STUDY OF THE REGULATION AND SIGNALLING OF CDK2-CYCLIN O COMPLEXES DURING APOPTOSIS

Doctoral thesis

This thesis is inscribed in the Departament de Ciències Experimentals i de la Salut of the Universitat Pompeu Fabra and the work described has been carried out in the IMIM-Hospital del Mar under the supervision of Dr. Gabriel Gil Gómez

Barcelona, 2008

Ramon Roset i Huguet
Doctorand

Dr. Gabriel Gil Gómez
Director

Als meus pares, infatigables!

Abstract

The aim of this thesis is the characterization of a protein involved in apoptosis. Our group has identified an early step common to different forms of intrinsic apoptosis stimuli. This step requires *de novo* synthesis of a novel Cyclin, Cyclin O, that upon apoptosis induction in lymphoid cells forms active complexes, primarily with Cdk2. Cyclin O expression precedes glucocorticoid and gamma radiation-induced apoptosis *in vivo* in mouse thymus and its overexpression induces 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 while leaving CD95 death receptor mediated apoptosis intact. This data demonstrates 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. In addition we have identified c-Myb a substrate of Cdk2-Cyclin O complexes and we show that c-Myb is downregulated during apoptosis of lymphoid cells.

INDEX

I. INTRODUCTION

1. Introduction to the molecular mechanisms of apoptosis

1.1. General overview of apoptosis

Multicellular organisms often need to get rid of cells that are in excess or that are potentially dangerous like cancer cells, cells infected with a virus or cells with highly damaged DNA. To this end, cells use a tightly regulated genetic program of cell death called apoptosis.

Apoptosis was discovered more than 35 years ago by different pathologists (1); while studying tissues by electron microscopy Kerr and colleagues realized that in a wide range of tissues there were many cells undergoing cell death with a characteristic pattern: chromatin condensation, cell shrinkage and membrane blebbing. They coined the term apoptosis in order to differentiate it from necrotic cell death, that is, the death resulting in cell blast and release of the cellular content.

Apoptosis is very important for the maintenance of the organism's cell homeostasis: cell division is compensated by cell death in order to ensure a proper cell turnover. It is also very important for proper body shaping during development. Extensive division and differentiation of a particular cell often precedes development of an organ or tissue, which is then "pruned" into the correct form by apoptosis.

In humans, both excessive and insufficient apoptosis can lead to severe pathological consequences. Suppression of the apoptotic machinery causes autoimmune diseases and is a hallmark of cancer (2;3). For example, the apoptosis-inhibiting protein Bcl-2 is overexpressed in several cancers (4) and the apoptotic protease activating factor (Apaf-1) is frequently inactivated in cancers such as malignant melanoma (5). On the other hand, abnormal upregulation of apoptosis is associated with neurodegenerative disorders (6).

The first genetic analysis of apoptosis came from the worm *Caenorhabditis elegans*. This nematode has an invariant pattern of cell death during development: every nematode eliminates the same 131 cells out of 1090 that form the immature organism. Subsequently, H. Robert Horvitz had the insight to mutagenize *C.elegans*, in order to identify genes regulating all 131 cell deaths (7). The first genes identified to be necessary for *C.elegans* apoptosis were *ced-3* and *ced-4*. Moreover, a gain of function mutation in *ced-9* was shown to be able to block apoptosis (8). The cloning of those genes revealed a high degree of conservation during evolution and the mammalian homologs were identified (Caspase-3, Apaf-1 and Bcl-2 respectively). The best example of evolutionary conservation was shown by Vaux DL and colleagues who demonstrated that human Bcl-2 could substitute for *C.elegans ced-9* protein (9).

Apoptosis consists basically in a proteolytic pathway that leads to the cell dismantling by the cleavage of several cellular proteins. First of all there is an initiator phase that senses the apoptotic signal and transduces the information to the next step, the executioner or effector phase. This first phase consists biochemically in the activation of an apical cystein-aspartate protease (Caspase), a type of proteases with a cysteine residue in the catalytic centre that cuts proteins after an aspartate residue. Active apical Caspases can cut directly effector Caspases, leading to their activation. The activation of effector Caspases (second step) is generally the point of no return so if effector Caspases get active, the cell will die inexorably. The role of effector Caspases is to cleave a large number of proteins including structural cellular components such as actin and lamin, regulatory proteins (inhibitor of Caspase activated DNAase, ICAD), other Caspases, etc. Interestingly, cleavage of ICAD leads to its dissociation from the DNAase CAD, which is then active and degrades the chromosomes into nucleosomal fragments (10). Altogether, those effects lead to the morphologic manifestation of apoptosis: cell shrinkage, chromatin condensation and membrane blebbing. This allows the dead cells to be efficiently phagocytosed and their components reused without releasing potentially harmful intracellular substances into the surrounding tissue and thus avoid a proinflammatory response.

1.2. Caspases, central players in apoptosis

Activation of Caspases is probably the most specific hallmark of apoptosis and is often used to differentiate apoptosis from other programmed cell deaths (11). To date, there have been 14 mammalian Caspases identified (Figure 1) and they are involved either in inflammation or in apoptosis. Although the first mammalian Caspase cloned, Caspase-1 or ICE (interleukin-1β converting enzyme), was identified to be an important regulator of inflammatory response, at least 8 of the 14 Caspases play important roles during apoptosis (12). Caspases involved in apoptosis are divided in two categories, the initiator (Caspase-2, -8, -9, -10, -12) and the effector or executioner Caspases (Caspase-3, -6, -7). All Caspases are produced in cells as catalytically inactive zymogens and must undergo proteolytic cleavage for their activation. Caspases are cleaved at two specific sites, producing an N-terminus inactive fragment and two active domains, p10 and p20. The active Caspase consist in dimers of two p10 and two p20 subunits.

The activation of effector Caspases is performed by an initiator Caspase through a specific cleavage that removes the self-inactivating N-terminal domain. On the other hand, apical Caspase activation is more complex. It was initially proposed that apical Caspases were activated by autocatalytic cleavage of the N-terminal domain, in a similar way than effector Caspases. However, it has been shown that cleavage is neither necessary nor sufficient for apical Caspase activation (13). It seems that activation occurs as a result of dimerization. Apical Caspases exist in the cell primarily as monomers and in this conformation lack activity. During apoptosis it is created a platform for apical Caspase activation that induce its dimerization and thus, activation. Examples of those platforms are DISC (Death-Inducing Signaling Complex) and apoptosome.

Caspases are tightly regulated since their activation is irreversible because cells do not possess a mechanism to re-ligate cut peptide bonds. The key step for Caspase activation is the formation of the apical Caspase activation platforms: DISC and apoptosome. However, there are also other mechanisms of regulation including transcriptional regulation, posttranslational regulation and protein degradation. In addition, the enzymatic activity of Caspases can be inhibited by IAPs (inhibitors of apoptosis).

IAP proteins were first identified in baculoviruses based on their ability to suppress apoptosis in insect cells (14). The functional unit of IAPs is the baculoviral IAP repeat (BIR), which is about 80 aminoacids length and is folded around a zinc atom (15). This domain is the responsible of binding to active caspases-3,-7 and -9 (but not others like -6 or -8) thus blocking substrate accessibility. Mammalian IAPs include XIAP (X-linked IAP), cIAP-1, cIAP-2, NAIP (Neuronal Apoptosis Inhibitory Protein), Survivin and Livin. Interestingly, IAPs can be sequestered by SMAC/DIABLO (Second Mitochondrial Activator of Caspases / Direct IAP Binding Protein with Low pI), Omi/HtrA2 (High Temperature Requirement Protein 2) and XAF1 (XIAP-associated factor-1).

Those proteins are in the mitochondrial intermembrane space and are released during apoptosis. Their binding to IAPs leads to the release of active Caspases. Genetic studies in *Drosophila* have shown that IAPs and its regulators play essential roles during apoptosis; however, their function in mammals seems to be less important or secondary to other functions. For example, Survivin seems to function in chromosome separation during mitosis (16).

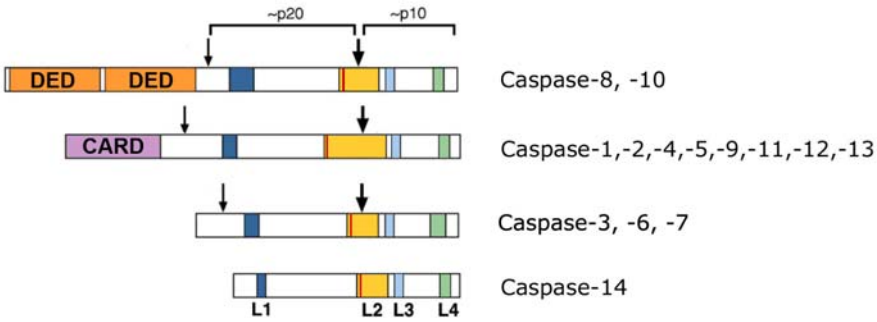


Figure 1. Schematic diagram of the mammalian Caspases. The position of the cleavage sites are highlighted with an arrow. The four surface loops (L1-L4) that shape the catalytic groove are indicated as colored boxes. The catalytic residue Cys is shown as a red line at the beginning of loop L2.(Adapted from Shi *et al*. (17)).

1.3. The apoptotic pathways

There are two main apoptotic pathways depending on the triggering signal: the extrinsic and the intrinsic pathway. The intrinsic pathway integrates signals generated by a variety of stressors, including DNA damage, endoplasmic reticulum stress, loss of adhesion, growth factor withdrawal, macromolecular synthesis inhibition and others. The extrinsic pathway, on the other hand, is originated when a specific cell surface receptor binds its cognate ligand. Each pathway diverges from the other in the initial steps whereas the executioner phase is the same in both: activation of effector Caspases.

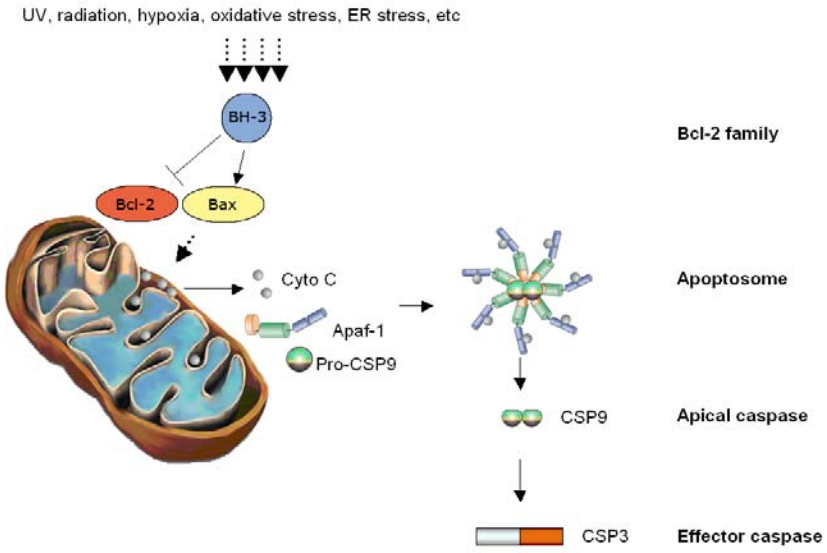


Figure 2. Schematic diagram of the intrinsic apoptotic pathway. Apoptotic stimuli induce mitochondrial permeabilization by modulating the activity of the Bcl-2 family of proteins. Upon mitochondrial permeabilization, Cytochrome *c* is released into the cytoplasm and catalyzes the assemblage of the Apoptosome. This consists of seven molecules of Apaf-1, seven of Cytochrome *c* and two of pro-Caspase 9, and are disposed as a star-like structure. Apoptosome formation induces Caspase-9 activation by dimerization and proteolytic cleavage. Active Caspase-9 can directly cleave Caspase-3, leading to the activation of the effector phase.

1.3.1. The intrinsic apoptotic pathway

The central step in the intrinsic apoptotic pathway is the regulation of mitochondrial permeabilization. This organelle can be seen as an arsenal that stores several pro-apoptotic proteins. Among them the most prominent is Cytochrome *c*. Cytochrome *c* is released into the cytoplasm in response to pro-apoptotic signals. Once in the cytoplasm it binds to Apaf-1 and induces an oligomerization of both proteins leading to the formation of a signaling platform called apoptosome, which recruits pro-Caspase-9. The formation of the apoptosome leads to the activation of Caspase-9 which activates the effector Caspases by direct cleavage of Caspase-3.

There are two interesting points in this pathway that I will further discuss: i) how are mitochondria permeabilized and ii) how Cytochrome *c* induces the formation of the apoptosome.

1.3.1.1. Regulation of mitochondrial permeabilization: the Bcl-2 family of proteins

Mitochondrion is the cell's powerhouse; it produces the majority of the cellular energy as ATP. As it is a double membrane organelle, it has two different spaces: the inner matrix and the intermembrane space. The intermembrane space is where the pro-apoptotic proteins are stored. Then, the sole permeabilization of the outer mitochondrial membrane (OMM) leads to the release of the proapoptotic factors into the cytoplasm and subsequent activation of the apoptotic cascade.

The Bcl-2 family of proteins regulates the permeabilization of mitochondria. Bcl-2 (B-cell lymphoma-2) was the former member of this family and was first identified as an oncogene that instead of promoting cell proliferation, it blocked cell death (18). The members of the family are divided in three groups: the antiapoptotic Bcl-2-like, the proapoptotic multidomain Bax (Bcl-2 associated X) and Bak (Bcl-2 homologous antagonist killer) and the proapoptotic BH3-only proteins. The way they regulate the mitochondrial outer membrane permeabilization (MOMP) could be summarized in the following way: Bax and Bak induce the MOMP either alone or with the collaboration of BH3-only proteins. Bcl-2-like proteins interact with Bax and Bak and keep them inactive. The role of BH3-only proteins is more controversial and currently two models have been proposed (Figure 3); the indirect model (19;20) proposes that BH3-only proteins bind to the anti-apoptotic Bcl-2 like proteins and thus release active Bax and Bak. And the direct model (21;22), which proposes a classification of the BH3-only proteins into activators and inactivators. The role of inactivators BH3-only is the same as proposed by the indirect model (Bcl-2 like inactivation). Conversely, the activators are proposed to bind to proapoptotic Bax and Bak and collaborate with them in the MOMP.

All BH3-only proteins can bind with high affinity to (at least some) pro-survival Bcl-2-like proteins and trigger apoptosis when overexpressed. They can be activated by several mechanisms like transcriptional control (Puma, Noxa, Bim)(23;24), phosphorylation (Bad) (25), Caspase cleavage (Bid) (26) or dissociation from microtubules (Bmf, Bim)(27;28). So the large variety of proapoptotic proteins and their complex regulation seems to have emerged to respond to the diverse apoptotic stimuli and to allow a fine regulation.

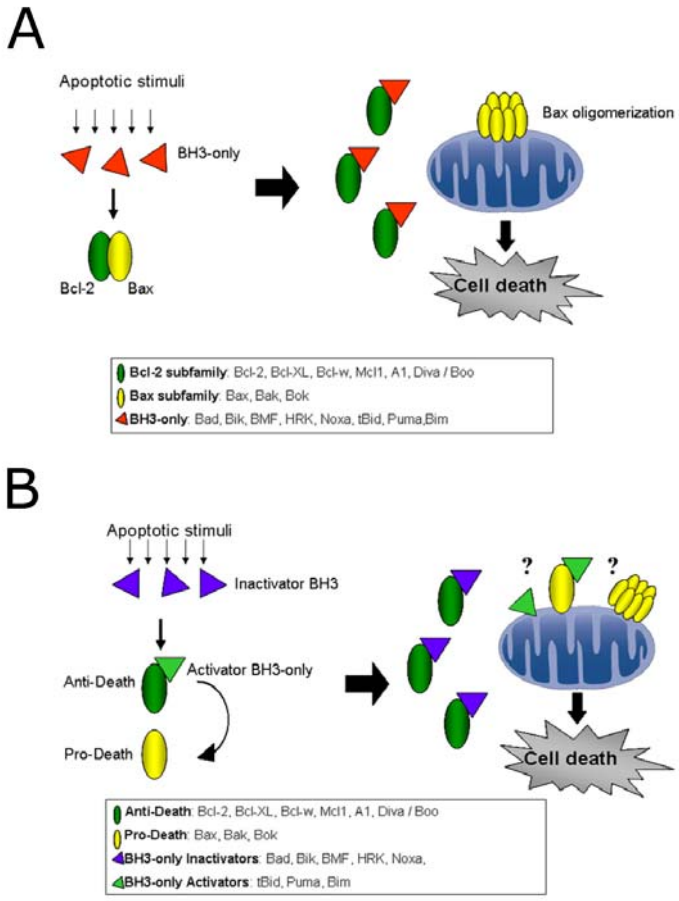


Figure 3. Models to explain the functions of the BH-3 only family of proteins. (A) The classical or direct model. Bax and Bak proapoptotic members are constitutively inhibited by the Bcl-2 like proteins (Bcl-2, Bcl-XL, Bcl-w, A1, Diva and Mcl-1). Upon arrival of the apoptotic signal, BH3-only proteins are activated and bind Bcl-2 like proteins releasing Bax and Bak. Bax and Bak can homo- and heterodimerize in the mitochondrial outer membrane, triggering the release of proapoptotic factors. (B) The hierarchy or indirect model. Anti-death proteins directly inhibit Activator BH3-only proteins. Upon arrival of the apoptotic signal, Inactivator BH3-only proteins (purple triangles) bind to Anti-death proteins, then releasing Activator BH3-only proteins (green triangles). These either directly activate Pro-death proteins or cooperate with them to release proapoptotic factors from the mitochondria. Picture from Roset R. et al.(29)

The mechanism whereby Bax and Bak induce MOMP is still controversial, despite intensive investigation. The predominant view is that upon commitment to apoptosis, Bax and Bak undergo conformational changes, oligomerize and form ion channels in the outer mitochondrial membrane that indirectly allow to the release of proteins across the outer mitochondrial membrane. The initial insight leading to the pore model was that the structure of Bcl-XL was similar to that of the T-domain of diphtheria toxin (30). This domain is able to form pores in artificial lipid bilayers and cell membranes. In support of this model, Bax and Bak are also able to form pores in artificial lipid bilayers. Moreover, during apoptosis induction Bax and Bak form oligomers in the outer mitochondrial membrane (OMM) (31). Inactive Bax exists mainly as a cytosolic monomer. Its activation results in a conformational change allowing its insertion into the OMM, and this process is required for MOMP induction. Presumably, the same applies to Bak, although the latter resides in the OMM also in its inactive state (32). It has been proposed that Bak is maintained in a monomeric inactive conformation due to interactions with VDAC2 (voltage-dependent anion channel-2), a transmembrane protein residing in the OMM. The conformational change leading to Bax and Bak activation would be induced by its release from Bcl-2 like proteins that is mediated by active BH3-only proteins.

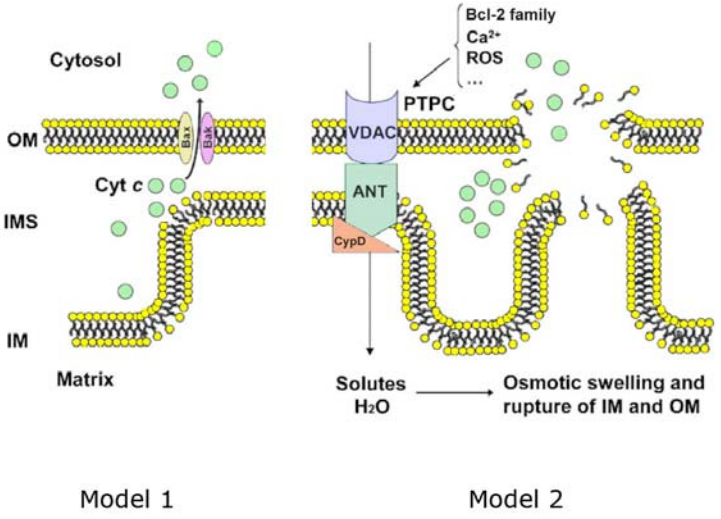


Figure 4. Models of mitochondrial permeabilization. Model 1 proposes Bax and Bak form pores in the outer mitochondrial membrane (OM) that are able to release Cytochome *c* from the intermembrane space (IMS). Model 2 proposes that several stimuli (Bcl-2 family of proteins, Ca^{2+} ions, Reactive Oxigen Species, etc) converge to induce PTPC opening thus leading to a massive entrance of water and solutes into the mitochondrial matrix that will lead to osmotic swelling and release of the proapoptotic factors.

