured other apoptotic parameters in a control clone (clone shG1) and in a cyclin O shRNA clone (clone 3.7). Cyclin O downregulation avoided plasma membrane phosphatidylserine exposure (Figure 6a) and mitochondrial transmembrane potential dissipation

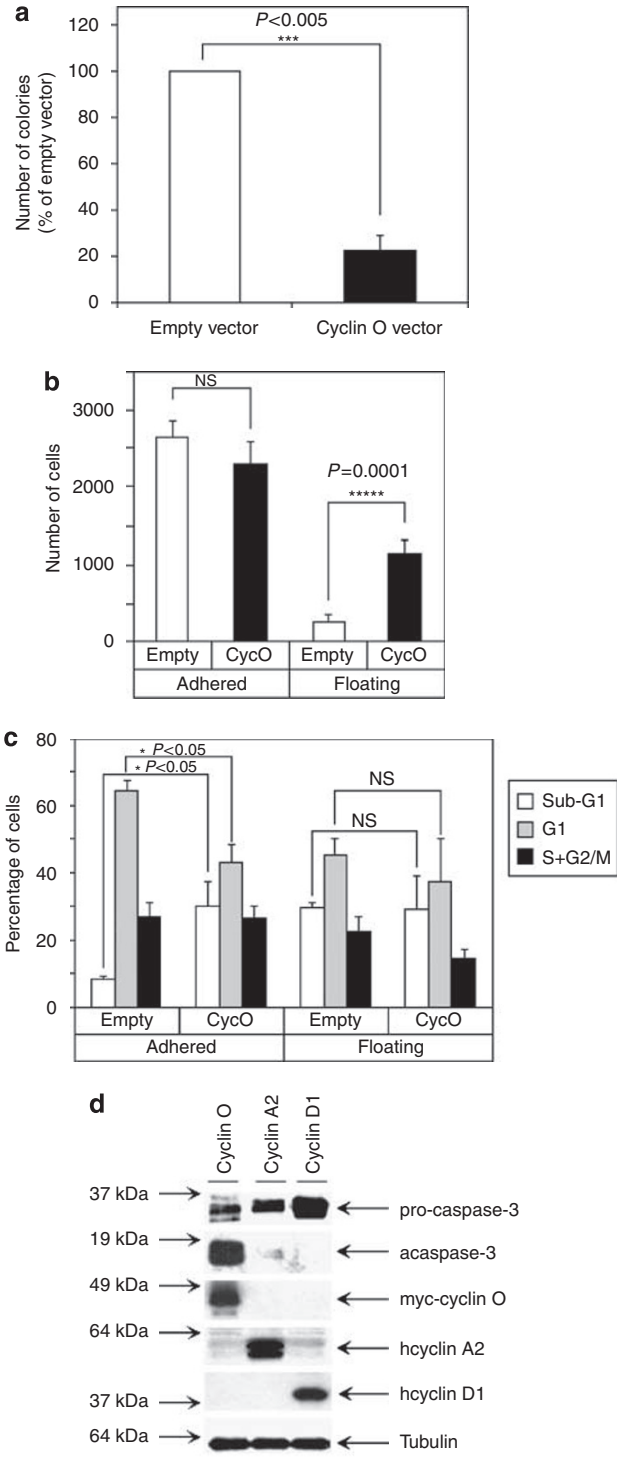
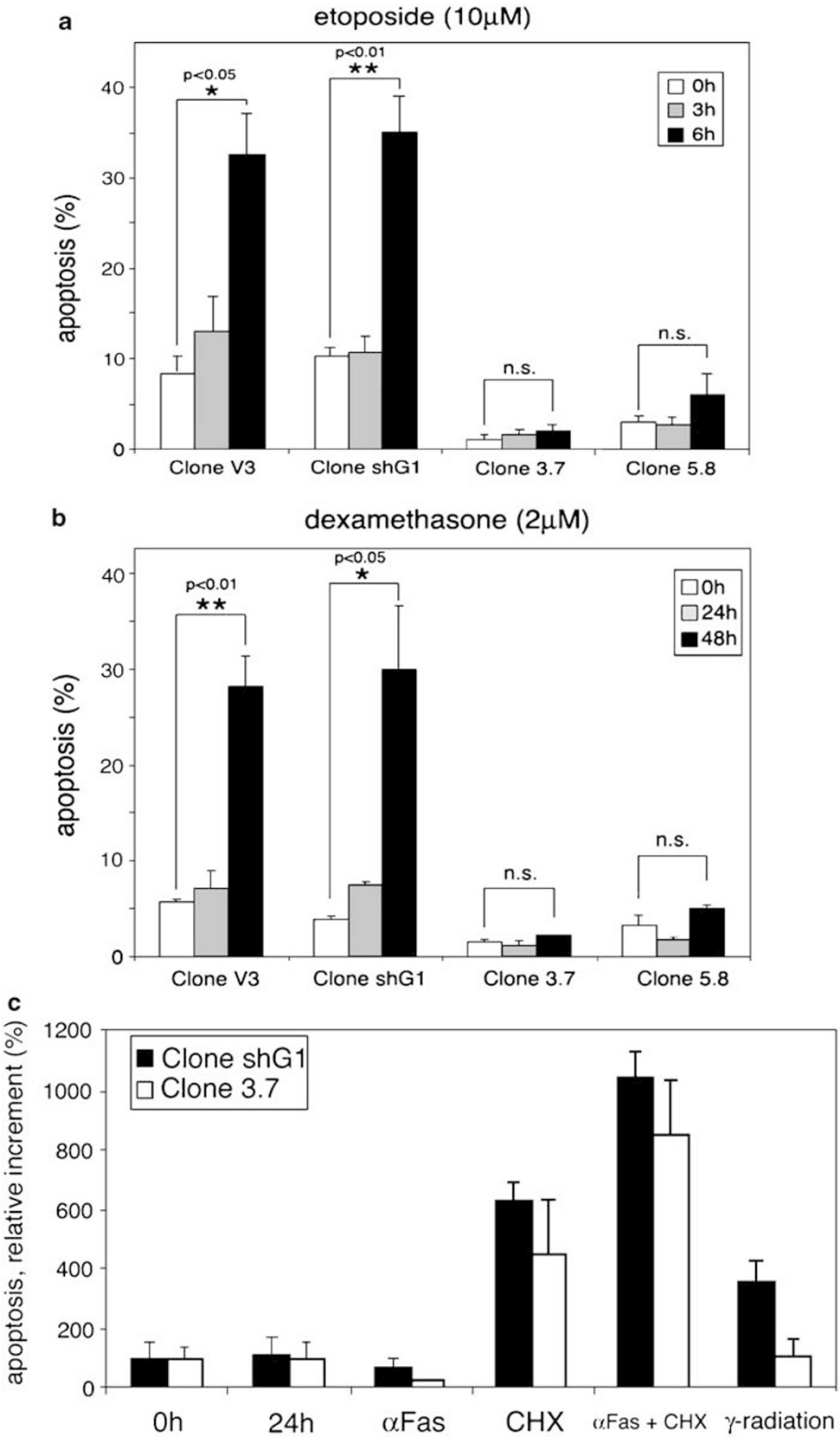


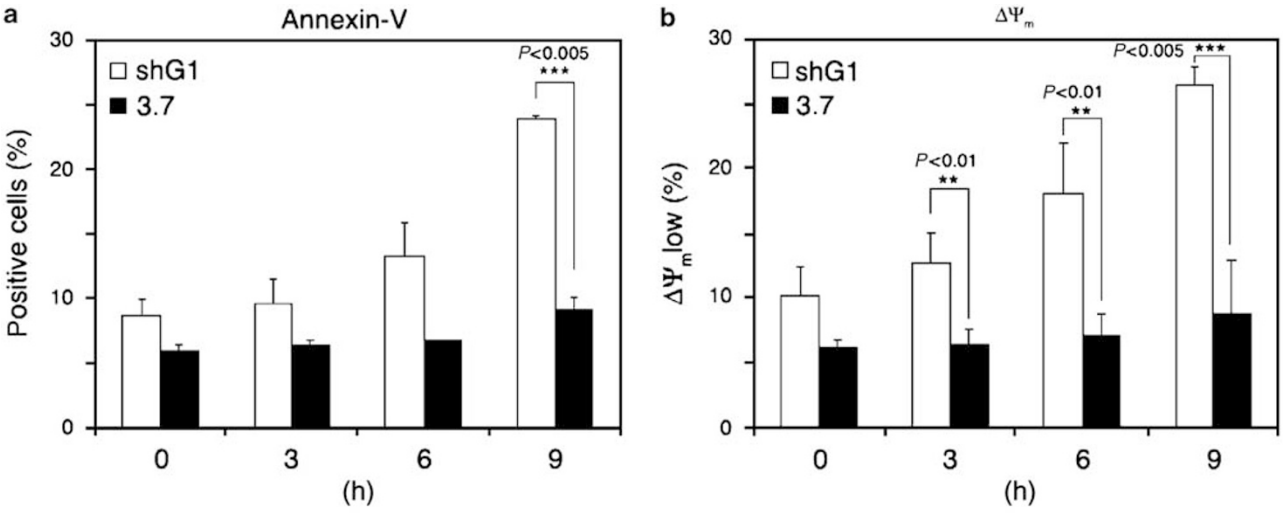
Figure 4 Mouse cyclin O overexpression induces apoptosis. (**a**) The plasmid pcDNA3 or pcDNA3-HAmcyclin O was linearised with *Ahd*I and transfected into U2OS osteosarcoma cells by calcium phosphate transfection. After 2 weeks of selection, the colonies were stained with methylene blue and counted. Represented are the mean percentage of variation in the number of colonies after transfection of the pcDNA3-HAmcyclin O plasmid (cyclin O vector), with respect to the transfection of the pcDNA3 empty vector ± the S.E.M. obtained from three independent experiments. (**b**) The plasmid pcDNA3 or pcDNA3-HAmcyclin O was transiently transfected into HEK293 cells and 72 h after the transfection, the cells floating in the culture medium or attached to the culture plate were counted. Data averaged from three independent experiments are shown. Error bars show the calculated S.E.M. (**c**) The attached and floating cells from (**b**) were stained with propidium iodide and the cell cycle analysed by flow cytometry. Represented are the mean values from three independent experiments and the calculated S.E.M. (**d**) HEK293 cells were transiently transfected with expression plasmids for myc-tagged cyclin O and human cyclins A2 and D1. After 48 h, floating and attached cells were harvested and the presence of apoptosis assessed by the detection of the processing of the pro-caspase-3 and the appearance of active caspase-3 (acaspase-3) by western blotting. Transfected myc-cyclin O and cyclins A2 and D1 were detected by western blotting as controls. Tubulin levels were measured as a loading control