The major problem of this model is that the pore size of Bax and Bak channels is not big enough to allow Cytochrome *c* release. For that reason it was proposed another model that implies the mitochondrial Permeability Transition Pore Complex (PTPC). PTPC is a large high-conductance multiprotein complex constituted of several components that connect the cytoplasm with the mitochondrial matrix. The exact composition has not been yet determined but is currently believed that the major PTCP components are VDAC2, ANT (Adenine Nucleotide Translocator), and cyclophilin D (CypD). According to this model, an apoptotic signal would open the PTPC allowing small solutes and water to enter the mitochondrial matrix. The resulting osmotic pressure will lead to mitochondrial swelling and releasing of its content (mainly, Cytochrome *c*).

Based on knockout experiments (that affect CypD as an essential PTCP component or Bax and Bak) it appears likely that both models are correct for distinct subsets of cell death. Moreover, it is reasonable to think that both mechanisms cooperate for MOMP. In that sense, Frezza et al. proposed a model where PTPC and Bax or Bak cooperate together with the mitochondrial fission machinery to achieve a total release of Cytochrome *c* (33).

1.3.1.2. The apoptosome

The apoptosome is the platform for activation of the apical Caspase Caspase-9, by the intrinsic apoptotic pathway. Caspase-9 activation requires the cofactor Apaf-1, which exists in a monomeric form in the absence of apoptotic stimulus. In the presence of Cytochrome *c*, Apaf-1 oligomerizes to form a wheel-shaped structure that recruits pro-Caspase 9. This triumvirate multiproteic complex is the apoptosome (Figure 5).

Apaf-1 is a multidomain protein that consists of three functional domains: an N-terminal Caspase-recruitment domain (CARD), a nucleotide-binding and oligomerization domain (NB-ARC) and a WD40-repeats domain at the C-terminus. In the absence of apoptotic stimulus, Apaf-1 is present in the cytoplasm as a monomeric protein; it is thought that in this situation the WD40-repeats domain folds back to the N-terminus of the protein and produces an auto-inhibited conformation. When Cytochrome *c* is released from the mitochondria, it binds to the WD40-repeats domain of Apaf-1, unfolding the structure. Strikingly, this binding is necessary in mammals but not in *Drosophila*, where Cytochrome *c* does not activate Apaf-1 (34). To achieve a full activation of Apaf-1 it is also necessary the presence of ATP (or dATP) (35). In the presence of ATP and Cytochrome *c*, Apaf-1 is completely active and oligomerizes. Structural studies show that active apoptosome consists in 7 Apaf-1 molecules arranged in a wheel-like structure. The WD40 regions with Cytochrome *c* protrude through the exterior and in the centre are the CARD domains. The CARD domains of Apaf-1 interact with the CARD domains of Caspase-9 through homotypic interactions. Once recruited to the apoptosome, Caspase-9 becomes activated by the process called 'proximity induced

dimerization'. The activation of Caspase-9 is solely a result of dimerization. Then active Caspase-9 can cleave itself and produce three fragments: the prodomain, and the catalytic active p10 and p20 fragments. However, this cleavage is a result of activation and is not necessary for activation itself since cleavage deficient mutants show no defect in Caspase activity (36). Active caspase-9 cuts and activates the effector Caspase-3, leading to the executioner phase of apoptosis.

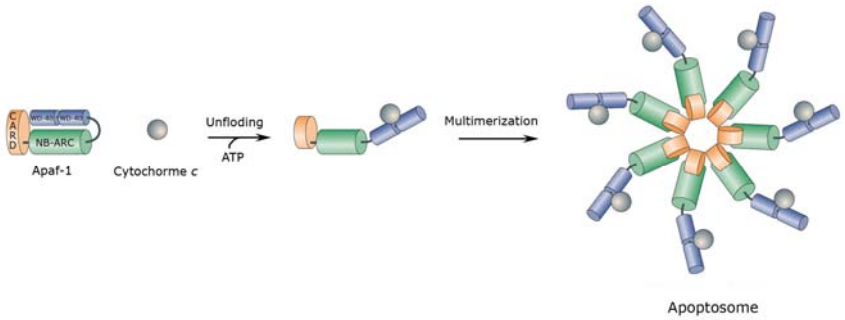


Figure 5. Mechanism of apoptosome formation. Apaf-1 is composed of four different domains, two WD-40 repeats domain (blue boxes), one NB-ARC domain (green boxes) and one CARD domain. In its inactive conformation Apaf-1 is thought to be folded in an autoinhibitory fashion. Upon Cyctochrome *c* bindin, Apaf-1 unfolds in an ATP dependent fashion and is able to oligomerize. Scheme adapted form Riedl and Salvesen (35).

1.3.2. The extrinsic apoptotic pathway

This apoptotic cascade is initiated with the engagement of a ligand to a specialized cell-surface receptor called death receptor (DR). DRs Family includes Fas/CD95/Apo1, TNF-αR (Tumor Necrosis Factor-alpha Receptor) and two receptors for TRAIL (TNF-α Related Apoptosis-Inducing Ligand), DR4 and DR5. The common feature of these transmembrane proteins is the presence of a cytoplasmic domain termed 'Death Domain' which is capable of binding to homologous domains, thus mediating protein-protein interactions.

The most paradigmatic receptor of this family is probably Fas/CD95. When Fas-Ligand (FasL) binds to Fas, it induces a trimerization of the receptor that is able to recruit the adaptor protein FADD (Fas-associated with Death Domain). The interactions are mediated by Death Domain–Death Domain homotypic contacts. Then FADD recruits pro-Caspase 8 through homotypic interactions with the Death Effector Domains (DEDs). This resulting assemblage of proteins is known as the Death-Inducing Signalling Complex (DISC) and is the platform that will activate Caspase-8.

The mechanism of Caspase-8 activation is the same as proposed for Caspase-9, 'proximity induced activation'(13). Recruitment of pro-Caspase 8 to the DISC juxtaposes multiple pro-Caspase 8 molecules, resulting in dimerization, cleavage of the prodomain, and activation of Caspase 8. This cleavage of the prodomain instead of a necessary event for the activation is merely a result of the activation.

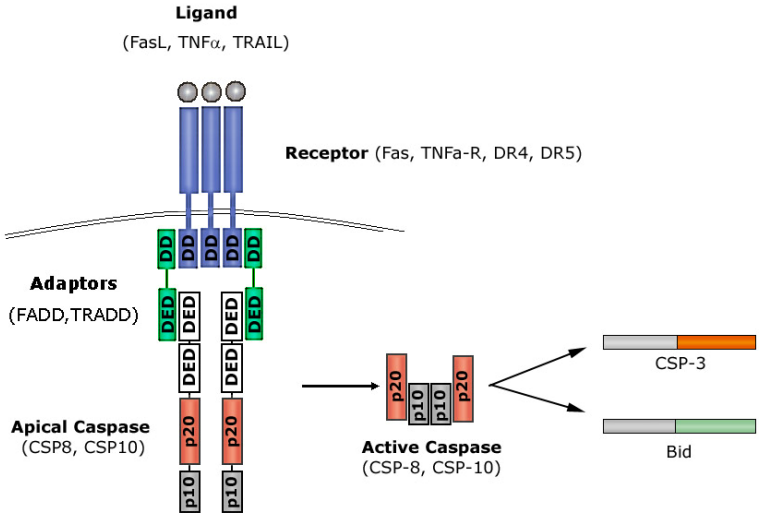


Figure 6. Schematic diagram of the extrinsic apoptotic pathway. Engagement of a ligand with the Death Receptor induces its oligomerization and recruitment of the adaptor proteins (FADD, TRADD) through homotypic Death Domain (DD) interactions. The adaptor proteins recruit Pro-Caspase 8 and 10 through homotypic Death Effector Domain (DED) interactions. This structure is called DISC (Death-Inducing Signaling Complex) and leads to Caspase 8 and 10 activation. This results in their autocatalytic cleavage in p10 and p20 subunits that conform the active apical Caspase. Active Caspase 8 and 10 activate the effector Caspase 3 by direct cleavage or by the activating the mitochondrial pathway through cleavage of Bid.

How Caspase-8 transduces the apoptotic signal depends on the cell type and on the quantity of active Caspase-8 produced in the DISC (37). In type I cells, a high production of active Caspase-8 can directly process the effector Caspase, Caspase-3, leading to its activation and ultimately to apoptosis. In type II cells, however, only a small amount of Caspase-8 is produced. The DISC in these cells is formed quite poorly, little FADD is recruited and little active Caspase-8 is produced in the DISC. Apoptosis in these cells is dependent, at least in part, on the cleavage of the BH3-only protein Bid, whose cleavage results in a proapoptotic fragment termed tBid (truncated Bid). tBid induces mitochondrial permeabilization and thus activation of effector Caspases

through the apoptosome. This pathway can also be seen as an amplification loop of the extrinsic pathway in type I cells. Biochemically those cells can be distinguished by the effect of Bcl-2 overexpression. In type I, where Caspase-3 is directly activated by Caspase-8, Bcl-2 overexpression does not protect from death receptor induced apoptosis. However, in type II cells, where the mitochondrial loop is necessary for death receptor induced apoptosis, Bcl-2 overexpression render cells resistant.

Other proteins have been found associated with the DISC. Of interest are Caspase 10 and c-FLIP (FLICE-like inhibitory protein). Caspase-10 is very similar to Caspase-8 and behaves in a similar way. Although it seems to be important in the human system, the lack of an orthologue in the mouse has complicated its study. c-FLIP is a protein similar to Caspase 8 but with a mutated catalytic site. For this reason it was proposed to function as a dominant negative, thus inhibiting Fas-induced apoptosis (38). Other studies, have reported that is able to hetero-dimerize with Caspase-8 producing active complexes, thus acting as a pro-apoptotic protein (39). Despite the huge number of reports on c-FLIP, it is not well established its function since knockout mice show embryonic lethality due to failure in heart development (40). This phenotype is similar to that of Caspase-$8^{-/-}$and $Fadd^{-/-}$ mice, suggesting a more complex function for the DISC proteins.

Fas has also been shown to activate not only the pro-apoptotic pathway, but pro-survival ones. NF-kappaB, ERK, JNK and p38 activation has been reported after FasL treatment in different cell models (41). In those apparently controversial effects of FasL could underlie the explanation of the phenotypes of mice deficient for DISC components. Moreover it could explain why in most cell systems the solely activation of Fas does not induce apoptosis. It is likely that multiple signalling pathways, including those activated by Caspases and NF-κB, are simultaneously triggered by ligation of Fas, and that only under certain conditions the signalling threshold is reached to induce apoptosis. This threshold could be modulated by the levels of Caspase-8, c-Flip, Bcl-2 etc.

1.4. The effector phase

Apoptotic cell death is executed by effector Caspases which cleave various vital proteins. To date more than 200 substrates have been identified for effector Caspases. A complete list of the substrates can be found online in a proteolytic database: http://cutdb.burnham.org.

Proteolysis of certain substrates can be linked to discrete morphological changes of cell death. For example, Lamins cleavage leads to nuclear envelope dismantling (42), ROCK-1 cleavage induces membrane blebbing (43), etc. In general, almost all cellular processes are inactivated by Caspase cleavage, including apoptosis regulators, cell adhesion proteins, cytoskeletal and structural components, nuclear structural proteins, ER and Golgi-resident proteins, Cell cycle regulatory proteins, DNA synthesis and repair proteins, transcription factors, RNA synthesis and splicing factors, protein translation factors, membrane receptors, adaptor proteins, signalling kinases and phosphatases, etc (44).

Probably the most characteristic event of the effector phase is the chromatin condensation and internucleosomal DNA cleavage. Some proteins have been identified as responsible of chromatin condensation and cleavage during apoptosis. The first protein characterized was CAD/DFF40 (Caspase-activated DNAase/DNA fragmentation factor 40 kDa) which was independently cloned by two groups in 1998 (45;46). CAD is an endonuclease that under normal conditions is localized in the cytoplasm in a complex with ICAD/DFF45 (inhibitor of CAD/ DNA fragmentation factor 45 kDa). This interaction blocks the endonuclease activity of CAD. In apoptotic conditions, ICAD is cleaved by Caspase-3 at two sites in such a way that no longer can bind CAD. CAD is then released and moves to the nucleus where it cuts DNA (Figure 7).

Despite the fact that mice lacking CAD show a dramatic reduction in DNA fragmentation during apoptosis, it is not completely abolished (47;48). This suggested that other proteins should be involved in DNA fragmentation. One interesting endonuclease identified is edonuclease G (endoG). This protein localizes to mitochondria but during apoptosis, when mitochondrion is permeabilized, endoG is released and goes to the nucleus where it cleaves DNA (49). Other proteins involved in DNA condensation and degradation include AIF (apoptosis inducing factor) a protein that is also released from mitochondria during apoptosis (50), Acinus (Apoptosis Chromatin Condensation Inducer in the Nucleus) (51) and Helicard (CARD-containing DNA helicase)(52), although their precise contribution is less clear.

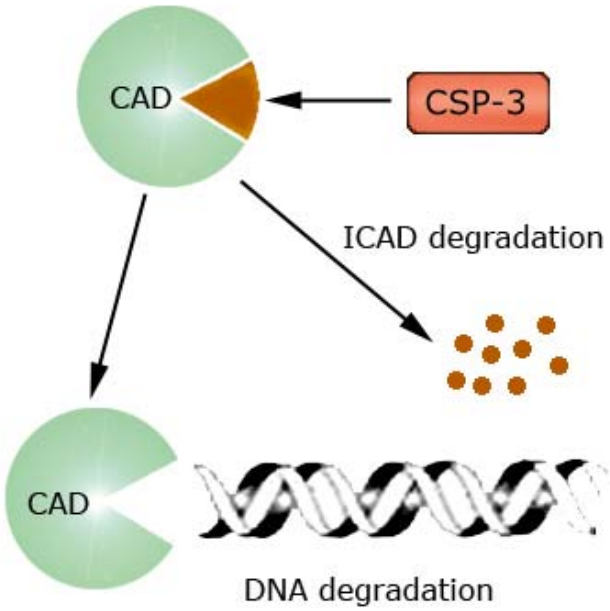


Figure 7. Scheme of Caspase Activated DNAase (CAD) activation during apoptosis. CAD is constitutively inactivated by Inhibitor of CAD (ICAD). During apoptosis, active Caspase-3 (CSP-3) cleaves ICAD thus releasing active CAD that degrades DNA in internucleosomal fragments.

1.5 Phagocytosis, the last step

Efficient elimination of apoptotic cells is crucial for tissue homeostasis in multicellular organisms, thus preventing the release of intracellular content into the surrounding tissue. Accordingly, defects in the clearance of apoptotic cells have been attributed to the onset of persistent inflammatory disorders and autoimmunity (53).

Phagocytosis of apoptotic cells can be done either by neighboring cells or by professional scavengers (macrophages) and is dependent on cell surface markers in the apoptotic cell. Those markers can be divided into 'eat-me' and 'don't eat-me' signals, which appear and disappear respectively in the surface of apoptotic cells.

The best known eat-me signal is the translocation of phosphatidylserine (PS) from the inner to the outer leaflet of the plasma membrane. PS can be recognized by a PS-receptor present in the phagocytes and trigger engulfment (54).

Apart from the active display of eat-me signals, the apoptotic cell loses the ability to present don't-eat-me signals. Brown and colleagues (55) demonstrated that the encounter of a viable cell with a phagocyte via the homophilic interaction of CD31 on both cell surfaces leads to the viable cell's repulsion from the phagocyte. This signaling is somehow disabled in apoptotic cells so that the apoptotic cell can not reject the macrophage. In addition, Lauber and colleagues (56) demonstrated that apoptotic cells secrete chemotactic signals in a Caspase-3 dependent fashion that induces attraction of monocytic cells. This chemotactic signal has recently been shown to be lysophosphatidylcholine (57).

The first insights into the engulfment machinery came from *C.elegans*. In this model, seven gene products have been described to be involved in apoptotic cell removal, each one with a homologous in higher organisms. These genes are divided into two partially redundant classes such that the most dramatic engulfment defect is seen when one gene of each category is altered in double mutant animals (58). *Ced-1* (which encodes an engulfment receptor), *ced-6* and *ced-7* constitute one cassette that helps apoptotic cell recognition. The second cassette influence cytoskeletal remodeling and contain *ced-2*, *ced-5*, *ced-10* and *ced-12*. *Ced-1* is a receptor for eat-me signals of the apoptotic cells and clusters around the dying cell in a manner that utilizes *ced-7* (59). Binding of the engulfment receptors to apoptotic cells ultimately signals cytoskeletal changes, mediated by the other cassette, that lead to the engulfment of the apoptotic cell.

2. The DNA-damage response

Cells exposed to ionizing radiation acquire multiple DNA lesions of which the most prominent are DNA strand breaks. DNA double strand breaks (DSB) are a very dangerous lesion since it can lead to chromosomal rearrangements and ultimately to cancer. Cells respond to ionizing radiation with a global reaction called DNA-damage response (DDR) that mediates cell cycle arrest, DNA repair and, if damage is severe, senescence or apoptosis.

The biochemical mediators of the DDR have been classically divided into four categories: sensors of the damage, mediators, transducers and effectors (60). Although the classification of a protein into a single category is sometimes difficult, this scheme is conceptually useful (Figure 8).

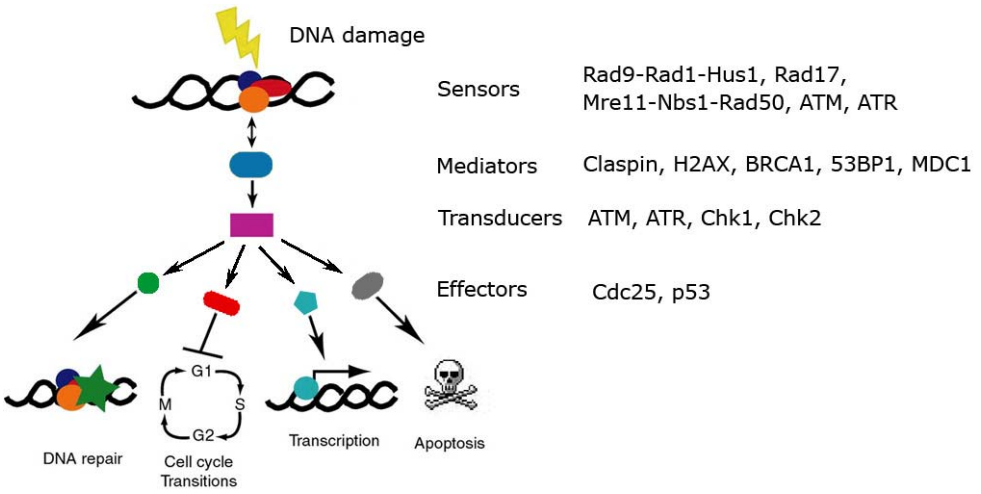


Figure 8. Scheme and classification of the major proteins involved in DNA damage response. (Classification based on Niida et al (60))

2.1. Sensors of DNA damage

The first step for the initiation of DNA damage response is the recognition of the damage. Studies in yeast and mammals revealed that there are at least two recognition machineries: one is the 9-1-1 complex, formed by three proteins, Rad9, Rad1 and Hus1. Upon DNA damage, 9-1-1 complex is recruited to DNA in a Rad17 dependent fashion and assembles with a structure resembling that of PCNA (61). The other sensor is the MRN complex, composed of three proteins: Mre11, Rad50 and Nbs1. This sensor seems to be the most relevant in DSB since recruitment of MRN complex to DSBs has been shown to be independent of any other protein examined so far (62). Moreover, Rad50 can directly bind DNA (63) and the C-terminus of Nbs1 in necessary for the recruitment of ATM to DSB lesions (64).