Figure 5 Downregulation of cyclin O abrogates apoptosis induced by intrinsic but not extrinsic stimuli. (**a**) WEHI7.2 cells were stably transfected with empty pSUPERpuro vector (clone V3), with pSUPERpuro expressing a shRNA against GFP (clone shG1) or two different shRNAs directed against mcyclin O exon 3 (clone 3.7 and clone 5.8) and single-cell clones isolated. The selected clones were treated with 10 μM etoposide. Samples were harvested at the indicated times and the percentage of apoptotic cells measured as sub-G1 DNA peak by propidium iodide staining by flow cytometry. Measures were performed in triplicate. Represented are the mean FACS values from three independent experiments. Bars correspond to the mean ± the S.E.M. (**b**) The same clones were treated with 2 μM dexamethasone and processed as in the preceeding section. Samples were harvested at the indicated times and the percentage of apoptotic cells measured as sub-G1 DNA peak by propidium iodide staining by flow cytometry. Measures were performed in triplicate. Represented are the mean FACS values from three independent experiments. Bars correspond to the mean ± the S.E.M. (**c**) To check the response of the clones to the apoptotic effect of the CD95 pathway, a control clone (shG1) and an shRNA clone (3.7) were cultured 0 and 24 h in the absence (0, 24 h) or presence of 0.5 μg/ml of the agonistic antibody Jo2 (αFas), 30 μg/ml of cycloheximide (CHX) or the combination of both treatments (αFas + CHX). Apoptosis was measured as described and the figure shows the relative increase in apoptosis of each condition relative to the value at 0 h (percentage). For comparative purposes, the response of the same cells to γ-radiation is also included. Error bars show the calculated error range for two independent experiments

(Figure 6b) in response to DNA damage. In agreement with this last observation, Bax and Bid (but not Bak) translocation from cytosol to mitochondria after γ-radiation (Figure 6c) and dexamethasone (data not shown) treatments is impaired in clone 3.7. As a consequence, cytochrome *c* was released to the cytosol in the case of the shG1 control clone but not in the clone with downregulated cyclin O.

All these observations indicate that cyclin O downregulation leads to an early, pre-mitochondrial block of the signalling of intrinsic stimuli.

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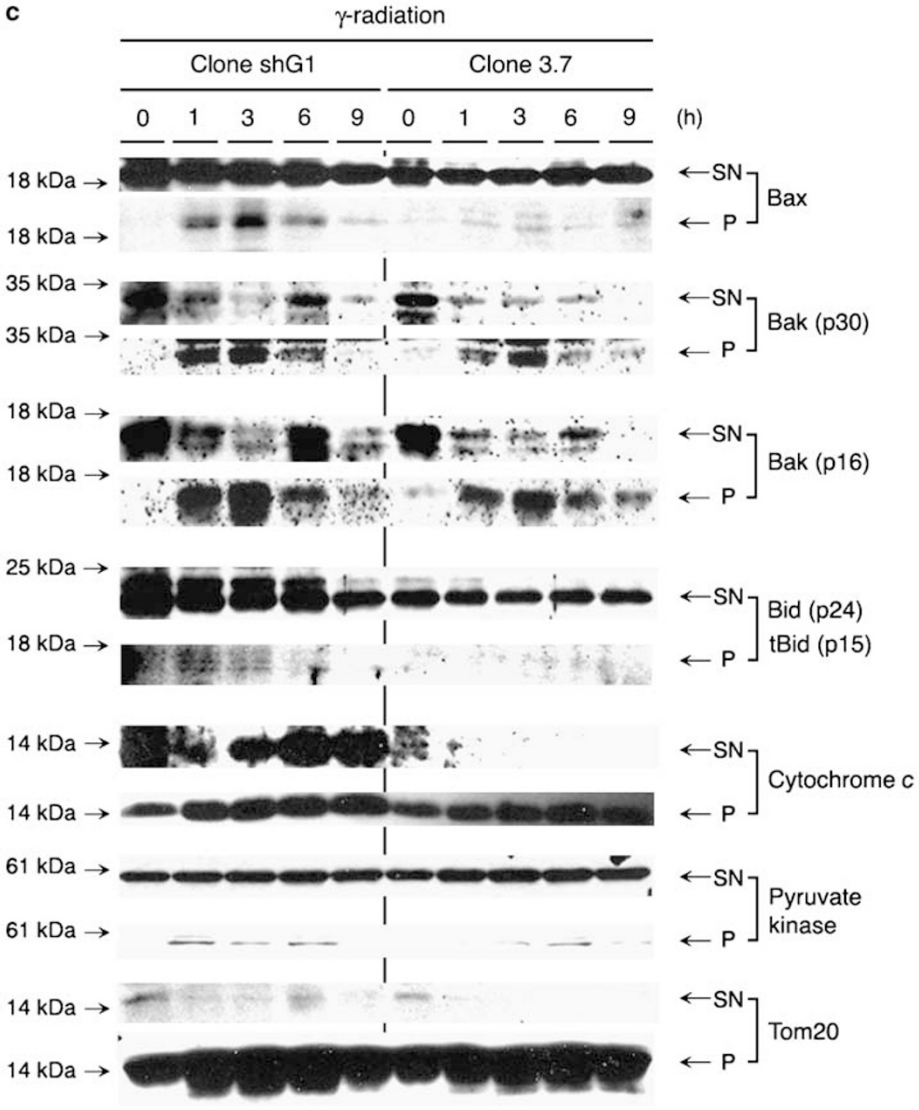
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Pyruvate kinase
Tom20

Cyclin O downregulation leads to a block in caspase activation. The lack of cytochrome *c* release from mitochondria precludes the formation of the apoptosome, the multiprotein complex responsible for the activation of caspase-9.[16] The treatment of clone shG1 with dexamethasone (Figure 7a) or γ-radiation (Supplementary Figure 6a) induces activation of the apical caspases, caspase-8 and -9, as well as caspase-3. However, the activation of all these caspases is completely abolished in the 3.7 clone, indicating defective apoptosis. The same phenotype is found in independent clones isolated in the original screening (Supplementary Figures 6b and c), ruling out a clonal effect.