2.2. Mediators and tansducers

The next step is the activation of the central components of the DDR, the phosphoinositide 3-kinase related kinases (PIKKs) ATM (ataxia telangiectasia-mutated), ATR (ATM and Rad3-related) and DNA-PK (DNA-dependent protein kinase), whose many substrates mediate cell cycle arrest, DNA-repair and apoptosis (65). For all these kinases, activation involves their recruitment to DNA lesions through direct interactions with the conserved C-terminal motifs in Nbs1, ATRIP and Ku80 which are required for ATM, ATR and DNA-PKs binding to DSBs respectively. In general, DNA-PK and ATM respond mainly to DSBs, whereas ATR is activated by single stranded DNA and stalled DNA replication forks (65). Activation of PIKKs by DNA-damage leads on one hand to induction of DNA repair and on the other hand to cell cycle arrest through activation of cell cycle checkpoints.

DSBs can be repaired by two distinct mechanisms, the error-free homologous recombination (HR) pathway and the error-prone non-homologous end-joining (NHEJ) pathway. HR involves the recognition of sister chromatids, the recession of the broken strand and the *de-novo* synthesis of the chain using sister chromatid as a template. NHEJ on the other hand, mediates the junction between two broken ends and is mediated by DNA-PK. This is error-prone since there is no way to recognize the ends to be ligated if several breaks are produced at the same time. The choice between pathways depends on two factors, the cell cycle phase and the nature of the break. NHEJ functions at all stages of the cell cycle, although it plays the predominant role in both G1 phase and in regions of unreplicated DNA in S-phase. HR on the other hand appears to predominate during S and G2 phases (66). Although it remains to be established how NHEJ and HR pathways are coordinated relative to one another, several works point into the same direction: NHEJ is the first choice pathway and if it fails to complete repair, HR is activated. Accordingly, mutations in the DNA-PK lead to increased utilization of HR whereas mutation in HR genes does not increase rates of NHEJ (67).

After ATM recruitment to DSB it is initiated a signaling cascade of protein phosphorylations and interactions that can be detected within less than a minute after DSB induction (68). Although the number of proteins involved in this cascade is large and the processes they govern are complicated, it could be summarized in the following way. DSB lesions are recognized by the multifunctional MRN complex, composed by Mre11, Rad50 and Nbs1 (69). The initial interaction of MRN complex with DSBs mediates the recruitment of ATM through the C-terminus of Nbs1 (64). This recruitment is able to activate ATM by a process still not understood that induces autophosphorylation on many serine residues including serine 1981 (70). Despite the fact that Ser1981 phosphorylation has been shown to be required for human ATM activation, it is dispensable for mouse ATM activity (71); nevertheless, Ser1981 phosphorylation serves as a marker of active ATM. Active ATM-MRN complex triggers two main pathways culminating in local rearrangements of DNA and the neighboring chromatin: i) ATM phosphorylates Histone H2AX on Ser139

which in turn serves as a platform for Mdc1 binding (72). Mdc1 has the particularity of binding both to γ-H2AX and Nbs1. This dual function induces the spreading of H2AX phosphorylation along the chromatin and creates the platform for assembly of other proteins. ATM is also able to phosphorylate other targets like Chk2 kinase. ii) MRN-ATM complex is also essential to initiate DSB repair by homologous recombination. The first event that trigger, is the resection and formation of single stranded DNA (ssDNA), the critical intermediate for homologous recombination repair. This event only occurs if cells are in S or in G2-phase of cell cycle (in G1 phase, the mechanism used preferentially is NHEJ which is mediated by DNA-PK). The ssDNA is then coated by RPA (Replication Protein A), which in turn facilitates recruitment of ATR-ATRIP (ATR interacting protein) complex. Full activation of ATR needs TopBP1, a protein that is also recruited to the ssDNA (73). At this point ATR phosphorylates most of its targets including the effector kinase Chk1.

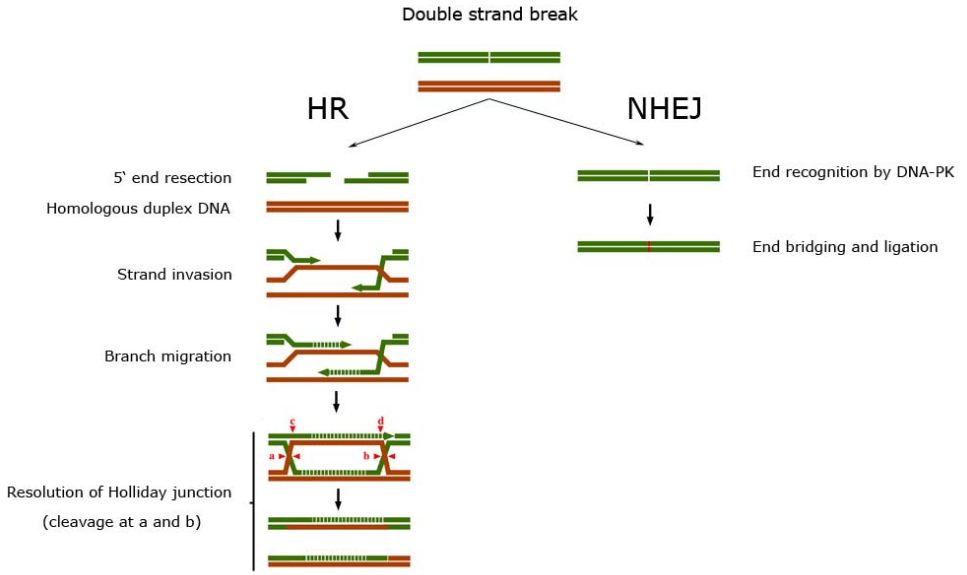


Figure 9. Mechanisms of double strand break repair. Double strand breaks are repaired by either homologous recombination (HR) or non homologous end-joining (NHEJ). Homologous recombination is a complex process that first of all resects 5' ends in the DNA break and then uses the homolog chromosome as a template to resynthesise the DNA strand. NHEJ on the other hand is a process dependent on DNA-PK that brings together two broken ends.

2.3. Effectors: Cell cycle checkpoints

Active ATM and ATR phosphorylate the effector kinases Chk2 and Chk1, leading to their activation. Although both ATM and ATR can phosphorylate both checkpoint kinases, ATM shows preference for Chk2 whereas ATR shows for Chk1 (74). Chk1 and Chk2 are responsible for the activation of the DNA-damage checkpoint by at least two different mechanisms. First, they can directly phosphorylate, and thus inhibit Cdc25 phosphatases, and second, they can activate the p53 response.

Cdc25 phosphatases serve as key activators of the Cdk/cyclins. The three human Cdc25 phosphatases (Cdc25A, Cdc25B, and Cdc25C) are responsible for dephosphorylating Cdks on P-Thr14 and P-Tyr15 residues of Cdk1 and Cdk2. This dephosphorylation triggers the final activation of Cdk/cyclin complexes during normal cell cycle progression (75). Cdc25A controls both the G1-to-S and G2-to-M transitions, whereas Cdc25B and Cdc25C are regulators of the G2-to-M transition.

In response to DNA-damage, Chk1 and -2 phosphorylate Cdc25A at the N-terminal domain inducing its degradation through the ubiquitin-proteasome pathway (76). This leads to inactivation of Cdk2 complexes and thus a block in S-phase. In addition, checkpoint kinases also phosphorylate Cdc25C in two residues at the N-terminal part of the protein creating a binding site for 14-3-3 proteins (77). This binding leads to cytoplasmatic retention of Cdc25C, which is unable to activate Cdk1-CyclinB complexes and thus cell cycle is blocked at G2/M phases. Although both Chk1 and -2 phosphorylate Cdc25 members, they are not completely redundant. For instance, Chk2 is unable to phosphorylate Cdc25A on Thr507 (78).

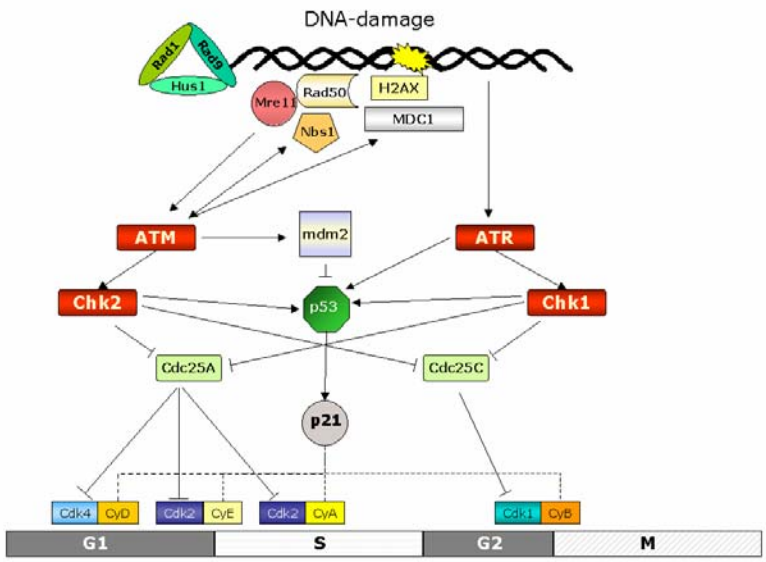


Figure 10. Scheme of the mammalian cell cycle checkpoints.

2.4. p53-mediated responses

The p53 tumor suppressor protein plays a central role in the decision of a cell to undergo either cell-cycle arrest or apoptosis in response to DNA-damage. The transcriptional activity of p53 is regulated by post-translational modifications such phosphorylation, sumorylation and acetylation.

In normal cells p53 protein levels are low due to Mdm2 (mouse double minute 2)-mediated ubiquitylation and degradation through the proteasome pathway. Upon DNA-damage ATM and ATR phosphorylate p53 in several serines including Ser15 and Ser20. Moreover, Chk1 and -2 also phosphorylate p53 at the same Ser20 and in Thr18 (79). In addition, the p53 negative regulator Mdm2 undergoes phosphorylation by ATM targeting the protein for degradation (80). Collectivelly all those phosphorylations lead to p53 stabilization and increased transcriptional activity.

The best understood functions of p53 are mediated by transcription of target genes, although it has been proposed other transcription-independent functions (81). The key target of p53-dependent checkpoint is $p21^{WAF1/Cip1}$, an inhibitor of Cdks. Accumulation of $p21^{WAF1/Cip1}$ after DNA-damage is capable of blocking CyclinE/Cdk2 complexes and thus maintains a long arrest in G1-phase. p53 on the other hand is also able to induce apoptosis if damage is severe. Target genes involved in apoptosis include Bax and the BH3-only proteins Noxa and Puma. How p53 is instructed to favor activation of growth-inhibitory genes in conjunction with repair or activation of an apoptotic program is still not well understood. Under conditions of limited damage, p53 associates with a set of transcriptional co-activators like Hzf (82) or YB1 (83) and induces the transcription of pro-arrest genes (like p21 and 14-3-3 sigma). On the other hand, in conditions of severe damage, p53 associates with other co-factors like Cas (82) thus leading to the transcription of pro-apoptotic genes (Bax, Noxa and Puma). It seems that the initial response of p53 is the activation of pro-arrest and repair genes and if damage is persistent, this allows p53 to switch to proapoptotic promoters. How this switch is produced include proteasome-dependent degradation of cofactors and specific phosphorylations, such as ser46 (84).

3. Mechanisms of glucocorticoid-induced apoptosis

Glucocorticoids (GCs) are a class of steroid hormones which display potent immuno-modulatory activities. Their ability to induce apoptosis in leukemia and lymphoma cells has been utilized by physicians for nearly a half-century. Today glucocorticoids are common components in many chemotherapeutic protocols for lymphoid malignancies, including multiple myeloma, acute lymphoblastic leukemia, chronic lymphocytic leukemia, and non-Hodgkin's lymphoma (85).

GCs exert most, if not all, of their effects through binding to the glucocorticoid receptor (GR). The GR is a ligand-activated transcription factor that belongs to the nuclear steroid hormone receptor family of zinc finger transcription factors. In the absence of hormone, GR resides in the cytoplasm in a multimeric complex of heat shock proteins and immunophilins (86). Upon ligand binding, the GR dissociates from at least some of its binding proteins, dimerizes and translocates into the nucleus to induce or repress the expression of a plethora of genes.

GR mediates gene regulation either directly by binding glucocorticoid response elements (GREs) on regulatory regions of target genes or indirectly through protein-protein interactions with other transcription factors, including NF-kappaB, AP-1, Stat5, NF-AT and CREB (87).

GR-mediated gene activation was shown to be essential since cycloheximide and actinomycin D blocked GC-induced apoptosis in all models studied so far (88). In addition, all mouse models reported to date support this idea; of note, cells form mice carrying the point mutation A458T, which impair dimerization and abolish GRE-dependent transactivation, are completely resistant to GC-induced apoptosis (89). Despite those evidences, the downstream events and the critical targets of GCs are still unclear.

Up to date, more that 900 different genes were reported as GC regulated, but of these only 70 appeared in more than one publication (90)(Table 1). Despite the technical differences in the studies, it still suggests that a distinct set of genes might be regulated in different cell systems and experimental conditions. This raises the possibility that multiple, cell-context-dependent mechanisms rather than a conserved canonical pathway may lead to GC-induced cell death. Accordingly, combined loss of the most promising pro-apoptotic target of GR, Bim, do not completely abolish apoptosis *in vivo*, although show some degree of protection (91).

Although the GR-target genes leading to apoptosis are not clear, it is generally accepted that apoptosis proceeds through the intrinsic apoptotic pathway. Probably the major evidences come from genetic studies in mice. Glucocorticoid-induced apoptosis in thymocytes from APAF-1 (92;93) and Caspase-9 (94) deficient mice is compromised (although not absent), and thymocytes from double knockout mice lacking the BH3-only molecules Bax

and Bak are GC resistant (95). Furthermore, overexpression of antiapoptotic Bcl-2 family members attenuated GC-induced cell death both in mouse T-cells and cell lines (90). Altogether those observations suggest that GC mediate their effect through the activation of the intrinsic apoptotic pathway. In that sense, the most promising target was Bim (24). However, even Bim is clearly induced in several models after GC treatment, loss of Bim alone or in conjunction with Puma only leads to partial protection from GC-induced apoptosis (91).

Description	Reg	Systems
• NFkB inhibitor α(IκB-α)	↑	PreB, S49, WEHI, MM, Jurkat, CEM, thymus
• FK506-binding protein 5 (FKBP 51)	↑	PreB, WEHI, MM, EoL, Jurkat, CEM
• BCL2-like 11 (Bim)	↑	PreB, S49, WEHI, CEM, thymus
• GILZ	↑	PreB, MM, CEM, thymus-2
• HIF-1 responsive RTP801 (dig-2)	↑	S49, WEHI, thymus, CEM
• Ubiquitin-like domain member 1	↑&↓	PreB, S49, WEHI, thymus
• Glucocorticoid receptor α	↑	PreB, S49, CEM
• Interleukin 7 receptor	↑	Jurkat, CEM, thymus-2
• Suppressor of cytokine signaling 1 (SOCS1)	↑	PreB, EoL, CEM
• Thioredoxin interacting protein (TXNIP)	↑	PreB, MM, CEM
• Absent in melanoma 1	↑	PreB, Jurkat, CEM
• Granzyme A	↑&↓	PreB, S49, WEHI
• Cyclin G2	↑&↓	PreB, S49, CEM
• Glutamine synthase	↑	PreB, MM, thymus-2
• Solute carrier family 16, member 1 (MCT-1)	↓	Pre B, Jurkat, CEM
• PIP-3-kinase, regulatory subunit (p85 α)	↑	S49, WEHI, EoL
• Tubulin βpolypeptide	↓	WEHI, Jurkat, CEM
• Acid phosphatase type 5	↑	S49,WEHI, thymocytes
• G protein-coupled receptor 65	↑	S49, WEHI, thymocytes
• TGF-β II Receptor α	↑	MM.1S, Jurkat, CEM
• Glutamate–cysteine ligase, modifier subunit	↑&↓	Pre B, Jurkat LS7, CEM
• CDK inhibitor 2D (p19, inhibits CDK4)	↑&↓	Jurkat, CEM, preB
• Hypothetical protein FLJ22833	↓	WEHI, S49, thymus
• Down syndrome critical region gene 1	↑	PreB, CEM
• Recombination-activating gene 1 (RAG 1)	↑&↓	PreB, CEM
• c-myc	↓	Jurkat, CEM
• CD53 antigen	↑	PreB, CEM
• BTG family, member 2	↑	PreB, CEM,
• Paralemmin 2	↑	PreB, CEM
• Integrin α4 (antigen CD49D)	↓	PreB, CEM
• HRY	↓	Jurkat, CEM

Table 1. Genes identified as targets of Glucocorticoids. (reg) regulation. Upwards arrow means that the gene is upregulated by glucocorticoids and downwards arrow that is downregulated. Cellular systems: Human: CEM, various subclones of the CCRF-CEM T-ALL cell line; PreB, PreB-697 B-ALL cells; MM; multiple myeloma cell line MM1s; Jurkat; T-ALL cell lines stably transfected with either rat GRwt or rat GRLS7 Mouse: WEHI, WEHI7.2 lymphoma cell line; S49, S49.A2 lymphoma cell line; thymus-1, normal C56BL/6 thymocytes; thymus-2, 18d fetal thymocytes from C57BL/6 wild-type mice or GR2KO mice. Adapted from (90).

4. Introduction to Cyclin Dependent Kinases

The Cyclin-Dependent Kinases (Cdks) are a family of serine/threonine protein kinases whose members are small proteins (~34–40 kDa). By definition, all Cdks share the feature that their enzymatic activation requires the binding of a regulatory Cyclin subunit. In most cases, full activation also requires phosphorylation of a threonine residue near the kinase active site. Although originally identified as enzymes that control cell-cycle events, members of the Cdk family are involved in other cellular processes as well. Animal cells, for example, contain at least nine Cdks, only four of which (Cdk1, 2, 4 and 6) are involved directly in cell-cycle control. Another family member (Cdk7) contributes indirectly by acting as a Cdk-activating kinase (CAK) that phosphorylates other Cdks. Cdks are also components of the machinery that controls basal gene transcription by RNA polymerase II (Cdk7, 8 and 9) and are involved in controlling the differentiation of nerve cells (Cdk5).

Cdks exert their effects on cell-cycle events by phosphorylating a large number of proteins in the cell. During mitosis in particular, when many aspects of cellular architecture and metabolism are altered, Cdks phosphorylate hundreds of distinct proteins. These Cdk substrates are phosphorylated at serine or threonine residues in a specific sequence context that is recognized by the active site of the Cdk protein. It is necessary that the target serine (S) or threonine (T) residue is followed by a proline (P); it is also highly favorable for the target residue to have a basic amino acid two positions after the target residue. The typical phosphorylation sequence for Cdks is [S/T*]PX[K/R], where S/T* indicates the phosphorylated serine or threonine, X represents any amino acid and K/R represents the basic amino acid lysine (K) or arginine (R).

In dividing cells, Cdk levels remain constant but levels of Cyclins and their subcellular localization vary in order to achieve a scheduled Cdk activation for proper completion of the cell cycle (Figure 11). In response to growth factor stimulation, cells synthesize D-type Cyclins that assemble with Cdk4 and 6. Therefore, CyclinD-Cdk complexes facilitate G1 progression by first phosphorylating Retinoblastoma protein, relieving trasnscriptional repression by the Rb-E2F complexes, and by sequestering Cip/Kip proteins, facilitating Cyclin E-Cdk2 activation. Cyclin E-Cdk2 mediated Rb phosphorylation disrupts the binding of Rb to E2F, allowing E2F activation and the transcription of genes necessary for S-phase entry and progression, including Cyclin E itself (96). Cyclin A expression appears at late G1 and contributes to the G1/S transition and S-phase progression by association with Cdk2 (97). G2-M transition and progression is regulated by the activity of CyclinB/cdk1 complexes. The first events that trigger Cyclin B-Cdk1 complexes are the disassembly of the nuclear envelope by phosphorylating nuclear Lamins (Margalit A 2005 j cel biochem). As mitosis proceeds Cyclin B-Cdk1 programs at least two functions: the mitosis specific phosphorylations of several proteins creating recognitions sites for other proteins and structural changes at G2/M that include centrosome separation and spindle assembly; and subsequently at the end of prophase the phosphorylation and activation of APC (Anaphase Promoting Complex) to allow

the assembly with its regulatory subunit Cdc20 (98). APC^{cdc20} then catalyzes ubiquitylation of substrates, including Cyclin B and securin, targeting them for proteasomal degradation (99). Cyclin B degradation and subsequent inactivation of Cdk1 is necessary for cell to exit mitosis.

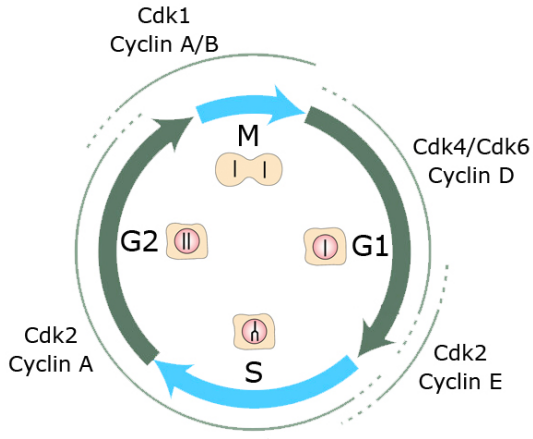


Figure 11. Cell cycle regulation by Cdks. Cyclin D associated with Cdk4 and Cdk6 regulates G1-phase progression. Entry into S-phase is regulated by Cycin E-Cdk2 complexes and S and G2 –phase progression by Cdk2-Cyclin A complexes. Mitosis (M-phase) is regulated by Cyclin B-Cdk1 complexes.

4.1. Regulation of Cdk activity

Regulation of Cyclin/Cdk protein kinase activity during cell cycle progression is accomplished by regulation of the timing of Cyclin protein accumulation and degradation, by binding of Cdk with inhibitory proteins and by phosphorylations and dephosphorylations of the Cdk (Figure 12).

Once complexed with their cyclin subunit, Cdk2 and Cdk1 must be phosphorylated on a regulatory threonine residue (Thr-160 and Thr-161 in mice and humans, respectively) to become active. This activating phosphorylation is accomplished by an activity known as the Cdk-activating kinase, or CAK, which is composed of Cdk7, cyclin H, and the RING-finger protein MAT1 (100).

Cdk1 and 2 molecules can also be phosphorylated on threonine 14 and tyrosine 15 amino acid residues by Myt1 and Wee1 respectively. These phosphorylations inhibit the activity of cyclin-Cdk complexes and are important to prevent premature activation of Cyclin B-Cdk1 complexes before entry into mitosis and are also targets of checkpoint regulation. Dephosphorylation of

Thr-14 and Tyr-15 and thus activation of the Cdk is done by the dual-specificity phosphatases of the Cdc25 family.

A third level of regulation of the Cdk kinase activity is the direct binding to the inhibitory proteins CKIs (Cdk Kinase Inhibitors). Depending on the structure and their specificity of substrate, CKIs are categorized into two families, the Cip/Kip and the Ink4. The Cip/Kip family contains three members ($p21^{Cip1}$, $p27^{kip1}$ and $p57^{kip2}$) and shows a broad spectrum of interaction, being able to inhibit all cell-cycle related Cdk-Cyclin complexes. The other family, INK4 is composed by $p16^{INK4A}$, $p15^{INK4B}$, $p18^{INK4C}$ and $p19^{INK4D}$. This family of CKIs is only able to bind and inhibit Cdk4 and 6. In contrast to Cip/kip proteins that bind both Cdks alone or in complexes impeding substrate accessibility, INK4 proteins bind directly to the Cdk and inhibit Cylin binding and thus activation.

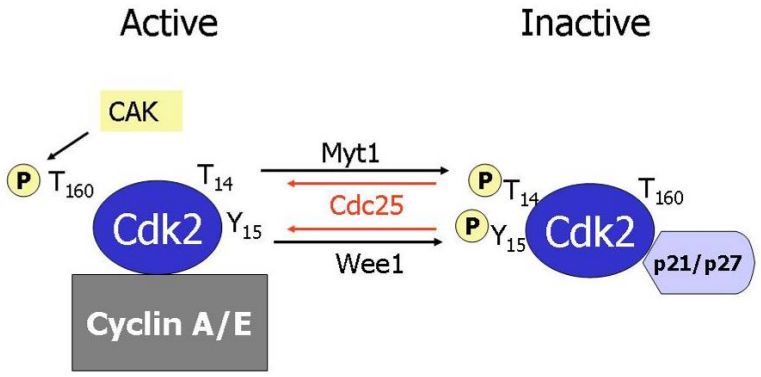


Figure 12. Mechanism of Cdk2 regulation. Cdk2 can be regulated by three different mechanism. First, by binding to its activating subunit, the Cyclin. All Cdks are activated by the binding of a Cyclin, in case of Cdk2 those are Cyclin A or E. Second, by phosphorylation. Cdk Activating kinase (CAK)-mediated phosphorylation on threonine 106 is necessary to get full activation. Moreover, Threonine 14 and Tyrosine 15 phosphorylation that is mediated by Myt1 and Wee1 kinases respectively, leads to inactivation. These residues can be dephosphorylated by Cdc25 proteins, leading to activation. And third, by binding to inhibitory proteins. p21Cip and p27Kip can bind to Cdks alone or in complex with a Cyclin and block the kinase activity.

5. Cell cycle and Cell death connections

Hypothesis suggesting a link between cell cycle and cell death appeared many years ago based on the common morphological aspects of both processes: cells release substrate attachments, condense their chromatin and disassemble the nuclear lamina. In that sense, in late 90's appeared a series of papers suggesting a connection between cell cycle and apoptosis. Several articles demonstrated an active role of cell cycle proteins (for example, Cdks, p27, c-myc, etc) in apoptosis and vice versa, apoptosis-related proteins were found to modify cell cycle (Bcl-2, Bax, etc). However, the underlying hypothesis was shown to be wrong since the biochemical machinery responsible for the morphological changes was different in cell cycle and apoptosis; for example, although both processes induce a breakage of the nuclear envelope, during cell cycle it is due to Lamins phosphorylation by Cdk1-CyclinB complexes (101), whereas during apoptosis it is due to Caspase-mediated cleavage of Lamins (102).

5.1. Role of Cdks in apoptosis

The first paper demonstrating a role for Cyclin dependent kinases (Cdks) in apoptosis appeared in 1994; Shi L. and colleagues found that Cdk1 activity was induced after treatment of a lymphoid cell line with several death-inducing compounds (103). Since then, a lot of reports have appeared implicating a certain Cdk or Cyclin in apoptosis. However, the precise role of Cdks in apoptosis in still unknown because no apoptosis-specific target has been identified so far, despite the efforts of several groups. Probably the most complete pathway comes from neuronal cells. Several data suggest that uncoordinated expression of cell cycle molecules and the consequent break of cell cycle checkpoints could be one of the primary mechanisms by which postmitotic neurons undergo apoptotic death. In that sense, several reports show activation of the E2F-Rb pathway during neuronal apoptosis. Upregulation of Cdk activity promotes successive phosphorylation and dysfunction of Rb family members, resulting in sequential E2F derepression and expression of selective E2F-responsive genes. Which are the actual proapoptotic targets of E2F is still unknown and could be a two-step sequential event by which E2F would induce the expression of other transcription factors responsible for the expression of the proapoptotic genes. Accordingly, Green LA proposed a model in which E2F induces the expression of b- and c-Myb proteins and those drive the transcription of the proapoptotic gene, Bim (104).

Which Cdk is activated during neuronal apoptosis is controversial and could depend on the model and stimulus used. Whereas some groups suggest that Cdk4/6 mediate Rb phosphorylation (105), others propose that is Cdk5 (106).

5.2. Cdk2 and apoptosis

Some studies involving Cdks in apoptosis, point Cdk2 as a critical regulator. Cdk2 activity in apoptosis has been demonstrated in proliferating cells lines such as BaF3 (107), MCF-7 (108), hepatoma cells FaO (109), gastric cancer cells SNU-16 (110), CHO (111), HeLA (112) and others, and in quiescent cells like thymocytes (107;113) and human umbilical vein endothelial cells (114).

In proliferating cells Cdk2 activity is very high, making it difficult to detect additional activation due to apoptosis induction. Therefore, the use of quiescent cells provides a useful system for the study of Cdks during apoptosis because the basal Cdk activity is almost undetectable. For that reason, thymocytes have been broadly used. Thymocytes from normal mice are a naturally occurring synchronized population since 90% of them are quiescent and do not progress through cell cycle (115). In thymocytes, activation of Cdk2 has been shown during antigen-mediated negative selection (116) and during dexamethasone or γ-radiation induced apoptosis (107). Its importance has been revealed by the fact that chemical inhibition of Cdk2 completely blocks apoptosis (107;117).

Since the activation of Cdk2 during thymocyte apoptosis has been shown to be a necessary step, our group has characterized it. First of all, it was discarded that Cdk2 activation was due to a cell cycle re-entry since the induction of apoptosis is not accompanied by other cell cycle hallmarks like Cdk4/6 activation, induction of Cyclin A and E or DNA-synthesis and is not followed by activation of the next Cdk of the cell cycle, Cdk1 (107). Moreover, *de novo* protein synthesis is required for activation of Cdk2 during apoptosis, since cycloheximide abolishes the increase in Cdk2 activity and can block thymocyte apoptosis induced by dexamethasone and γ-radiation. In agreement with this requirement of *de novo* gene expression, Cdk2 activation is downstream of p53 in the DNA damage-induced apoptosis. In addition, Cdk2 activation parallels with the degradation of its inhibitor $p27^{kip1}$. Interestingly, Bcl-2 and Bax have been shown to modulate $p27^{kip1}$ levels both during cell cycle and apoptosis. Bcl-2 overexpression delays G1 to S phase transition in cell lines (118) and in activated T-cells (115) whereas Bax speeds it up, and this effect has been shown to be mediated by $p27^{kip1}$ levels. During apoptosis the same effect has been seen: Bax overexpressing cells degrade more rapidly $p27^{kip1}$, show higher levels of Cdk2 activity and die more rapidly. On the other hand, Bcl-2 overexpression, works on the opposite way (107).

Although degradation of the Cdk2 inhibitor $p27^{kip1}$ parallels the activation of Cdk2, cycloheximide (that prevents Cdk2 activation) does not block this degradation. Therefore, $p27^{kip1}$ degradation is not sufficient for Cdk2 activation during thymocyte apoptosis. Along with the data showing that Cyclin A and E do not mediate Cdk2 activation during thymocyte apoptosis, this strongly suggests that a new activator should be synthesized downstream of p53 and the GR. The identity of this activator has been strongly pursued by our group. We have recently identified a new protein, Cyclin O, which we propose to be

the Cdk2 activator during apoptosis. The molecular and functional characterization of this Cyclin is the aim of this thesis.

Another important issue addressed by our group is the molecular positioning of Cdk2 activation in the apoptotic pathway. What is clear is that both dexamethasone and DNA-damage mediate apoptosis through the intrinsic apoptotic pathway. Cdk2 clearly precedes mitochondrial permeabilization and after Cdk2 activation it is necessary gene transcription for apoptosis to proceed. This strongly suggests that Cdk2 is controlling some transcription(s) factor(s) that would activate apoptosis. Unfortunately, up to date its identity is still unknown. Nevertheless, although the direct targets of Cdk2 are not still identified, what is known is that before mitochondrial permeabilization there is activation of the apical Caspase, Caspase-8, and cleavage of its target, Bid. Altogether those observations favour a model in which Cdk2 would induce the activation of apical Caspases by a mechanism still unknown and active Caspases would transduce the signal through cleavage of Bid. (Figure 13)

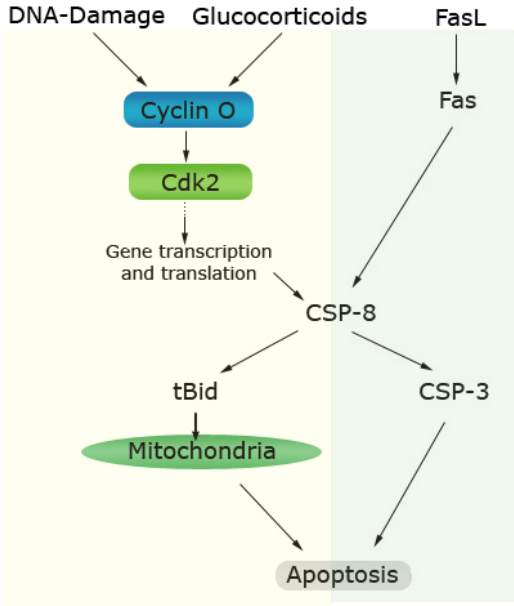


Figure 13. Positioning of Cdk2 during thymocyte apoptosis. Scheme based on Granés F. et al (117).

II. OBJECTIVES

Our group is interested in the study of the molecular pathways that govern apoptosis in quiescent cells. Using thymocytes as a model, we have shown that the protein kinase Cdk2 plays an important role during apoptosis. Since Cdk2 should bound a Cyclin to be active and neither Cyclin A nor Cyclin E are the responsible of Cdk2 activity during apoptosis, our group suggested that another Cyclin (ARCA, Apoptosis Related Cdk2 Activator) would activate Cdk2 during apoptosis. We have identified an uncharacterized Cyclin, Cyclin O that we thing that could be ARCA. Given that, we have proposed the following objectives:

1. General characterization of Cyclin O.
    a. Analyze the pattern of expression of Cyclin O in tissues and cell lines.
    b. Characterize Cdk partners of Cyclin O.

2. Check if Cyclin O corresponds to ARCA.
    a. Analyze the pattern of expression of Cyclin O after induction of apoptosis.
    b. Analyze the effects of Cyclin O downregulation by means of shRNA.

3. Identify and characterize Cyclin O-Cdk2 specific substrates.

III. MATERIALS AND METHODS

1. Anti-Cyclin O antibodies

Four different antibodies against Cyclin O were generated in rabbits either using full-length recombinant protein or different peptides. The sequences of the peptides and their localization are summarized in Figure 1.

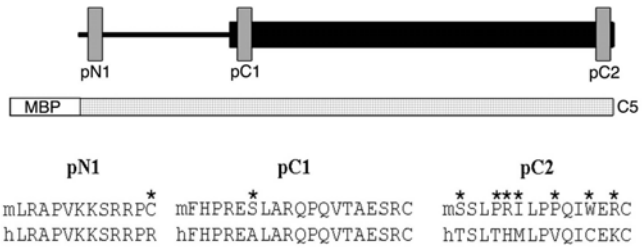
N1 peptide was coupled with glutaraldehyde whereas C1 and C2 were coupled with 3-maleimidobenzoic acid N-hydroxysuccinimide ester (MBS, Sigma-Aldrich, St. Louis, MO, USA) to keyhole lympet haemocyanine (KLH). New Zealand Rabbits were used for immunisation and were kept at the animal facility of the Facultat de Farmàcia, Universitat de Barcelona.

The N1, C1 and C2 sera were affinity purified using the peptide bound to an EAH-sepharose column according to the manufacturer (GE Healthcare, USA). The affinity purified antibodies were checked for different applications and their specificity carefully determined by several independent tests, such as ELISA, block of the immunohistochemical staining by incubation with the antigen (N1, C5 and C1 antibodies).

In order to demonstrate the specificity and to characterise the performances of the antisera raised against different regions of Cyclin O, we carried out several standard tests. In all the cases, the production of the specific antibody in the different batches of sera was confirmed and titrated by Elisa, affinity purified using antigen columns and finally, the titres adjusted with PBS containing bovine serum albumin to a final concentration of 1mg/mL. Affinity purified antibodies were aliquoted and kept at -20ºC until use. The antigen columns were prepared using the corresponding peptide or, in the case of the C5 sera, the recombinant protein GST-Cyclin O, in order to eliminate the antibodies directed against the Maltose Binding Protein present in the antigen preparation. Given the fact that the 3-maleimidobenzoic acid N-hydroxysuccinimide ester (MBS) used to crosslink the C1 and C2 peptides to the keyhole lympet haemocyanine (KLH) can elicit a potent antibody response in rabbits, the antigens used to coat the Elisa plates were crosslinked to the carrier ovoalbumin using glutaraldehyde (Harlow and Lane, 1999). In the case of the C5 sera, the GST-Cyclin O protein was bound directly to the Elisa plate.

The lack of immunoreactivity using N1 antibody in cells containing an shRNA against Cyclin O can be considered as an specificity test. Moreover, the fact that the anti-Cyclin O antibody C2 fails to immunoprecipitate significant kinase activity from the same shRNA clone also supports the specificity.

A



B

	Elisa	WB	IHC	IP
C5	+	+	+	-
N1	+	-	+	-
C1	+	-	+	-
C2	+	+	+/-	+

Figure 1. Cyclin O antigens and uses of the antibodies. (A) Black region represents the coding sequence of murine Cyclin O (352 amino acids). Black box denotes the Cyclin Box domain of the protein. Grey rectangles represent the location of the peptides chosen to generate the antibodies (peptides pN1, pC1 and pC2). The sequence of the peptides used and the equivalent human sequences are showed below. (m) murine sequence, (h) human sequence. The asterisks denote mismatched residues between the mouse and the human sequences. The white rectangle represents the Maltose Binding Protein (MBP) fused to the whole sequence of the murine Cyclin O (grey gridded rectangle); this protein was the antigen used to generate the C5 antiserum. (B) Uses of the affinity purified antibodies. Only the C2 antibody efficiently immunoprecipitated murine Cyclin O. (WB) western Blot, (IHC) immunohistochemistry, (IP) immunoprecipitation.