To confirm the lack of executory caspase activity, the DEVDase cleavage activity of control shG1 and shcyclin O clone 3.7 was measured after γ-radiation or dexamethasone treatment. No detectable increase in activity was measured in the 3.7 clone after treatment with any of the two apoptotic stimuli (Figure 7b). In agreement with this, extracts from dexamethasone-treated clone 3.7 did not contain the caspase cleavage product (p85) of the substrate protein PARP (Figure 7c).

To further demonstrate that the extrinsic apoptosis pathway is intact, we measured caspase-8 activation, full-length Bid cleavage and generation of tBid in the shG1 control and 3.7 cyclin O shRNA clones (Figure 7d). As expected, no significant differences were detected. These experiments demonstrate that cyclin O expression is necessary for DNA damage- and glucocorticoid-induced apoptosis, but not for CD95 signalling.

All these results strongly suggest that, in agreement with previous data from thymocytes using Cdk2 chemical inhibitors, lack of activation of the cyclin O–Cdk2 complexes during DNA damage- or glucocorticoid-induced apoptosis results in the absence of apical and executory caspase activation and, hence, a specific lack of the apoptotic response of the lymphoid cells.

Discussion

We previously suggested that a novel protein may be responsible for Cdk2 activation during apoptosis of thymocytes, as the levels of the canonical Cdk2 cyclins (cyclins A and E) are barely detectable by western blotting and the Cdk2 kinase activity detected is not associated with cyclin A or E.[3] We have characterised cyclin O as the most likely candidate to be the so-called apoptosis-related Cdk2 activator.

Cyclin O is mostly similar to the A- and B-type cyclins, although we have no evidence that it can participate in cell cycle regulation. A cDNA highly homologous to human cyclin Oγ locus was reported to encode a protein with uracil-DNA glycosylase activity.[17] However, the correct sequence of both the genomic locus and several ESTs shows that this transcript does not encode the reported protein. Neither purified recombinant cyclin Oα nor cell extracts obtained after its transient transfection into HEK293 cells show detectable uracil-DNA glycosylase activity as a consequence of its overexpression as measured by an enzymatic assay[18] (data not shown).

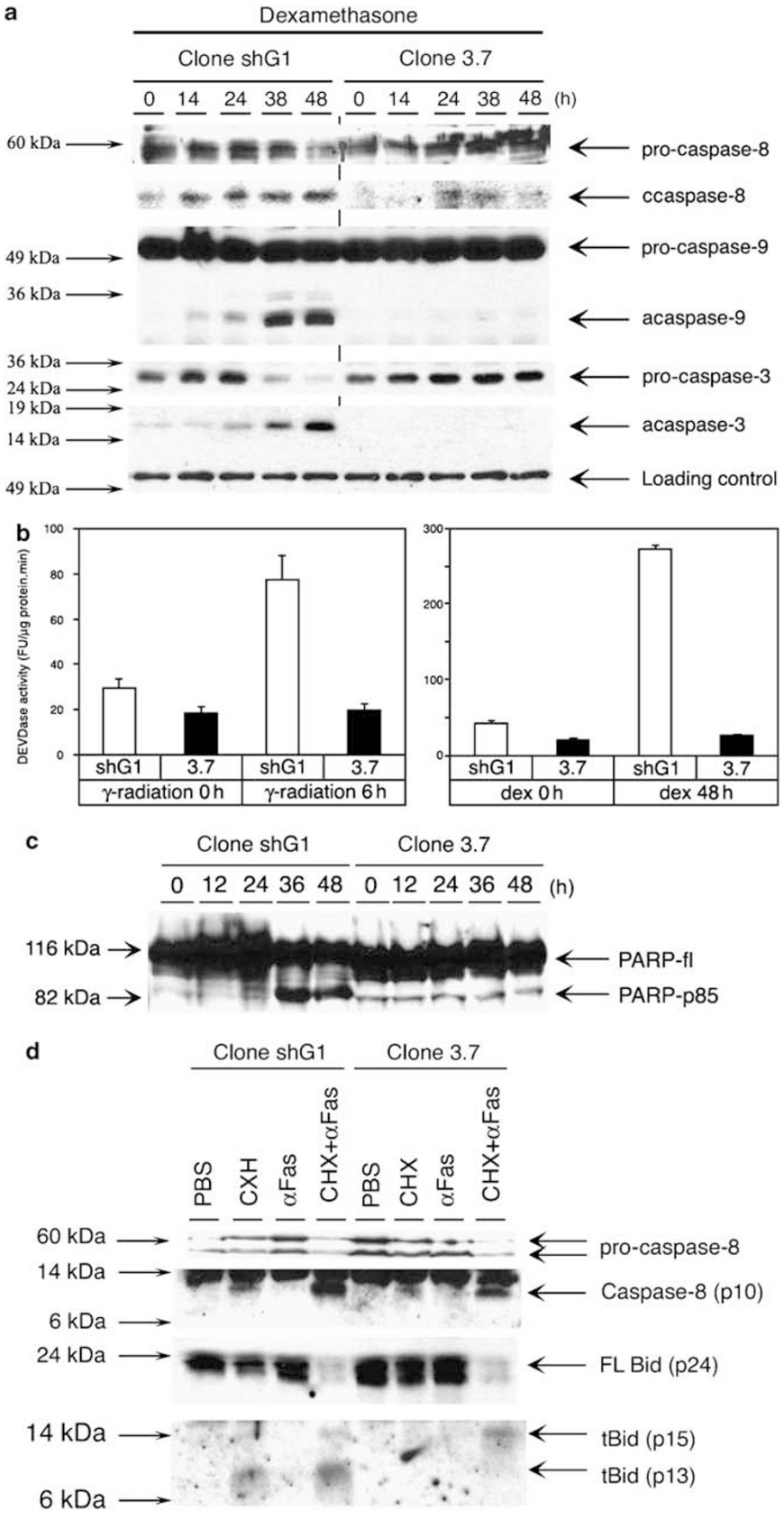
Cyclin O binds to Cdk1 and Cdk2, leading to their activation (Figure 3). It is clear that in wild-type mouse fibroblasts, where both kinases are expressed, Cdk2 is the main kinase forming complexes with cyclin O. However, in the absence of Cdk2, Cdk1 binds and gets activated by cyclin O, which further confirms the observations of the redundancy of Cdk2 as found in Cdk2 KO mice.[11] Binding of a cyclin to different Cdks is not a surprising finding, given the well-characterised high degree of redundancy in the Cdk family.[19]