2. Other antibodies

All the antibodies used are listed in the following table:

Protein	Antibody	Source	Supplier
Cdk1	A-17	Mouse monoclonal	Beckton-Dickinson (BD Biosciences, San José, CA, USA)
Cdk2	M2	Rabbit polyclonal	Santa Cruz (Santa Cruz Biotechnology, CA, USA)
Cdk4	C-22	Rabbit polyclonal	Santa Cruz (Santa Cruz Biotechnology, CA, USA)
Cdk5	C-8	Rabbit polyclonal	Santa Cruz (Santa Cruz Biotechnology, CA, USA)
Cdk7		Rabbit polyclonal	Santa Cruz (Santa Cruz Biotechnology, CA, USA)
Cdk9	H-169	Rabbit polyclonal	Santa Cruz (Santa Cruz Biotechnology, CA, USA)
Cdc25A	F-6	Rabbit polyclonal	Santa Cruz (Santa Cruz Biotechnology, CA, USA)
Cdc25C	C-20	Rabbit polyclonal	Santa Cruz (Santa Cruz Biotechnology, CA, USA)
$P21^{Cip1}$	C-19	Rabbit polyclonal	Santa Cruz (Santa Cruz Biotechnology, CA, USA)
Cdk2	55	Mouse monoclonal	BD Biosciences (San José, CA, USA)
Myc-Tag	9E10	Mouse monoclonal	Hybridoma from American Type Culture Collection (ATCC, Rockville, MD, USA)
HA-Tag	12CA5	Mouse monoclonal	Hybridoma from American Type Culture Collection (ATCC, Rockville, MD, USA)
Caspase-8	5D3	Rabbit polyclonal	MBL (MA, USA)
Bid	559681	Rabbit polyclonal	BD-Pharmingen (BD Biosciences, CA, USA)
Cleaved Caspase-3	Asp175, 5A1	Rabbit monoclonal	Cell Signal (Cell Signalling Technology, Boston, MA, USA)
phospho-ATM	Ser1981, 10H11.E12	Mouse monoclonal	Cell Signal (Cell Signalling Technology, Boston, MA, USA)
phospho-Chk1	Ser345, 133D3	Rabbit monoclonal	Cell Signal (Cell Signalling Technology, Boston, MA, USA)
phospho-p53	Ser15	Rabbit polyclonal	Cell Signal (Cell Signalling Technology, Boston, MA, USA)
Bim	14A8		Alexis (Lausane, Switzerland)
α-Tubulin		Mouse monoclonal	Sigma (St. Louis, MO, USA)
CD95-agonistic	Jo2	Hamster monoclonal	Beckton-Dickinson (BD Biosciences, San José, CA, USA)
c-Myb	1.1	Mouse monoclonal	Upstate Biotechnology (Lake Placid, NY, USA)

Table 1. Antibodies used.

3. Semiquantitative and quantitative RT-PCR

RNA was prepared from mouse tissues using Trizol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer instructions.
RT-PCR reactions were done using SuperScript III One-Step RT-PCR with Platinum Taq (Invitrogen Carlsbad, CA, USA) using 0.5μg of RNA per reaction and the PCR reactions described in Table 3. Quantitative RT-PCR was performed using QuantiTect Sybr Green reagent (Qiagen) using 100ng of RNA/well in a final volume of 10 μl. Samples were analyzed in three independent triplicates and the data was analysed using SDS2.1 software (Applied Biosystems). PCR cycles are described in table 2.

Semiquantitative RT-PCR		
50ºC	30 min	
95ºC	5 min	
94ºC	30 sec	
55ºC	30 sec	40 cycles*
68ºC	1min	
68ºC	10 min	

Quantitative RT-PCR		
50ºC	30 min	
95ºC	10 min	
94ºC	30 sec	
55ºC	30 sec	40 cycles
72ºC	30 sec	
72ºC	10 min	

Table 2. RT-PCR conditions. *40 cycles for Cyclin O detection. For HPRT only 30.

Technique	mRNA	Oligo 1	Oligo 2
RT-PCR	Cyclin O	5′-CGCTTGCAAGCAGGTAGAGG-3′	5′-CTACCTCGTGATGGACTTCG-3′
RT-PCR	HPRT	5′-GGCCAGACTTTGTTGGATTTG-3′	5′-TGCGCTCATCTTAGGCTTTGT-3′
RT-PCR	c-Myb		
qRT-PCR	Cyclin O	5′-CGCTTGCAAGCAGGTAGAGG-3′	5′-TGAGTGAAGTGCTCCAGGAAG-3′
qRT-PCR	HPRT	5′-GGCCAGACTTTGTTGGATTTG-3′	5′-TGCGCTCATCTTAGGCTTTGT-3′

Table 3. Primers used in RT-PCR reactions

4. Cell fractioning

$2*10^6$ cells were collected by scrapping from a 10cm plate and washed once with cold PBS. Cell pellet was ressuspended in 50μl of ice-cold Dig 0x buffer (25mM Tris-HCl pH7, 68mM sucrose, 220mM mannitol, 1mM DTT, 2μg/ml aprotinin, 2μg/ml leupeptin, 2μg/ml antipain, 20μg/ml soybean trypsin inhibitor, 1mM Pefablock™, 1mM sodium orthophosphate, 0.2mM ortovanadate, 1mM sodium fluoride and 1mM β-glicerophosphate). Cells were then permeabilized by addition of 50μl of Dig 2x buffer (Dig 0x buffer with 0.1% Digitonin). Lysates were mixed by tapping and after 30 seconds, were centrifuged for 2 minutes at 20,000xg and the supernatant, containing the cytosolic fraction, was carefully removed (SN). In some cases the pellet was ressuspended again with 50μl of Dig 0x buffer and cytosolic extraction repeated with 50 μl of Dig2x, obtaining a more diluted cytosolic franction (SN2). The remaining pellet, containing basically the nuclear, mitochondrial and membrane fraction, was extracted with 100 μl of RIPA Buffer (50mM Tris HCl pH 8, 150mM NaCl, 1% NP-40, 0.5% sodium Deoxycholate, 0.1% SDS, 1mM DTT, 2μg/ml aprotinin, 2μg/ml leupeptin, 2μg/ml antipain, 20μg/ml soybean trypsin inhibitor, 1mM Pefablock™, 1mM sodium orthophosphate, 0.2mM ortovanadate, 1mM sodium fluoride and 1mM β-glicerophosphate) during 20 minutes on ice and then centrifuged at 20,000xg 20 minutes. The supernatant was the nuclear fraction (P). The fractions were quantified by Bradford reagent (Bio-Rad GmbH, Munchen, Germany) and processed for immunoblotting.

5. Immunoblotting

Cells used for immunoblotting were washed with cold PBS and freezed at -80ºC until used. Cell pellets where extracted routinely with RIPA buffer and quantified using Bradford reagent. Equal amounts of protein were loaded on each lane (ranging from 30 to 60 μg) and separated by SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE). Proteins were transferred into a nitrocellulose membrane (Protran, Schleicher & Schuell GmbH, D-37582 Dassel, Germany). Membranes were blocked with 5% non-fat milk prepared in TBS-T (20mM Tris HCl pH 7.6, 137mM NaCl, 0.1% Tween 20) either for 1 hour at room temperature or overnight at 4ºC with constant agitation. Primary antibodies were diluted in TBS-T containing 2% Bovine Sera Albumin (BSA) (Sigma-Aldrich, St Louis, MO 63178, USA) and were incubated for 1h at room temperature or overnight at 4ºC. After extensive washing with TBS-T, membranes were incubated for 1 hour with the corresponding secondary HRP-conjugated antibody (all purchased form DAKO, DK-2600, Glostrup Denmark). Membranes where then blotted using either the standard ECL chemiluminiscent substrate (Pierce, Rockford, IL, USA) or SuperSignal West Pico (Pierce, Rockford, IL, USA) for low abundant proteins like Cyclin O.
For stripping, membranes were incubated for 30 minutes at 65ºC with 62.5 mM Tris-HCl pH 6.8, 2% SDS, 100 mM β-mercaptoethanol.

6. Immunohistochemistry

6.1. From tissues

Immunohistochemical analyses of tissues were performed using 3 μm sections of paraformaldehyde-fixed paraffin-embedded mouse tissue blocks. Antigen retrieval was done 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 27ºC. As secondary antibody, the EnVision^R anti-rabbit system was applied (Dako, Goldstrup, Denmark). Sections were counterstained with haematoxylin, dehydrated and mounted.

6.2. From adherent cells

Cells were grown over glass coverslides and when reached the desired confluence, where fixed with paraformaldehyde. Cells were washed with PBS and permeabilized with PBS containing 0.5% Triton-X100 during 10 minutes. Blocking and antibody incubations were done as in tissue samples.

6.3. From cells growing in suspension

$50*10^6$ cells were pelleted and washed with PBS. Cell pellet was fixed with paraformaldehyde, embedded in paraffin and processed as tissues.

7. Production of recombinant proteins in *E.coli*

7.1 Vectors and strains

For the production of recombinant proteins we used the *E.coli* strain BL21 (DE3) transformed with the corresponding expression vector. For GST fusion proteins we used the pGEX vectors (Amersham Biosciences, Piscataway, NJ) whereas for MBP fusion proteins we used pMALC (New England Biolabs, Beverly, MA) with the desired protein cloned in the correct frame.

7.2 Production of bacterial lysate

A saturated culture of bacteria was diluted 1:1000 and incubated at 37ºC with constant agitation at 200 rpm for 3h in LB plus the corresponding antibiotic. At that time, recombinant protein expression was induced by adding 1mM IPTG (Isopropyl-beta-D-thiogalactopyranoside) to the media. After 1-3 hours of protein expression, culture was harvested centrifuging at 6000 rpm for 15 minutes. Cell pellet was frozen at -80ºC for at least for 1h. Bacteria were then ressuspended with 1/10 volume of NTEN (20mM Tris-HCl pH=8, 150mM

NaCl, 1mM EDTA, 0,5% NP40, 1mM DTT, 2μg/ml aprotinin, 2μg/ml leupeptin, 2μg/ml antipain, 20μg/ml soybean trypsin inhibitor, 1mM Pefablock™) and lysed by sonication. To obtain a clear lysate, the extract was centrifuged at 10.000 rpm for 10 minutes and the pellet was discarded. The supernatant was aliquoted and stored at -80.

7.3. Purification of recombinant proteins

For GST fusion protein purification, we incubated 1 ml of the cleared lysate with 50 μl of glutathione-sepharose beads (GE Healthcare, USA) during 3h with constant agitation at 4ºC. Beads where extensively washed with NTEN buffer and either used for next step (pull-down or kinase substrate) or eluted with 100mM Tris-HCl pH=8, 120mM NaCl, 20mM reduced Glutation. For MBP fusion protein purification the protocol was the same but using amylose-Sepharose beads (New England Biolabs, Ipswich, MA, USA) and for the elution, 20 mM maltose instead of Glutation. To check the quality and quantity of the proteins, an aliquot was run on a SDS-PAGE and stained with coomassie blue.

8. *In vitro* purification of Cyclin O-Cdk2 complexes

We expressed MBP-Cyclin O, Cdk2wt-GST and Cdk2DN-GST in *E.coli* as described before. 2 ml of *E.coli* lysate containing MBP-Cyclin O was incubated with 100 μl of amylose-Sepharose beads for 3h at 4ºC with constant agitation. Beads were then extensively washed with NTEN and split in two aliquots. One was incubated with 1 ml of *E.coli* lysate containing GST-Cdk2wt protein, and the other, with a lysate containing GST-Cdk2DN protein, during 3h at 4ºC. The bound proteins were eluted with 500 μl NTEN plus 20 mM maltose and incubated with 50 μl of Gluatione-Sepharose beads. After 3h with constant agitation at 4ºC, beads were extensively washed with NTEN, eluted with Laemli's Buffer (10% Glycerol, 50 mM Tris pH 6.8, 5% β-Mercaptoethanol, 0.1% Bromophenol Blue, 2% SDS) and the eluted proteins were loaded on a SDS-PAGE. To asses the stochiometric ratio, gel was stained with silver following an standard protocol.

9. Cell culture and transfections

The human HEK293, U2OS, HT29 cell lines (obtained from ATCC) and immortalized MEFs were maintained in Dulbecco's modified Eagle's medium supplemented with antibiotics and 10% foetal calf serum. Both wild-type and $Cdk2^{-/-}$ immortalized MEFs were kindly provided by Sagrario Ortega (CNIO, Madrid, Spain). The mouse T-cell lymphoma cell line WEHI7.2 (obtained from Dr. Roger Miesfeld, University of Arizona, Tucson, Arizona, USA) was grown in low glucose (1 g/L) Dulbecco's modified Eagle's medium supplemented with antibiotics and 10% foetal calf serum. The EL4 lymphoid cell line was also obtained from ATCC and maintained in RPMI supplemented with antibiotics and 10% foetal calf serum.

U2OS and HEK293 cells were transfected using the calcium phosphate method. Cells were seeded the previous day at a density of 10^6 cells in a 10 cm plate. For the preparation of the precipitate, 10 μg of plasmid DNA were adjusted with pure water to 450 μl and then 50 μl of 2.5M CaCl2 were added. This mix was added drop wise over 500 μl of HBS 2X (50mM HEPES, 280mM NaCl, 1,5mM Na2HPO4) with constant agitation by bubbling. After 10 minutes, this mix was added directly over the cells. Next day media was replaced and cells were harvested by scrapping 48 hours after transfection unless otherwise stated.

WEHI7.2 and EL4 cells were transfected by electroporation as described. 10^7 cells were washed with PBS and ressupended with 320 μl of CITOMIX (120mM KCl, 0.15mM CaCl2, 10mM K2PO4/KH2PO4, 25mM HEPES pH 7.6, 2mM EGTA, 5mM MgCl2). Then 40μl of 50mM Glutatione and 40μl of rATP-Na 20mM were added. Linearized plasmid DNA was adjusted to a concentration of 1mg/ml and 25μl were added. Cells were electropored using a 0.2 cm BioRad electroporation cuvette at 950μF, 250V, 20msec. Cells were immediately transferred into 20ml of fresh media, left to recover during 24h, and antibiotic was added for selection.

MEFs cells were transfected with Lipofectamine™ (Invitrogen) according to the manufacturer instructions using 10μg of linearized DNA per each 10cm plate.

10. Immunoprecipitation and kinase assays

10^6 cells were lysed with Lysis Buffer (50mM Tris HCl pH 7.5, 150mM NaCl, 20mM EDTA, 0.5% NP-40, 1mM DTT, 2μg/ml aprotinin, 2μg/ml leupeptin, 2μg/ml antipain, 20μg/ml soybean trypsin inhibitor, 1mM Pefablock™, 1mM sodium orthophosphate, 0.2mM ortovanadate, 1mM sodium fluoride and 1mM β-glicerophosphate) and a specific Cdk or Cyclin was immunoprecipitated using 2μl of the corresponding antibody and 25 μl of protein A-Sepharose Beads. Beads were washed extensively with Kinase Buffer (50mM Tris-HCl pH=7.5, 10mM MgCl2, 1mM DTT) and finally ressuspended in 20 μl of Hot Mix (50mM Tris-HCl pH=7.5, 10mM MgCl2, 20μM ATP, 10μCi [γ−32P] dATP, 1mM DTT, 2μg Histone H1). In some specific kinase reactions instead of using Histone H1, other substrates were used. In all these cases the substrates were purified from *E.coli* as described and 1μg of recombinant protein bound to beads was used in each reaction. Kinase reactions were performed during 30 minutes at 30ºC and then were stopped adding 20 μl of Laemli's Buffer 2X. 20 μl of the reaction were loaded on a SDS-PAGE, coomassie stained, drained and subjected to autoradiography.

11. Pulldown-elution-IP-Kinase assay

Cell extracts from wild type MEFs, MEFs cdk2$^{-/-}$ or mouse brain were prepared using Lysis Buffer and quantified using Bradford reagent. 500μg of this extract were incubated with 10 μg of purified MBP-Cyclin O previously bound to 75μl of amylose-Sepharose beads at 4ºC. After 4 hours of incubation, the beads were extensively washed and a 1/100 aliquot was removed ('beads' fraction). Then, the remaining beads, which contain MBP-Cyclin O together with associated proteins, were eluted by incubation with 420μl of Lysis Buffer supplemented with 15 mM maltose. After 30 min of elution, the eluate was split in 4 aliquots of 100μl and one of 20μl ('eluate' fraction) that was left on ice until used for kinase assay. 100μl aliquots were immunoprecipitated with 1 μg of normal rabbit IgGs (Sigma), 2μl of anti-Cdk1, 2 μl of anti-Cdk2 (M2) or 2 μl of anti-Cdk5 together with 20μl of Protein-A Sepharose, during 3 hours at 4ºC with constant agitation. The immunoprecipitates and the 'beads' fraction were extensively washed and ressuspended with 20μl of Hot Mix and processed as described in kinase assays. The 'eluate' fraction was mixed with 20μl of Hot Mix and processed the same way.

12. Cyclin O-Cdk2 coimmunoprecipitation

10^6 HEK293 cells were transfected with a myc-tagged Cyclin O expression vector using the calcium phosphate method as described. 48 hours after transfection cells were collected by scrapping, washed with PBS and lysed with Lysis Buffer. For the immunoprecipitation first, 50 μl of the supernatant of the 9E10 anti-myc hybridoma was bound to 25 μl of protein-G Sepharose beads. As a nevative control, 50 μl of an irrelevant hybridoma was also used. Cell extract was then mixed with the antibody beads and incubated during 3h at 4ºC with constant agitation. Beads were extensively washed, eluted with Laemli's Buffer and separated by SDS-PAGE. Western blot was performed against Cdk1, Cdk2, Cdk4 and Cdk5 in different membranes using the corresponding polyclonal antibody. Membranes were then stripped and proved against Cyclin O using the polyclonal anti-Cyclin O antibody C2.

For endogenous Cyclin O-Cdk coimmunoprecipitation, we used the anti-Cyclin O C2 serum crosslinked to Protein G-Sepharose beads. 2μl of C2 serum was incubated with 50μl of Protein G-Sepharose in a final volume of 500μl of PBS for 1 hour at RºT with constant agitation. Beads were washed with 0.2M Sodium Borate pH 9, and then proteins were crosslinked by adding 100μl of 20mM Dimethylpimelimidate. Reaction was carried out for 30min at RºT. Beads were whased with PBS and free ends were blocked by incubation with 0.2M ethanolamine during 2 hours at RºT. Crosslinked Antibody-ProteinG beads were mixed with 1mg of MEFs extract prepared with Lysis Buffer. Immunoprecipitation was done at 4ºC during 3 hours with constant agitation. Beads were extensively washed with Lysis Buffer, eluted with Laemli's Buffer and processed for immunoblotting.

13. Mice

CD2Baxα, EμBcl2 transgenics and wild type mice were kept in SPF conditions in the mouse facility of PRBB with free access to water and normal chow 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.

14. shRNA cell clones

To downregulate mCyclin O, we used the following oligos to make shRNA constructs based on the pSuper (119) plasmid system (provided by Reuven Agami, The Netherlands Cancer Institute, Amsterdam, The Netherlands): shEx3: 5'- GCGCCCACCATCAACTTC-3' and shC5: 5'-GCTCTAGAGGCTCAAACCC-3'; shRNA against GFP: 5'-GCTGACCCTGAAGTTCATC-3'; shRNA against Luciferase: 5'-GAGAGATCCTCATAAAGGCT-3'.

WEHI7.2 and EL4 cells were transfected by electroporation with the pSuper-based shRNA constructs. Single cell clones were isolated by serial dilution and selected with 1 μg/mL of puromycin (Sigma). MEFs clones were transfected with Lipofectamine™, diluted 1/50 and selected with 1 μg/mL of puromycin. Isolated colonies were carefully removed and considered as single cell clones.

15. Citometry

15.1. Measurement of cell cycle and apoptosis by DNA content

In order to analyze cell cycle profile and apoptosis, adherent cells (10^6 cells) were tripsinized and ressuspended with 200 μl of PBS. Cell suspension cultures (10^6 cells) were harvested by centrifugation and ressuspended in 200 μl of PBS. Cells were then fixed by adding 2ml of 70% ethanol and leaved at 4ºC for at least 24 hours. For the staining, cells were washed twice with PBS and ressuspended with Staining Solution (50μg/ml RNAase, 5μg/ml propidium idodide, prepared in PBS) at a final concentration of 10^6 cells/ml. Samples were incubated for 30 minutes at 37ºC and then analyzed by flow cytometry using FACScan (Beckton Dickinson, CA, USA). Analysis was done using BD CellQuest™ software.

15.2. Measurement of phosphatidylserine exposure and $\Delta\Psi_m$.

Clones G1 and 3.7 were resuspended at 1.5 x 106 cells/ml in PBS with the indicated fluorophores: AnnexinV-FLUOS (Roche, Switzerland; concentration titrated according to manufacturer protocols) for phosphatidylserine exposure or CMX-Ros (Mitotracker, Molecular Probes, 100 nM) for $\Delta\Psi_m$ measures. Cells were incubated for 10 min at 37ºC and analysed in a FACScan (Beckton Dickinson, CA, USA). Analysis was done using BD CellQuest™ software.

16. Yeast two hybrid screening

We used the vector pBRIDGE (Clontech) with Cdk2 fused with Gal4BD and Cyclin O cloned in the additional expression site, and a pADGal4 (Clontech) based library made from cDNAs of apoptotic thymocytes. The yeast strain Y190 was transformed with the bait vector and used for the screening. This strain was transformed with 500μg of a plasmid DNA Library and plated over selection media (SD-Trp-Leu-Met-His +25 mM 3-AminoTriazole). $6*10^6$ clones were analyzed. Colonies appeared by day 10 after transformation and positive for β-galactosidase activity were further considered (121 colonies). DNA was amplified by PCR and sequenced.

17. Luciferase assays

We used a luciferase reporter vector that contained the minimal promoter of TK and three c-Myb binding sites in tandem. This vector was constructed by cloning the following oligonucleotide: 5'-AGCTTGCATTATAACGGTCTTTAACGGTCTTTAACGGTCTTTTAGCGCC-3' into the pTK-luc vector, kindly provided by Carme Caelles (Universitat de Barcelona, Barcelona).
For luciferase assays, 10^5 HEK293 cells were plated in triplicate in 24-well plates (Nunclon). Next day each well was transfected with 2μg of plasmid DNA using calcium phosphate method as described before. Luciferase activity was measured after 48 hours using Dual Luciferase Reporter Kit (Promega), according to the manufacturer instructions.

IV. RESULTS

1. Cyclin O expression

Our group is focused on the study of apoptosis in quiescent cells using thymocytes as a model. In this model it has been shown that Cdk2 plays a central role during apoptosis (107;113). Interestingly, previous results from our group suggested that an unidentified Cyclin could be responsible for the activation of Cdk2 during apoptosis. This fact could explain why Cdk2 can play a role both in cell cycle and in apoptosis. That is, during cell cycle Cdk2 would be activated by its canonical Cyclins (A and E) and then phosphorylate cell cycle-specific substrates and during apoptosis, another Cyclin (ARCA, Apoptosis Related Cdk2 Activator) would be the activator and thus lead to the phosphorylation of apoptosis-specific substrates.

We hypothesized that ARCA was a non-characterized Cyclin. We searched the whole genome in order to identify undescribed Cyclins. We identified one Cyclin, located in human chromosome 5 called Cyclin O (120). Preliminary results pointed on the direction that this protein could be ARCA and thus encouraged us to further characterize it.

The human Cyclin O has 350 amino acids and is a highly conserved gene present in all vertebrate genomes sequenced so far. About two thirds of the protein sequence encompasses a highly conserved Cyclin box, sharing about 28% of identity with human Cyclins A2 and B1. The N-terminal part of Cyclin O is less conserved; it is rich in basic amino acids and contains several conserved putative regulatory motifs, suggesting a regulatory role. It can be identified a Nuclear Localization Signal (NLS), a box for the binding of the Ubiquitin-ligase enzyme β-TrcP, a GSK3β phosphorylation site and several Cdk and MAPK phosphorylation sites (Figure 1).

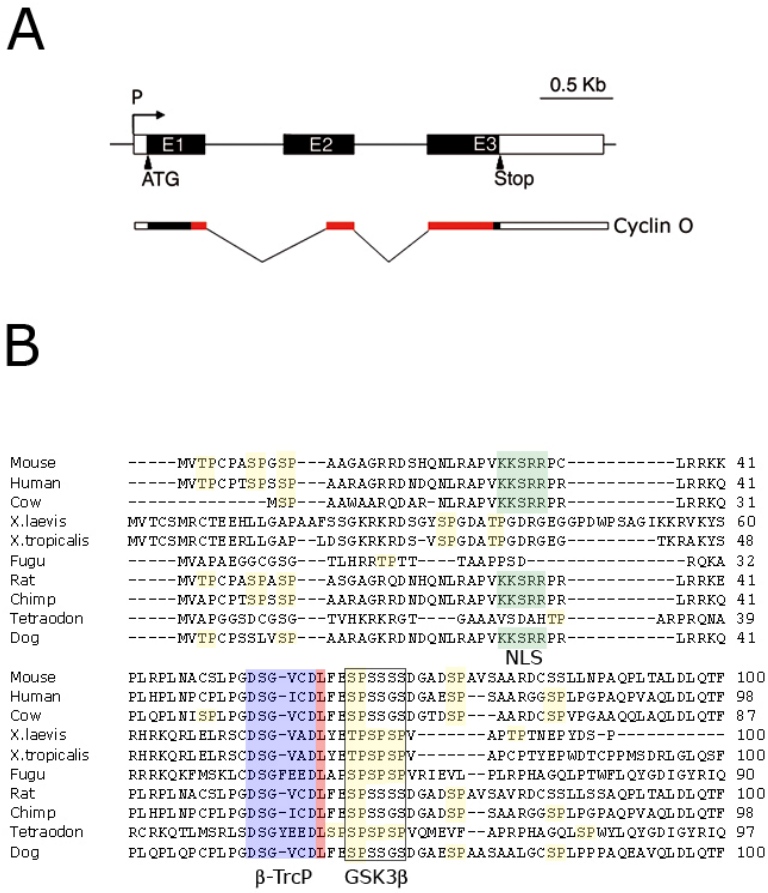


Figure 1. Scheme of Cyclin O. (A) Scheme of the murine Cyclin O locus, with the three exons colored black and the Cyclin box domain colored in red. (B). Alignment of the first 100 aminoacids of Cyclin O of different species showing high degree of homology. Several conseved motifs are highlighted: putative Cdk and MAPK phosphorylation sites (yellow), Nuclear Localization Signal (NLS), β-TrcP binding box (purple) with the ubiquitinable lysine highlighted in red, and a GSK3β phosphorylation site.

Since Cyclin O has not been described before, first of all we determined the pattern of expression in mouse tissues by semi-quantitative RT-PCR (Figure 2). The mRNA of Cyclin O is expressed in several tissues although at low levels, and the highest expression is found in testis. Interestingly, tissues that show no expression of the mRNA (i.e. spleen and thymus) the expression can be induced by some stimuli like γ-radiation (see below).

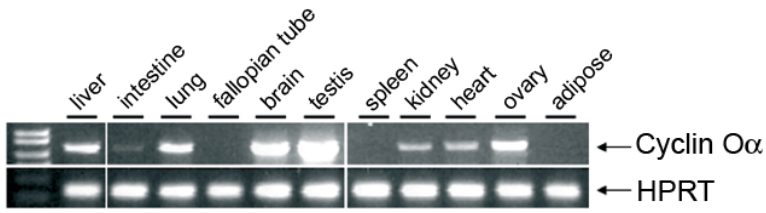


Figure 2. Cyclin O expression in mouse tissues. Expression of Cyclin O was measured by semi-quantitative RT-PCR in mouse tissues. As a loading control, HPRT levels were measured.

We also attempted to confirm the expression pattern by western blot. Since no commercial antibody exists against Cyclin O, we have generated four different antibodies against Cyclin O. Unfortunately, none of them was able to detect the endogenous protein by western blot even in testis or brain where the RNA levels are the highest. We think that this could be due to the low levels of expression in mouse tissues. We also checked some cell lines for the expression but we have only been able to detect the endogenous protein in cytosol enriched samples from human tumor cell lines or in mouse fibroblasts (Figure 3).

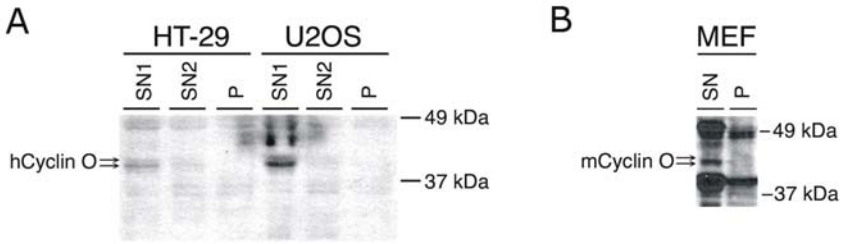


Figure 3. Detection of Cyclin O by western blot in the cytosolic fraction of cells. (A) The human colon adenocarcinoma cell line HT29 and osteosarcoma U2OS, were fractioned as described in materials and methods. Two consecutive cytosolic extractions where done, SN1 and SN2 respectively. The remaining fraction was designed as P and corresponds mainly to nuclei. The C5 antibody was used for detection. (B) Mouse fibroblast (MEFs) where subjected to one cytosolic extraction (SN). The remaining extract was designated P and corresponds mainly to nuclei. The C2 antibody was used for detection.

To confirm the RNA expression and subcellular fractionation data, we performed immunohistochemistry experiments in normal mouse tissues and in cell lines. Cyclin O is expressed in some tissues, although at low levels, in agreement with the RT-PCR data. In the majority of tissues as well as in cell lines, the protein shows a cytoplasmatic punctated pattern (Figure 3). This is in agreement with the subcellular fractionating data of figure 3 where Cyclin O

is only detected in the cytosolic fraction. Moreover, from the imunohistochemisty pictures of Figure 4 we can also conclude that the cell lines analyzed express higher levels of endogenous Cyclin O than brain; this fact could explain why we have only been able to detect the protein by western blot in those cells.

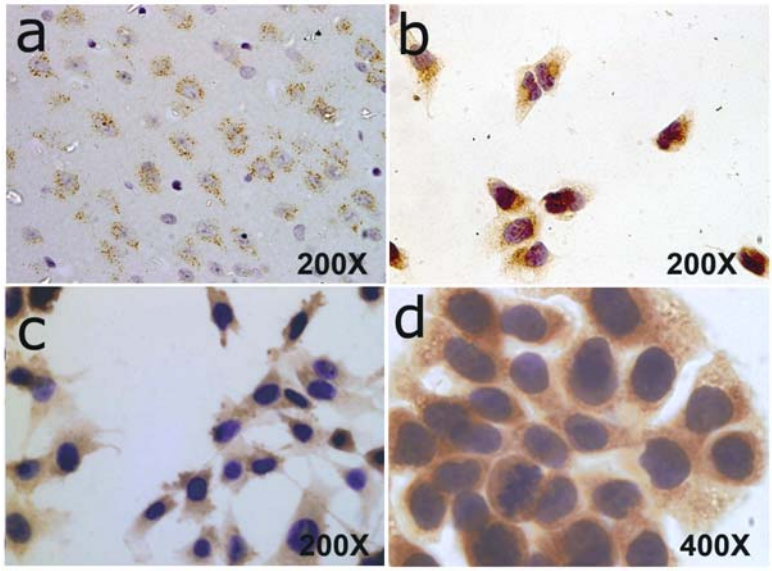


Figure 3. Detection of Cyclin O in mouse brain and in cell lines by immunohistochemistry. Cyclin O was detected by immunohistochemistry in murine brain cortical neurons (a), in human U2OS osteosarcoma cell line (b), in mouse embryonic fibroblasts (c) and in human HT29 colon cancer cell line. Cyclin O was detected using the N1 antibody and the EnVision™ system with the colorimetric substrate DAB.

2. Biochemical characterization of Cyclin O

We further characterized biochemically Cyclin O because we thought that this protein could be the activator of Cdk2 during apoptosis (ARCA). If Cyclin O is ARCA, then should have at least three properties: i) bind and activate Cdk2 ii) should be induced during thymocyte apoptosis iii) must be necessary for thymocyte apoptosis.
Thus, first of all we tested whether recombinant Cyclin O is able to bind and activate purified Cdk2. MBP-Cyclin O can bind stochiometrically to Cdk2 and is able to activate its kinase activity as shown by phosphorylation of the generic substrate Histone H1. However, a Cdk2 point mutant that lacks kinase activity, although it can bind Cyclin O at the same 1:1 stochiometric ratio, it shows no kinase activity (Figure 5). This mutant is unable to bind the cofactor Mg^{2+} necessary for ATP hydrolysis because the Aspartic residue that mediates this effect is replaced by an Alanine (121). Thus, it behaves as a dominant negative protein because it can bind to substrates and to Cyclins but is unable to phosphorylate substrates.

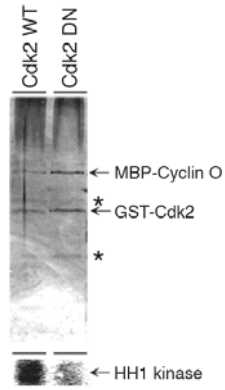


Figure 5. Cyclin O-Cdk2 recombinant complexes are active. Complexes of recombinant MBP-Cyclin O and either GST-Cdk2 WT or GST-Cdk2 DN purified from *E.coli*, were assembled *in vitro* and the (1:1) stochiometry was assessed by SDS-PAGE and silver staining (upper panel). Asterisks denote degradation bands of MBP-Cyclin O. The activity of the complexes was measured by a kinase assay using Histone H1 (HH1) as a substrate (lower panel).

Although all Cdks phosphorylate Serine and Threonine residues followed by a Proline, they show different substrate specificities. Cdk4 and 6 phosphorylate efficiently Retinoblastoma protein (Rb) whereas Cdk1, 2, 3 and 5 phosphorylate Histone H1 and Cdk7, 8 and 9 phosphorylate the C-terminal domain of RNA polymerase II (122).

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 kinase assays of the immunoprecipitates were carried out using either purified Histone H1 or recombinant GST-RNA polymerase II C-terminal domain (GST-CTD) as exogenous substrates. The Cyclin O complexes 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 Histone H1 (Figure 6A).

Since Cdk2 complexes immunoprecipitated from apoptotic thymocytes are inhibited by the ATP analogue roscovitine (107), we tested if Cyclin O immunocomplexes are also inhibited by this drug. Myc-tagged Cyclin O was immunoprecipitated from transiently transfected HEK293 cells. The immuno-complexes were assayed for Histone H1 kinase activity at different concentrations of roscovitine. As a control, we used the same amount of kinase activity of Cdk1 and Cdk2 immunoprecipitated from the same cells. As shown in Figure 6B, Cyclin O complexes have a comparable sensitivity to the inhibition by roscovitine as Cdk1 or Cdk2. From all Cdks tested, only Cdk1, Cdk2, Cdk5, Cdk7 and Cdk9 are known to be efficiently inhibited *in vitro* by roscovitine (123), so they are the most likely candidates to be Cyclin O partners. Given the fact that Cyclin O complexes poorly phosphorylate GST-CTD, this excludes Cdk7 and Cdk9, as they are efficient CTD kinases (122).

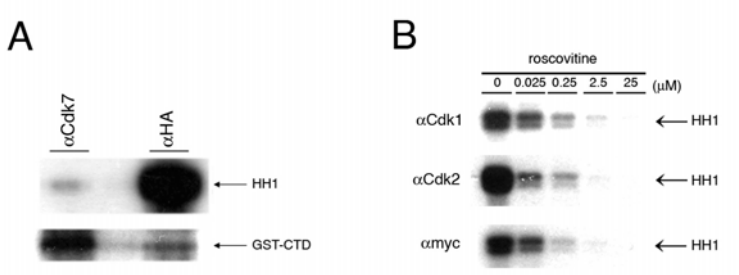


Figure 6. Biochemical properties of Cyclin O complexes. (A) Cyclin O-containing complexes preferentially phosphorylate Histone H1 over RNA Polymerase II C-terminal domain. HA-tagged Cyclin O was immunoprecipitated from HEK293 cells transiently transfected with an expression vector using the anti-HA tag monoclonal antibody and the immunoprecipitates assayed for Histone H1 (HH1) and RNA polymerase II C-terminal domain (GST-CTD) kinase activity (upper panel). The same experiment was performed with immunoprecipitates against Cdk7 using a polyclonal antibody against endogenous Cdk7 (lower panel). (B) Cyclin O-containing complexes are sensitive to roscovitine. HEK293 cells were transiently transfected with an expression vector for myc-tagged Cyclin O and 48 hours after transfection, whole cell extracts were immunoprecipitated with antibodies against Cdk1, Cdk2 and anti-myc tag. The immunoprecipitated kinase activity was measured in the presence of the indicated concentrations of roscovitine or equivalent amounts of the solvent DMSO.

To find out which Cdks bind Cyclin O *in vivo* we performed coimmunoprecipitation experiments using HEK293 cells transiently transfected with myc-tagged Cyclin O. As it can be seen in Figure 7A IP(1), only Cdk1 and Cdk2 can efficiently coimmunoprecipitate with Cyclin O. In order to discard an unspecific effect due to the myc-Tag (e.g. immunoprecipitation of the endogenous c-Myc protein), we performed the same immunoprecipitation without transfecting Cyclin O (Figure 7A, IP(2)). As expected no Cdk coimmunoprecipitated.

To detect the interaction of endogenous Cyclin O with Cdk2 and Cdk1, we used MEFs since they were the murine cells with highest levels of Cyclin O protein we have found. Whole cell extracts from MEFs were immunoprecipitated either with normal rabbit IgGs (rIgG) as a negative control or the anti-Cyclin O C2 antibody (C2) that efficiently immunoprecipitates the native Cyclin O-Cdk complexes. The immunoprecipitates were then analysed by Western blotting against Cdk1 and Cdk2 with the corresponding mouse monoclonal antibodies. The membranes were stripped and probed with the anti-Cyclin O antibody C2 as a control. As shown in Figure 7B, Cyclin O coimmunoprecipitates both with Cdk1 and Cdk2. From these experiments it can be deduced that endogenous Cyclin O clearly forms stable complexes *in vivo* with Cdk1 and Cdk2.

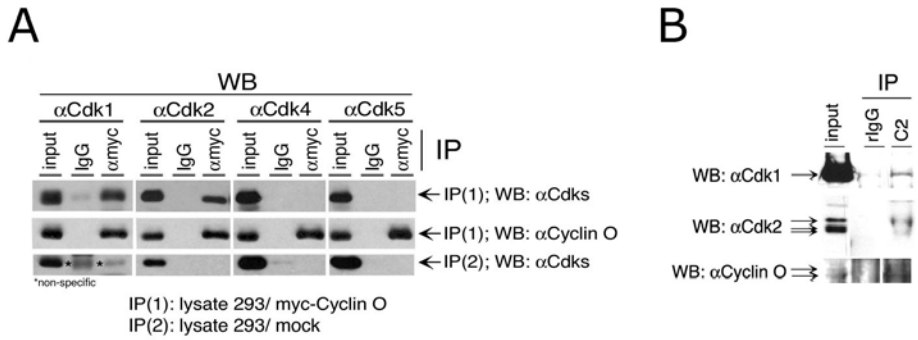


Figure 7. **Cyclin O binds Cdk1 and Cdk2 *in vivo*.** (A) Protein extracts of HEK293 cells transiently transfected with an expression plasmid for myc-tagged mouse Cyclin O were immunoprecipitated with a control rabbit IgG (IgG) or with a monoclonal antibody against the myc-tag (9E10) covalently bound 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, Cdk4 and Cdk5 (upper pannel) and, after stripping of the membranes, with antibodies against mouse Cyclin O (C2, middle pannel). As a control, 2.5% of the input was run in the gel and processed. To rule 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, Cdk4 and Cdk5 (lower pannel). The asterisks indicate non-specific bands. (B) Extracts from wild type 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 with the mouse monoclonal antibodies A-17 and clone 55, respectively, and SuperSignal West Pico chemiluminiscent substrate. As a control, the membrane was stripped and mouse Cyclin O was detected by western blotting with the antibody C2 and SuperSignal West Pico chemiluminiscent substrate.