The expression of cyclin O is rapidly elevated in the thymus and spleen of the mouse and in WEHI7.2 cells after irradiation or dexamethasone treatment. This pattern, especially in the thymus, is compatible with the expression of the cyclin preceding apoptosis induction. Thus, the kinetics of cyclin O expression after apoptosis induction is compatible with a causal role in apoptosis, a role demonstrated by the use of RNA interference both in WEHI7.2 cells (Figure 5a and b) and in EL-4 lymphoid cells (data not shown).

As we have shown before in different experimental models,[3,6] CD95 is an example of extrinsic stimuli that is independent of Cdk2 activation, and anti-CD95-induced apoptosis cannot be blocked by Cdk2 chemical inhibitors. We show that in WEHI7.2 cells, CD95-induced apoptosis is completely independent of protein synthesis. In fact, it is necessary to add the protein synthesis inhibitor, cycloheximide, to uncover the pro-apoptotic action of anti-CD95 antibody. Inhibiting protein synthesis blocks cyclin O production and so (Figure 5c), CD95-induced apoptosis is indistinguishable between a control clone (shG1) and a cyclin O-interfered clone (3.7).

We have previously positioned the activation of Cdk2 in DNA damage- and glucocorticoid-induced thymocyte apoptosis as an early step preceeding phosphatidylserine exposure, mitochondrial dysfunction and caspase activation.[5] We now show that cyclin O downregulation leads to a complete apoptosis block that is characterised by a lack of phosphatidylserine exposure, mitochondrial dysfunction and a total absence of activation of the apical caspases, caspase-8 and -9 and the executory caspase-3. In addition to this, the induction of cyclin O mRNA after γ-radiation treatment is independent of Bax or Bcl2, indicating that its action lies upstream of the mitochondrial action of the Bcl2 family proteins. However, the magnitude of induction of the cyclin mRNA is increased by transgenic expression of Bax and

Figure 6 Cyclin O acts at a pre-mitochondrial step before the loss of plasma membrane asymmetry in the signalling of intrinsic apoptotic stimuli. (**a**) Clones shG1 (white bars) and 3.7 (black bars) were treated with 10 Gy of γ-radiation using a ^{137}Cs irradiator and the percentage of cells exposing the phospholipid phosphatidylserine at the outer plasma membrane measured by means of their ability to bind to (fluorescein-labelled) annexin-V and quantitation by flow cytometry in triplicate. Represented are the means ± the S.E.M. of the percentage of annexin-V-positive cells. (**b**) The same experiment was performed but the dissipation of the mitochondrial transmembrane potential was measured by means of the quantitation by flow cytometry of the percentage of cells with low fluorescence for the mitochondrial potential-sensitive dye CMX-Ros ($\Delta\Psi m^{low}$) in triplicate. Represented are the means ± the S.E.M. of the percentage of $\Delta\Psi m^{low}$ cells. (**c**) Clones shG1 and 3.7 were treated with 10 Gy of γ-radiation and at the indicated times, cells were harvested and subcellular fractions obtained. Bax, Bak, Bid and cytochrome *c* abundance was measured by western blotting in cytosol (supernatant, SN)- and mitochondria (pellet, P)-enriched fractions. p16Bak is the product of an alternatively spliced Bak mRNA. As a control of the subcellular fractionation, pyruvate kinase was used as a cytosolic marker and Tom20 as a mitochondrial membrane marker


a
Dexamethasone
Clone shG1
Clone 3.7
0 14 24 38 48 0 14 24 38 48 (h)
60 kDa
pro-caspase-8
ccaspase-8
49 kDa
pro-caspase-9
36 kDa
acaspase-9
36 kDa
24 kDa
pro-caspase-3
19 kDa
acaspase-3
14 kDa
49 kDa
Loading control
b
DEVDase activity (FU/µg protein.min)
shG1 3.7 shG1 3.7
γ-radiation 0 h
γ-radiation 6 h
dex 0 h
dex 48 h
c
Clone shG1
Clone 3.7
0 12 24 36 48 0 12 24 36 48 (h)
116 kDa
PARP-fl
82 kDa
PARP-p85
d
Clone shG1
Clone 3.7
PBS
CXH
αFas
CHX+αFas
CHX
60 kDa
pro-caspase-8
14 kDa
Caspase-8 (p10)
6 kDa
24 kDa
FL Bid (p24)
tBid (p15)
tBid (p13)

diminished by Bcl2, perhaps reflecting a regulation of the apoptosis amplification feedback loop.[20]