2.1. Cyclin O binds and activates preferentially Cdk2

The coimmunoprecipitation experiments showed that both Cdk1 and Cdk2 can interact with Cyclin O *in vivo*. However, with those experiments we can not know if this interaction leads to activation of Cdk1, Cdk2 or both. To address this question we used an *in vitro* approach to isolate complexes of Cyclin O with a single known Cdk (Figure 8A). We incubated the MBP-Cyclin O fusion protein bound to amylose beads with whole cell extract from wild-type fibroblasts. 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 then immunoprecipitated using normal rabbit IgGs or antibodies against Cdk1, Cdk2 or Cdk5, and kinase assay were performed in the immunoprecipitates using Histone H1 as exogenous substrate. Only the antibodies against Cdk2 recovered significant amounts of kinase activity from wild type fibroblasts (Figure 8C upper panel).

Then we wondered what would happen in Cdk2 null fibroblasts or in brain where there is neither Cdk1 nor Cdk2 (Figure 8B). As shown in Figure 8A middle panel, in Cdk2 null fibroblast Cyclin O associated kinase activity is recovered in the anti-Cdk1 immunoprecipitates. Moreover, if neither Cdk1 nor Cdk2 is present, then the activity is recovered in the anti-Cdk5 immunoprecipitates. It is interesting to note that in all the extracts used in this experiment, the levels of Cdk5 where high, however, Cyclin O predominantly associated with either Cdk1 or Cdk2.

This experiment strongly suggests that, at least *in vitro*, Cdk2 is the preferred binding partner of Cyclin O leading to the formation of kinase-active complexes. Additionally, it also suggests that if Cdk2 is not present, it can form active complexes with Cdk1. The activation of Cdk5 by Cyclin O was unexpected since in the coimmunoprecipitation experiments they did not interact. This suggests that this interaction is limited to tissues where the levels of Cdk5 are high and where Cdk1 and Cdk2 are not present, therefore it could be relevant in brain cells.

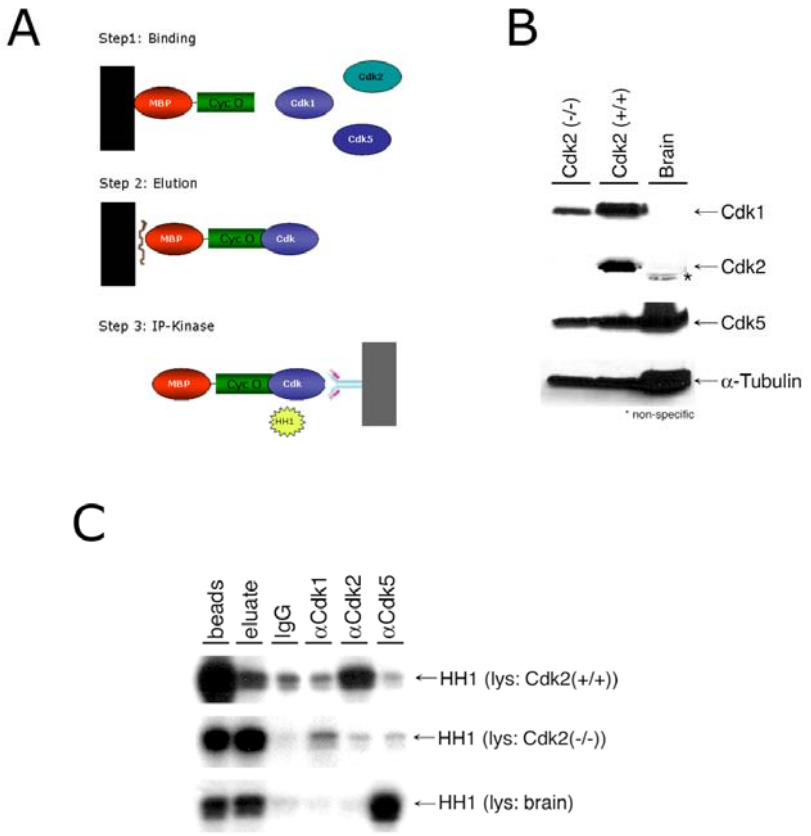


Figure 8. Cyclin O forms active complexes preferentially with Cdk2. (A) Scheme of the biochemical approach designed to obtain specific Cdk-Cyclin O complexes. Step 1: MBP-Cyclin O fusion protein was bound to amylose beads (black rectangle) and incubated with a cell extract. Step 2: MBP-Cyclin O with associated proteins were eluted from the amylose beads with maltose. Step 3: the eluted fraction was divided in four aliquots and immunoprecipitated with normal rabbit IgGs or antibodies specific for Cdk1, Cdk2 or Cdk5. The immunoprecipitates were then assayed for kinase activity over purified Histone H1 (HH1). Grey rectangle represents Protein A-Sepharose beads. (B) Cell extracts from mouse fibroblasts Cdk2 wild type (+/+) or knockout (-/-) and from wild type mouse brain (Brain) were analysed by western blotting for the presence of Cdk1, Cdk2 and Cdk5. As a loading control, α-Tubulin levels were measured. (C) The experiment explained in A, was carried out using whole cell extracts from mouse fibroblasts (Cdk2 +/+), fibroblasts from Cdk2 knockout mice (Cdk2 -/-) and mouse brain. An aliquot of step1 (beads) and step 2 (eluate) were processed directly for kinase assay.

3. Apoptotic stimuli induce Cyclin O

Our model for the study of apoptosis is based on the observation that upon apoptosis induction in thymocytes, there is an upregulation of Cdk2 activity. If it is due to Cyclin O, then this protein should be induced in apoptotic conditions. We subjected mice to whole body irradiation and then we analyzed the levels of Cyclin O mRNA in the thymuses. As shown in Figure 9A (white bars), the levels of expression of Cyclin O show a time-dependent induction, peaking at 3 hours after stimulus.

Our previous results indicated that thymocytes from Bax transgenic mice activated Cdk2 at higher levels than non-transgenic littermates during DNA-damage and dexamethasone-induced apoptosis, and the opposite effect was found for Bcl2 transgenics (107). We speculated that this could be related to the levels of Cyclin O; that is, Bax transgenic mice may induce Cyclin O at higher levels and Bcl-2 transgenic ones may behave in an opposite fashion. To investigate if this was the case, we measured the kinetics of induction of the Cyclin O mRNA after γ-irradiation in mice transgenic for Bax or Bcl-2 and compared it with non-transgenic littermates. As it is shown in Figure 9A, transgenic overexpression of Bax or Bcl2 significantly modify the levels of expression of Cyclin O as expected. However, noticeably both show induction of Cyclin O. These results suggest that the induction of Cyclin O is independent of Bax and Bcl-2 but these proteins can modulate the levels of expression at late time points. In conclusion, we show that the levels of Cyclin O mRNA correlate with the levels of Cdk2 kinase activity reported by Gil-Gomez et al.(107).

Additionally to the mRNA levels we have also analyzed the expression of the protein by immunohistochemistry. Wild type mice were treated with 10Gy of γ-radiation or injected intraperitoneally with 2mg of dexamethasone phosphate. Thymuses were removed 3 and 6 hours after stimulus was administered and immediately fixed in a solution of paraformaldehyde. Paraffin sections of the thymuses were incubated with antibodies against Cyclin O (N1) and active Caspase-3 as apoptosis marker. As a control, a section of each condition was stained with haematoxilin-eosin (H&E). As expected, both Cyclin O and active Caspase-3 staining are negative at time 0 (Figure 9B). However, both radiation and dexamethasone treatment leaded to a time-dependent increase in the expression of Cyclin O in the cytoplasm of the thymocytes, in parallel with increasing number of apoptotic cells positive for active Caspase-3 and showing pyknotic nuclei in the H&E staining. Dexamethasone induced apoptosis, however, shows a slower kinetics. The thymus, 6 hours after the administration of the steroid does not show so much apoptosis as the radiation treated animals as measured by active Caspase-3 or by the number of pyknotic nuclei. Accordingly, the levels of Cyclin O are also lower.

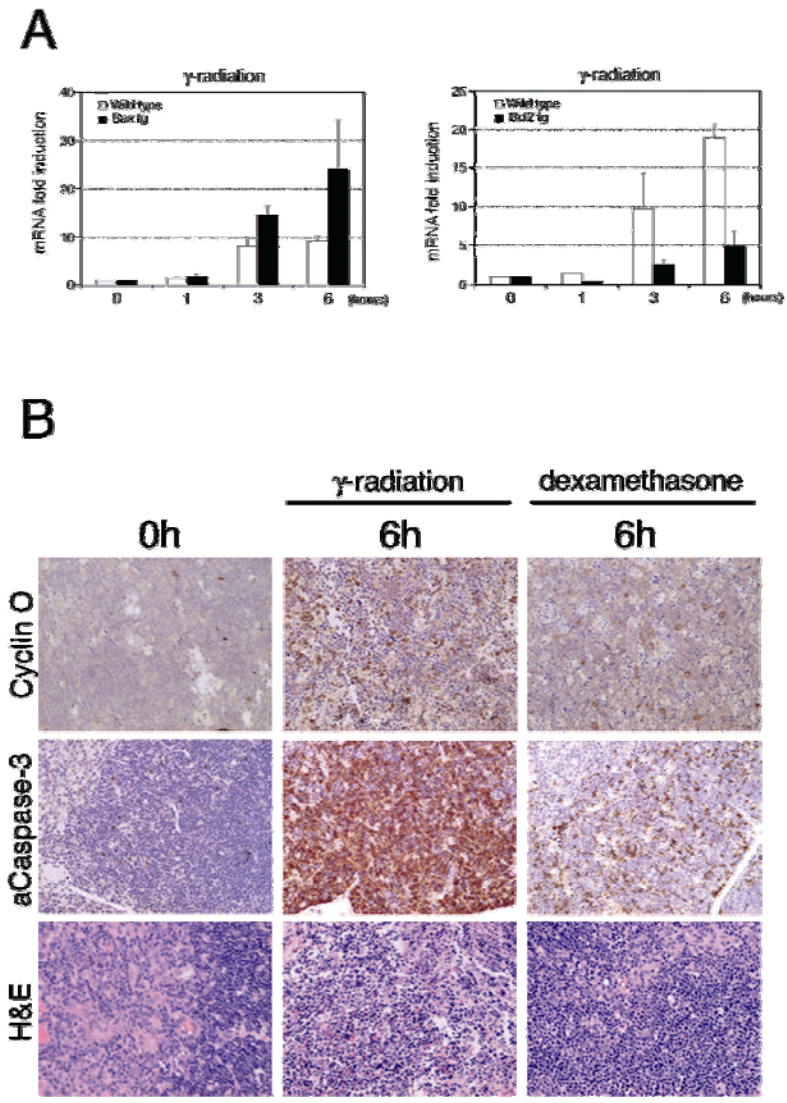


Figure 9. Mouse Cyclin O is induced during thymocyte apoptosis *in vivo* and is regulated by Bax and Bcl2. (A) Mice transgenic for Bax (left panel) and Bcl-2 (right panel) and non-transgenic littermates (wild type) were treated with 10Gy of gamma radiation using a ^{137}Cs irradiator. At the indicated times, mice were sacrificed and the RNA isolated. The levels of Cyclin O 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 done in triplicate is shown (mean plus standard deviation) as fold induction over the levels of the non-transgenic mice at time 0. (C) Thymuses from mice irradiated as before or intraperitoneally injected with 2 mg of dexamethasone (dexamethasone phosphate, Fortecortin^{R}) were fixated in paraformaldehyde and processed for Cyclin O and active Caspase-3 (aCaspase-3) detection by immunohistochemistry. Samples were counterstained with haematoxylin. A section of the same samples were stained with haematoxylin-eosin (H&E) as a control. Cyclin O was detected by using the N1 antibody and the EnVision^{TM} system with the colorimetric substrate DAB. In the case of the active Caspase-3, no EnVision^{TM} signal enhancing system was used.

4. Cyclin O expression is necessary for apoptosis induced by intrinsic stimuli

We have shown that Cyclin O is induced by apoptotic stimuli in thymocytes with the same kinetics as reported for activation of Cdk2. Since Cdk2 activity has been shown to be necessary for glucocorticoids and DNA-damage induced apoptosis, we reasoned that if we somehow eliminate Cyclin O then, we would avoid Cdk2 activation and, consequently, thymocyte apoptosis would be abrogated. Unfortunately, manipulate gene expression in thymocytes is difficult since they have a short life span in culture that makes impossible to manipulate them (i.e. before you get the expression, all thymocytes have died). Moreover, the infection efficiencies are very low even with lentiviral vectors. Given all this difficulties, we decided to move to a cell line where genetic manipulation is easier. We sought for a cell line with a similar behavior to thymocytes in terms of apoptosis sensitivity and Cyclin O regulation. We finally decided to work with the lymphoid cell line WEHI7.2. This is a cell line derived from a mouse T-cell lymphoma that is sensitive to glucocorticoids and DNA-damaging agents (both signaling through the intrinsic apoptotic pathway) and to FAS agonistic antibodies that induce apoptosis by the extrinsic pathway (124).

We analyzed the regulation of Cyclin O in this cell line. As seen in Figure 10, Cyclin O levels are almost indetectable in basal conditions; however, Cyclin O is dramatically upregulated following treatment with either genotoxic stimulus (etoposide) or the glucocorticoid dexamethasone. The kinetics of Cyclin O induction closely parallels those found in thymocytes (Figure 9A).

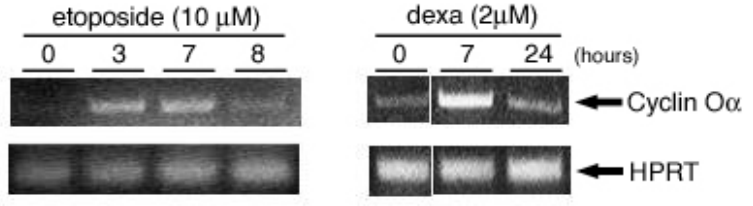


Figure 10. WEHI7.2 mouse lymphoid cells induce Cyclin O upon intrinsic apoptotic stimuli. WEHI7.2 cells were treated with 10μM etoposide or with 2μM dexamethasone and samples collected at the indicated times. RNA was extracted from the samples and the levels of Cyclin O analysed by semi quantitative RT-PCR. As a loading control, the mRNA levels of HPRT were measured in the same samples.

To address the question of whether Cyclin O is necessary for apoptosis, we downregulated its expression in WEHI7.2 cells. We isolated single cell clones stably expressing one of two different shRNA constructs directed against different regions of Cyclin O mRNA. The clones obtained were designated "3" or "5" according to the construct transfected and followed by the number of the clone. As a control, we isolated clones expressing either the empty vector pSuperpuro (Brummelkamp 2002) (and designated "V" clones) or vector expressing an shRNA against GFP ("G" clones). Since western blot has proven to be unsuccessful for the detection of Cyclin O, most likely due to the low levels of expression of the protein in lymphoid cells, we adopted two complementary strategies to demonstrate the effectiveness of the shRNAs. On one hand, we measured the levels of Cyclin O in control and shRNA clones by immunohistochemistry with the N1 antibody after apoptosis induction (Figure 11A). On the other hand, we immunoprecipitated native Cyclin O complexes with the C2 antibody from etoposide treated cells and assayed the immunocomplexes for kinase activity over Histone H1 (Figure 11B). Both experiments show that the shRNAs against Cyclin O efficiently prevented its expression whereas control clones induced Cyclin O in response to apoptotic stimuli.

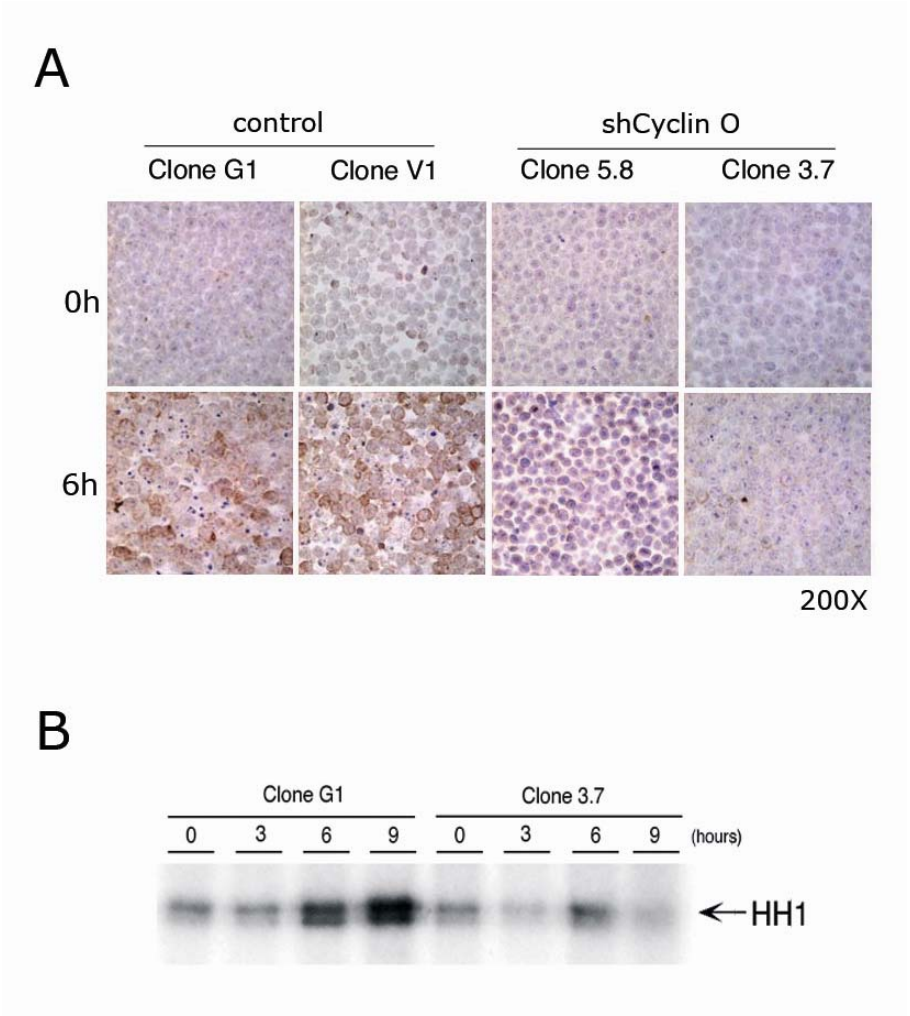


Figure 11. Validation of Cyclin O shRNA clones. (A) Absence of immunoreactivity to anti-Cyclin O antibodies in etoposide-treated Clones expressing a shRNA against Cyclin O (clones 3.7 and 5.8). Clones V1 (empty vector), G1 (shRNA against GFP), 3.7 and 5.8 were treated with 10μM etoposide, samples were harvested at 0 and 6 hours and processed for Cyclin O detection by immunohistochemistry. Both control clones V1 and G1 show very low expression of Cyclin O in untreated cultures, whereas Cyclin O levels rise dramatically after 6 hours of etoposide treatment, showing a clear punctuate cytoplasmic staining. In the case of clones 3.7 and 5.8, almost no staining is detected even after treatment with etoposide. (B) Absence of Cyclin O assossiated kinase activity in 3.7 clone after etoposide-induced apoptosis. Clones G1 and 3.7 were harvested 0, 3, 6 and 9 hours after apoptosis induction with etoposide (10μM). Cell extracts were obtained and immunoprecipitated with the C2 antibody that preserves the activity of the Cyclin O associated kinase(s). Etoposide treatment induces time dependent, Cyclin O-associated Histone H1 kinase activity, whereas this effect is strongly diminished in the cells expressing the Cyclin O shRNA vector (clone 3.7)

We then characterized the response of these clones to apoptosis. If our hypothesis was true, then clones with Cyclin O downregulated should be resistant to apoptosis. Three to five independent clones from each construct were analyzed for their response to apoptosis. All the clones tested had the same phenotype and we chose a representative clone of each group to show the results of the tests carried out.
In contrast to the control clones (V1 and G1), clones with Cyclin O downregulated (clones 3.7 and 5.8) were completely refractory to apoptosis induced either by etoposide or dexamethasone (Figure 12). Noteworthy, both shRNA constructs behaved very similarly, discarding an unspecific effect over other mRNAs (off-target effects).