The cyclin O shRNA clone 3.7 shows normal Bak translocation to the mitochondria, whereas Bax is not translocated. However, genetic evidences show that tBid generation is necessary for the oligomerisation of Bak or Bax and to induce cytochrome *c* release.[21] Owing to the defect in caspase-8 activation, clone 3.7 is unable to generate tBid and, therefore, to induce cytochrome *c* release, in spite of the normal translocation of Bak to the mitochondria (Figure 6). Defective cytochrome *c* release to the cytosol explains the lack of caspase-9 and -3 activation due to the inability to assemble the apoptosome.[16]

Our findings support the hypothesis that cyclin O is the apoptosis-related Cdk2 activator protein predicted for thymocytes to undergo apoptosis by intrinsic stimuli. The evidence also suggests that the only role of cyclin O–Cdk complexes in lymphoid cells is the regulation of apoptosis through the control of activation of apical caspases such as caspase-8. Previous evidence from our laboratory showed that *de novo* gene expression is required in thymocytes downstream of Cdk2 activation and before caspase-8 cleavage.[5] These results would be compatible with cyclin O–Cdk2 complexes activating latent transcription factor(s), resulting in the transcription of genes necessary for the activation of the apical caspases.

The fact that cyclin O–Cdk1/2 complexes are involved in the activation of caspases opens the possibility that they may also regulate their activation during other non-apoptotic functions, such as tissue differentiation, cell proliferation or maturation of pro-inflammatory cytokines (reviewed in Lamkanfi *et al*.[22,23]).

Induction of apoptosis by unscheduled activation or expression of cell cycle regulators such as cyclins or Cdks has been extensively described (see Golsteyn[24] for a recent review). Inappropriate activation of a Cdk such as Cdk1 perturbs the normal cell cycle progression, leading to checkpoint activation and apoptosis induction.[25] Expression of a cyclin in a quiescent tissue, in the presence of a mitogenic stimulus such as serum, has been shown to lead to an abortive attempt to re-enter cell cycle, leading to apoptosis.[26] However, these cases are based solely on the forced overexpression of components of the normal cell cycle machinery in altered conditions. We report here the characterisation of a novel cyclin for which the physiological function is to participate in the signalling of the intrinsic apoptotic stimuli at least in lymphoid cells. Its normal expression precedes apoptosis induction and if it is down-regulated, apoptosis is blocked. We propose cyclin O to be a novel apoptosis-specific regulator of Cdk activity.

Materials and Methods

***In silico* cyclin O identification and cloning.** Human cyclin A2 N-terminal cyclin box domain (amino acids 200–360 from gi 85396865) was used to identify new cyclins *in silico* by screening the first draft of the human genome using the tblastn program (http://www.ncbi.nlm.nih.gov/Blast) with default stringency parameters given by the program. We detected two previously undescribed cyclin-like sequences in chromosomes 5 and 19. For the sequence in chromosome 5, later on named cyclin O,[7] we could complete the structure of the gene with the help of ESTs present in the GenBank database. We then searched the first draft of the mouse genome and found the orthologue gene in mouse chromosome 13. The cyclin-like sequence of chromosome 19 was not further characterised.

The mcyclin O-coding region was amplified by PCR using as a template DNA from a cDNA library constructed with RNA purified from apoptotic thymocytes and using the oligonucleotides 5′-GGAATTCCATGGTTACCCCTTGCCCTGCCAG CCC-3′ and 5′-GCGAATTCGCTTATTGCCAACTCTGGGGCAGGCTGC-3′. The PCR products amplified (corresponding to the α and β forms) were gel purified, digested with *Eco*RI and cloned in suitable vectors.

The Gene ID from cyclin O in the mouse genome corresponds to 218630 and in the human genome to 10309.

Mouse cyclin Oδ locus transcript is represented by the EST gi:58060079. Rat cyclin Oδ is represented by the EST gi:49903553. Two representative human ESTs that encode the cyclin Oγ-specific exon E1γ are gi: 14569066 and gi: 14817125.

Antibodies. Anti-cyclin O antibodies. The N1 and C2 antibodies were generated against the peptides H-LRAPVKKSRRPC-NH_2 (N1) coupled with glutaraldehyde and H-SSLPRILPPQIWERC-NH_2 (C2) coupled with 3-maleimidobenzoic acid *N*-hydroxysuccinimide ester (MBS; Sigma-Aldrich, St. Louis, MO, USA) to keyhole lympet haemocyanine. The production of the specific antibody in the different batches of sera was confirmed and titrated by ELISA. The N1 and C2 sera were affinity purified using the peptide bound to an EAH-sepharose column according to the manufacturer (GE Healthcare, USA). Affinity-purified N1 antibody was biotinylated using biotin N-hydroxysuccinimide ester. The affinity-purified antibodies were checked for different applications and their specificity carefully determined by several independent tests (Figure 1 for western blotting; Supplementary Figures 4 and 8 for immunohistochemistry, immunoprecipitation–kinase and ELISA). All the antibody techniques were performed as described.[27]

New Zealand Rabbits were used for immunisation and were kept at the animal facility of the Facultat de Farmàcia, Universitat de Barcelona.

Other antibodies were from commercial suppliers: Lamin B1 (Ab16375) was from Abcam (Cambridge, UK); anti-Bak (Ab-2) was from Calbiochem; Cdk2 (M2), Cdc25A (F-6), Cdc25C (C-20), Bax (N-20), Tom20 (FL-145) and p21Cip1 (C-19) were from Santa Cruz (Santa Cruz Biotechnology, CA, USA); monoclonal antibody against Cdk2 (clone 55; BD Biosciences, San José, CA, USA); monoclonal antibody anti-myc tag 9E10 was from a hybridoma from the American Type Culture Collection (ATCC, Rockville, MD, USA); cleaved caspase-3 (Asp175, 5A1 rabbit mAb), caspase-9 (C9 mAb), phospho-ATM (Ser1981, 10H11.E12 mAb), phospho-Chk1 (Ser345, 133D3 rabbit mAb) and phospho-p53 (Ser15 rabbit antibody) were from Cell Signal (Cell Signalling Technology, Boston, MA, USA), anti-pyruvate kinase was from Chemicon International (Temecula, CA, USA); anti-Bim (14A8) was from Alexis (Lausane, Switzerland), mouse anti-α-Tubulin clone DM1A was from Sigma (St. Louis, MO, USA), rabbit anti-caspase-8 (Ab-4) was from Neomarkers (Fremont, CA, USA) and the anti-Cdk1 (A-17), anti-cytochrome *c* (7H8.2C12), anti-Bid (559681) and the CD95 agonistic antibody Jo2 were from Beckton-Dickinson (BD Biosciences). Monoclonal antibodies against PARP were kindly provided by Dr. José Yelamos (IMIM, Barcelona).

Figure 7 Downregulation of cyclin O abrogates apoptosis induced by intrinsic stimuli due to a failure to activate caspases. (**a**) Similar to the case of γ-radiation-treated cells (Supplementary Figure 6a), cyclin O downregulation leads to a complete lack of activation of apical caspases, caspase-8 (cleaved caspase-8, ccaspase-8) and caspase-9 (active caspase-9, acaspase-9), and executory caspase-3 (active caspase-3, acaspase-3) upon dexamethasone treatment. As a control, the inactive proforms of the caspases are shown. (**b**) Clones shG1 and 3.7 were treated with 10 Gy of γ-radiation using a ^{137}Cs irradiator (left panel) or treated with 2 μM dexamethasone (right panel) and cultured for 6 h. Cell extracts were obtained at 0 and 6 h and the DEVDase activity measured in triplicate using the fluorogenic substrate Ac-DEVD-AFC. Represented are the means ± the S.E.M. of the fluorescence units generated per minute and per microgram of protein. (**c**) Clones shG1 and 3.7 were treated with 2 μM dexamethasone and cells harvested at the indicated time points. Cell extracts were analysed by western blotting for PARP full-length (p116) and cleaved (p85) expression. (**d**) Clones shG1 and 3.7 were cultured 24 h in the absence (PBS) or presence of 0.5 μg/ml of the agonistic antibody Jo2 (αFas), 30 μg/ml of cycloheximide (CHX) or the combination of both treatments (αFas + CHX). Whole-cell extracts were analysed by western blotting for the presence of cleaved caspase-8 (p10), downregulation of full-length (FL) p24 Bid and the appearance of the cleaved forms of Bid, tBid p13/p15

Cell fractionation and immunoblotting. Cell fractionation was performed essentially as described.[5] SN1 is the result of the first centrifugation and is highly enriched in cytosol. SN2 is the supernatant of the second centrifugation and contains diluted cytosolic extract. The pellet (P) contains mainly nuclei, membranes and mitochondria.

Semiquantitative and quantitative RT-PCR. RNA was prepared from mouse thymus and spleen using Trizol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions.

Forward primers used for quantitative RT-PCRs were for mcyclinOα, 5′-CGCTTG CAAGCAGGTAGAGG-3′ and for mcyclinOβ, 5′-CGCGCCAGCCA CAAGTAGAGG-3′. For both isoforms, the reverse primer was 5′-TGAGTGA AGTGCTCCAGGAAG-3′. For mcyclinOδ, the forward primer was 5′-CCCATG GCTCCCCTAGGTG-3′, and the reverse 5′-GCTGCCCGGGCCGCCTTCAGC-3′. In the case of HPRT, the primers used were 5′-GGCCAGACTTTGTTGGATTTG-3′ and 5′-TGCGCTCATCTTAGGCTTTGT-3′.

Quantitative RT-PCR was performed using QuantiTect Sybr Green reagent (Qiagen) according to the manufacturer's protocol and the data were analysed using SDS2.1 software (Applied Biosystems).

Immunohistochemistry. Immunohistochemical analyses were performed using 3 μm sections of paraformaldehyde-fixed paraffin-embedded mouse tissue blocks or cultured cell pellets. Antigen retrieval was performed by boiling the slides in 10 mM sodium citrate (pH 6) for 10 min. Slides were blocked with filtered 5% non-fat milk dissolved in PBS. Affinity-purified antibody against cyclin O was incubated for 90 min at 27°C. As secondary antibody, the EnVision^R anti-rabbit system was applied (DakoCytomation, Goldstrup, Denmark). Sections were counterstained with haematoxylin, dehydrated and mounted.

Cell culture and transfections. The human HEK293 and U2OS cell lines were obtained from ATCC and maintained in Dulbecco's modified Eagle's medium supplemented with antibiotics and 10% foetal calf serum. The mouse T-cell lymphoma cell line WEHI7.2 (obtained from Dr. Roger Miesfeld, University of Arizona, Tucson, AZ, USA) was grown in low-glucose (1 g/l) Dulbecco's modified Eagle's medium supplemented with antibiotics and 10% foetal calf serum. U2OS and HEK293 cells were transfected using the calcium phosphate method as described.[28] The total cell counting by flow cytometry was carried out using the Cell Count Fluorospheres method according to the manufacturer (Beckman Coulter, Fullerton, CA, USA). WEHI7.2 cells were transfected by electroporation as described.[29]

Pull-down–elution–IP–kinase assay. Purified MBP-mcyclin O (10 μg) was bound to 75 μl of amylose beads (New England Biolabs, Ipswich, MA, USA) and incubated with a cell extract of HEK293 at 4°C. After 4 h of incubation, the beads were extensively washed, and the MBP-cyclin O together with associated proteins was eluted by incubation with 15 mM maltose. After 30 min, aliquots of the eluate were immunoprecipitated with 1 μg of normal rabbit IgGs (Sigma), 2 μl of anti-Cdk1 or 2 μl of anti-Cdk2 (M2) and kinase assay performed in the immunoprecipitates using 1 μg of histone H1 as exogenous substrate. As controls, an aliquot of the amylose beads before elution (beads) or from the eluate was processed directly by the kinase assay.[30]

Cyclin O-Cdk2 co-immunoprecipitation. Cell extracts were prepared from HEK293 cells transiently transfected with a myc-tagged cyclin O expression vector and then was immunoprecipitated with 50 μl of the supernatant of the 9E10 hybridoma (monoclonal antibody directed against the anti-myc epitope) bound to protein-G sepharose beads (GE Healthcare). After washing steps, myc-cyclin O and associated proteins (Cdk2) were detected by western blotting of the beads (M2 antibody). As a negative control, an immunoprecipitation with the corresponding amounts of purified non-immune rabbit immunoglobulins was carried out in both cases.

Mice and treatments. CD2Baxα (Bax18 line[31]) and EμBcl2 (Eμ-*bcl-2-25* line[32]) transgenics bred to FVB/N wild-type mice were kept in SPF conditions in the mouse facility of PRBB with free access to water and food during all the procedures. Mice used in the experiments came from one or two litters from the same parents and only non-transgenic littermates were used as controls. In case two different litters were used, for each time point, the control and transgenic mice came from the same litter.

Irradiation procedure: mice were treated with 10 Gy of γ-radiation using a ^{137}Cs irradiator and kept with free access to water and food during the time course of the experiment. At the indicated time points, mice were killed, the organs removed and either fixated in paraformaldehyde for immunohistochemistry or immediately frozen in liquid nitrogen for RNA isolation.

Dexamethasone treatment: mice were intraperitoneally injected with 2 mg of dexamethasone (dexamethasone phosphate, Fortecortin^R) and processed as indicated for the irradiation procedure.

Colony-forming assays. U2OS osteosarcoma cells (5×10^5 per well) were plated in a six-well plate. Next day, three wells were transfected with pcDNA3 empty vector (Invitrogen) and three more with pcDNA3-HAmcyclin O (2 μg per well). All the plasmids used were previously linearised with *Ahd*I. Forty-eight hours after transfection, cells were trypsinised, each well was diluted 1/100 and plated in triplicate in six-well plates in the presence of 1 mg/ml of G418. Selection media were replaced every 3 days. Two weeks after transfection, cells were fixed with 4% paraformaldehyde, stained with Coomassie Blue and counted.

shRNA cell clones. To downregulate mcyclin O in WEHI7.2 cells, we used the following oligos to make shRNA constructs based on the pSuper[13] plasmid system (provided by Reuven Agami, The Netherlands Cancer Institute, Amsterdam, The Netherlands): shEx3: 5′-GCGCCCACCATCAACTTC-3′ and shC5: 5′-GCTCTA GAGGCTCAAACCC-3′; shRNA against GFP: 5′-GCTGACCCTGAAGTTCATC-3′.

WEHI7.2 cells were transfected by electroporation with the pSuper-based shRNA constructs described above and single-cell clones isolated. After selection with 1 μg/ml of puromycin (Sigma), apoptosis was induced in the selected clones with etoposide (10 μM), dexamethasone (2 μM) or with the CD95-agonistic antibody Jo2 (0.5 μg/ml plus 30 μg/ml of cycloheximide). Immunoprecipitation and kinase assay were performed as described[5] using the C2 antiserum. Apoptosis was measured by quantitation of the sub-G1 cell population by flow cytometry after propidium iodide staining of ethanol-fixed cells.

Statistical analysis. Except where indicated, data were expressed as the mean ± S.E.M. of the values from the number of experiments as indicated in the corresponding figures. Data were evaluated statistically by using the Student's *t*-test. Significance levels: $P = 0.05$, *; $0.05 < P < 0.01$, **; $0.01 < P < 0.005$, ***; $0.005 < P < 0.001$, *** and $0.001 < P < 0.0005$, *****.

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Supplementary Information accompanies the paper on Cell Death and Differentiation website (http://www.nature.com/cdd)