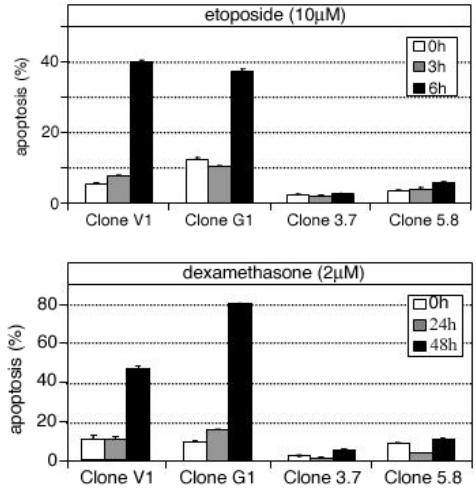


Figure 12. Downregulation of Cyclin O abrogates etoposide and dexamethasone-induced apoptosis. WEHI7.2 cells were stably transfected with empty pSUPERpuro vector (clone V1), with pSUPERpuro expressing an shRNA against GFP (clone G1), or two shRNAs directed against mCyclin O Exon 3 (clone 3.7 and clone 5.8) and single cell clones isolated. The selected clones were treated with 10μM etoposide (upper panel) or 2 μM dexamethasone (lower panel). 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 done in triplicate. Bars correspond to the mean plus the standard error of the measure.

Our previous work has ruled out the participation of Cdk2 in the signal transduction pathway mediated by Fas (107;116). In fact, Fas signalling leads to direct activation of Caspase-8 through the intermediate protein FADD. As the activation of Caspase-8 is medidated by protein-protein interactions and depends on a proteolytic cleavage, neither transcription nor translation is necessary for this pathway to activate apoptosis. Moreover, protein translation inhibitors are usually necessary to ensure apoptosis after Fas agonistic treatment since survival pathways are also activated by FAS and mask the apoptotic effect (41). To investigate if downregulation of Cyclin O expression had any effect on the signalling by the extrinsic stimuli, we studied the response of the clone 3.7 to the FAS agonistic antibody Jo2 in the presence of cycloheximide. The apoptotic response to anti-FAS remains intact in the presence of downregulated Cyclin O expression (Figure 13A). To further demonstrate that the extrinsic biochemical pathway is intact, we measured Caspase-8 activation in the G1 control and 3.7 Cyclin O shRNA clones and the cleavage of its substrate Bid (Figure 13B). In agreement with the results of Figure 13A, no significant differences were detected.

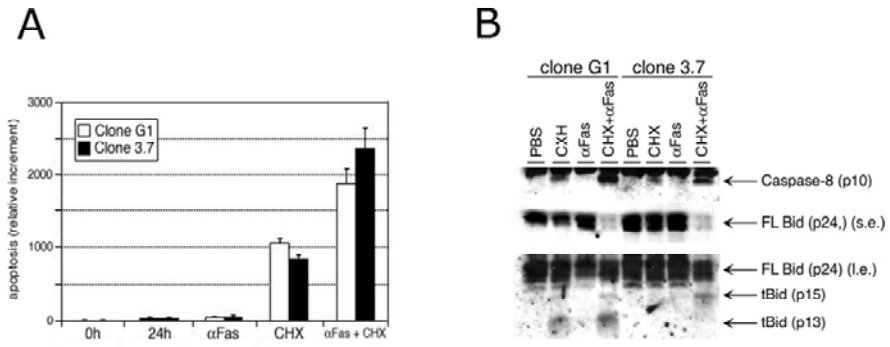


Figure 13. Downregulation of Cyclin O does not affect extrinsic apoptotic signalling. (A) To check the response of the clones to the apoptotic effect of the FAS pathway, a control clone (G1) and an shRNA clone (3.7) were cultured 24 hours in the presence of 0.5μg/mL of the agonistic antibody Jo2 (αFas), 30 μg/mL of cycloheximide (Chx) or the combination of both treatments (αFas+Chx). 24h after treatment, cells were collected and one aliquot was used to measure apoptosis and the other for western blot. (A) Apoptosis was measured in triplicate as described in Figure 12 and the relative increase in apoptosis of each condition relative to the value at 0 hours is shown. Error bars show the standard deviation of the mean. (B) 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. (s.e.): short exposure; (l.e.): long exposure.

5. Cyclin O downregulation only affects the apoptotic response to DNA damage and glucocorticoid treatment in lymphoid cells

The failure of the cells with downregulated Cyclin O to undergo apoptosis could be due to a defect in the signal transduction pathway of the apoptotic stimuli used. In other words, we may have selected clones intrinsically resistant to apoptosis due i.e. to mutations in the glucocorticoid receptor and in the DNA-damage response pathway.

DNA-damage response pathway is a signal transduction pathway that detects DNA lesions and transduces the signal to the effector mechanisms that constitute the checkpoint response. To check for the integrity of this pathway, we compared the kinetics of activation of sensors, transducers and effector proteins after γ-radiation treatment of the control G1 clone and the Cyclin O shRNA clone 3.7.

As seen in Figure 14A, the kinetics of phosphorylation of the sensor protein ATM and the signal transductor protein Chk1 are the same in the Cyclin O shRNA clone than in the control clone. Moreover, the activation of the effector proteins are not significatively changed as a result of the downregulation of Cyclin O: downregulation and re-synthesis of the cell cycle checkpoint mediator phosphatase Cdc25A, the distribution of the different phosphorylated bands of Cdc25C, the kinetics of p53 activation by phosphorylation and the upregulation of its target gene $p21^{Cip1}$. However, activation of Caspase-3 is completely abolished, indicating defective apoptosis. In agreement with this, the DNA profile of the cells 9 hours after treatment with γ-radiation shows a complete lack of a subdiploid population in the clone 3.7 while the alive cells show a profile similar to clone G1 cells with a prominent G1, intra-S and G2 arrest, indicating that checkpoints are working properly.

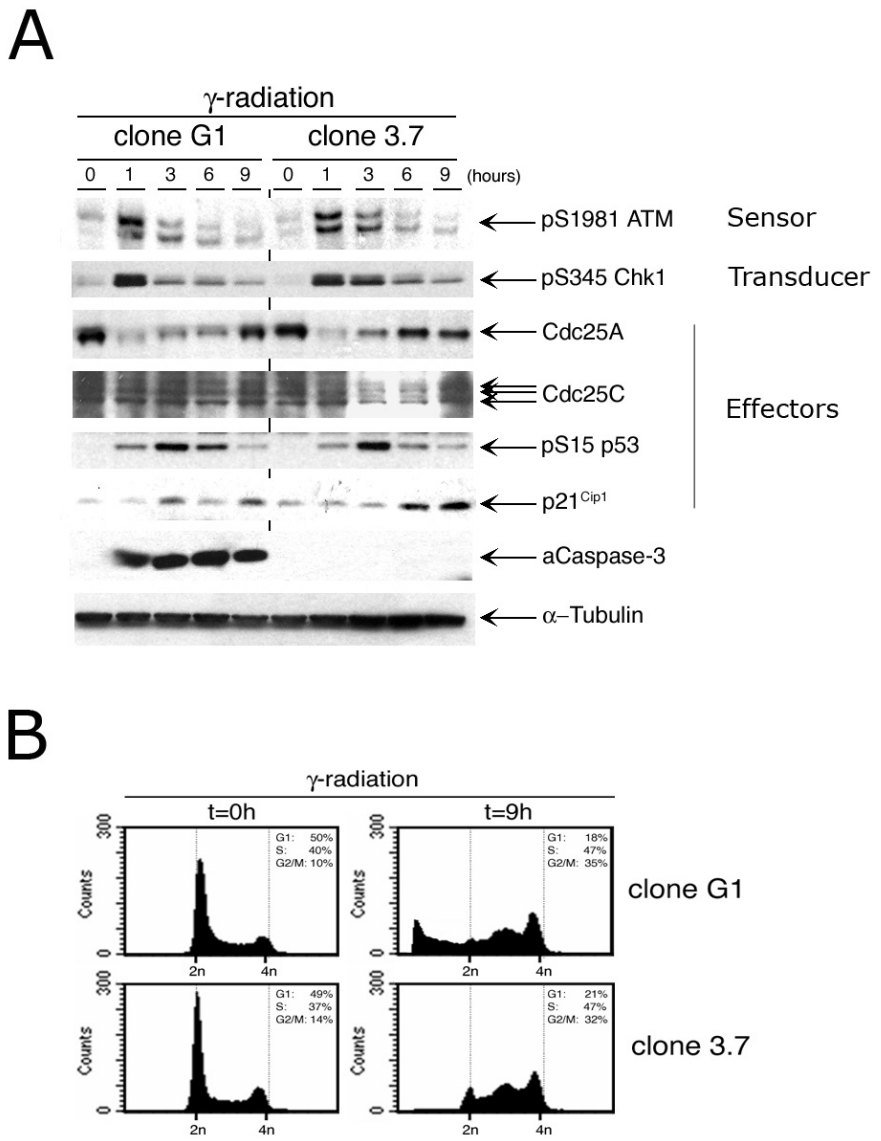


Figure 14. DNA-damage response is not affected by Cyclin O downregulation. (A) The integrity of the DNA damage response was evaluated in the control clone G1 and the Cyclin O deficient clone 3.7 by measuring by western blotting the kinetics of activation of ATM and Chk1, the checkpoint regulatory proteins Cdc25A and C and the activation of p53 and its target gene $p21^{Cip1}$. The activation of Cyclin O downstream mediator Caspase-3 was detected by western blotting in order to check the apoptotic response to γ-radiation. As a loading control, the membranes were probed with α-Tubulin. (B) DNA profile of the control clone G1 and the Cyclin O shRNA clone 3.7 cells after γ-radiation. Only the G1 clone shows a sub-G1 DNA peak, but the cell cycle distribution of the live cells looks similar, indicating the integrity of the cell cycle effector mechanisms of the checkpoint in the absence of Cyclin O.

To confirm that the general glucocorticoid response was not affected by Cyclin O downregulation, we further characterized it in clones G1 and 3.7. We first analyzed the transcriptional activity of the glucocorticoid receptor. We have focused on one of its best characterized target genes, the BH3-only Bcl-2 family member Bim (24). As it is shown in Figure 15A, dexamethasone treatment of clone G1 and 3.7 cells leads to a similar induction of the major Bim isoform (BimEL), indicating a normal transcriptional response to the hormone.

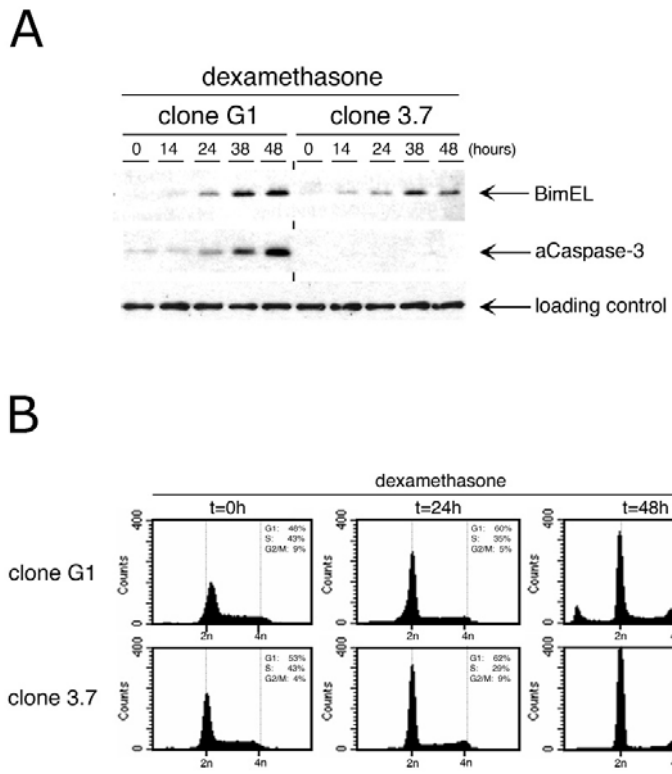


Figure 15. Glucocorticoids response is not affected by Cyclin O downregulation. (A) Control clone G1 and shRNA Cyclin O clone 3.7 were treated with 2 μM dexamethasone and cells were collected at the indicated times. Cell extracts were prepared and analyzed by western blot for the presence of Bim (BimEL is the major isoform) and active Caspase-3 as a marker of apoptosis. Cyclin O downregulation does not affect the kinetics of Bim induction but abolishes Caspase-3 activation. (B) Time course of the DNA profile of the control clone G1 and the Cyclin O shRNA clone 3.7 cells after treatment with dexamethasone (2μM)

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 (125). As it can be seen in the DNA profile shown in Figure 15B, dexamethasone treatment of clones G1 and 3.7 leads to a time-dependent arrest mainly in G0/G1 that is almost complete in clone 3.7 after 48 hours, while in clone G1 appears the sub-G1 apoptotic DNA peak superimposed to the cell cycle arrest. However, activation of Caspase-3 is, again, totally absent in clone 3.7 (Figure 15A).

All these results strongly suggest that, lack of activation of the Cyclin O-Cdk2 complexes during DNA damage or glucocorticoid-induced apoptosis results in the absence of Caspase activation and, hence, a specific lack of the apoptotic response of the cells. This is in agreement with previous data from thymocytes where it has been shown that chemical inhibition of Cdk2 abrogates Caspase activation and consequently results in apoptosis blockade (117).

6. Cyclin O downregulation blocks apoptosis upstream mitochondria

With the experiments using Cyclin O shRNA clones we have demonstrated that apoptosis is blocked because there is no DNA degradation and no Caspase-3 activation. DNA degradation is the last step in the apoptotic cascade and Caspase-3 activation is the previous step, just after mitochondria permeabilization and Caspase-9 activation. Furthermore, we have also shown that the initial response either to genotoxic damage or to glucocorticoids is intact. In order to position more precisely the role of Cyclin O in the apoptotic pathway, we analyzed mitochondrial permeabilization and phosphatidylserine exposure after apoptosis induction. Clones G1 and 3.7 were treated with 10 Gy of γ-radiation and the percentage of cells exposing the phospholipid phosphatidylserine at the outer plasma membrane (annexin-V positive) and cells with low mitochondrial potential ($\Delta\Psi_m^{low}$) was measured by flow cytometry (Figure 16). Clone 3.7 shows significatively less cells with low mitochondrial potential and positive for phosphatidylserine than the control clone G1 9 hours after irradiation. Since knocking down Cyclin O avoids mitochondrial permeabilization, this positions its function upstream of the mitochondria. These results are also in agreement with our previous work with thymocytes where we showed that inhibition of Cdk2 blocked apoptosis in a very early step before phosphatidylserine exposure and mitochondrial outer membrane permeabilization (117).

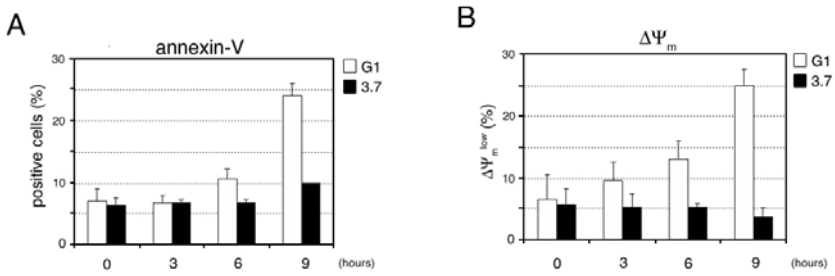


Figure 16. Downregulation of Cyclin O avoids phosphatidylserine exposure and mitochondrial transmembrane potential dissipation after γ-radiation. (A) Clones G1 and 3.7 were treated with 10 Gy of γ-radiation using a ^{137}Cs irradiatior and the percentage of cells exposing the phospholipid phosphatidylserine at the outer plasma membrane were measured by means of their ability to bind to (fluorescein-labeled) annexin-V and quantitated by flow cytometry. (B) The same experiment was performed but as a marker of apoptosis was used the dissipation of the mitochondrial transmembrane potential by means of the quantitation by flow cytometry of the percentage of cells with low fluorescence for the mitochondrial potential-sensitive dye CMX-Ros ($\Delta\Psi_m^{low}$).

7. Cyclin O is necessary for apoptosis of non-lymphoid cells

Although the results obtained using the WEHI7.2 cell line were in agreement with the data obtained from thymocytes, we wanted to rule out that this was a cell-type specific effect. To address this question we decided to analyze the effect of knocking down Cyclin O in another lymphoid cell line (EL-4) and in a completely different system, immortalized mouse embryonic fibroblasts (MEF). With this experiment we could find out whether Cyclin O has a proapoptotic effect only in cells from lymphoid origin or if its role is more general.

We isolated single cell clones stably expressing the shRNA against Cyclin O previously designed as "3" or an shRNA against Luciferase as a control. Several clones were isolated both in EL-4 cells and in MEF. In order to assess the effectiveness of the shRNA in EL-4 clones, we selected two representative clones, one with shRNA against Cyclin O (L3-2) and one control (Luc2). Since we found that the basal expression of Cyclin O in EL-4 cells was low, we treated cells with 10Gy of γ-radiation. Then we collected cells every 12 hours and analyzed Cyclin O associated kinase activity (Figure 17A). Luc2 clone shows a dramatic peak of Cyclin O associated kinase activity 12 hours after irradiation, indicating that Cyclin O was induced. However, L3-2 clone with Cyclin O downregulated did not show this early peak; instead, it shows a peak of Cyclin O activity 36 hours after irradiation but of a lower magnitude. This indicates that the shRNA against Cyclin O prevented its expression in EL-4 cells, at least at early time points after irradiation. Once we had demonstrated the effectiveness of the shRNA, we analyzed the response to radiation in four randomly selected EL-4 cells. We irradiated the selected clones with 10 Gy of γ-radiation and quantified the amounts of apoptosis at different time points until 48 hours. As shown in Figure 17B, clones with downregulated Cyclin O show less basal apoptosis and less apoptosis induced by γ-radiation.

We have seen that the basal expression of Cyclin O in MEF can be detected by immunohistochemistry (Figure 3B). Then we checked the efficiency of the shRNA in MEF clones by immunohistochemistry. Two control clones LucC and Luc D, and two shCyclin O clones (sh3C, sh3D) were analyzed by immunohistochemistry using the N1 antibody (Figure 17C). Although is difficult to quantitate the expression levels of a protein by immunohistochemistry, the results shown in Figure 17C are clear enough to conclude that the expression of Cyclin O is lower in clones expressing an shRNA against Cyclin O. Then we subjected these clones to different doses of γ-radiation. As seen in Figure 17, those clones with Cyclin O downregulated show less apoptosis.

Altogether these results suggest that Cyclin O participates in the apoptotic program not only in lymphoid cells but also in other cell types such us mouse fibroblasts. However, the effect that we have seen knocking down Cyclin O in MEF or in EL-4 cells is not as strong as seen in WEHI7.2 cells. This could be due to the different efficiencies of the shRNA. In WEHI7.2 clones the

downregulation is almost complete (Figure 11) whereas in EL-4 and in MEF is only partial (Figure 17).

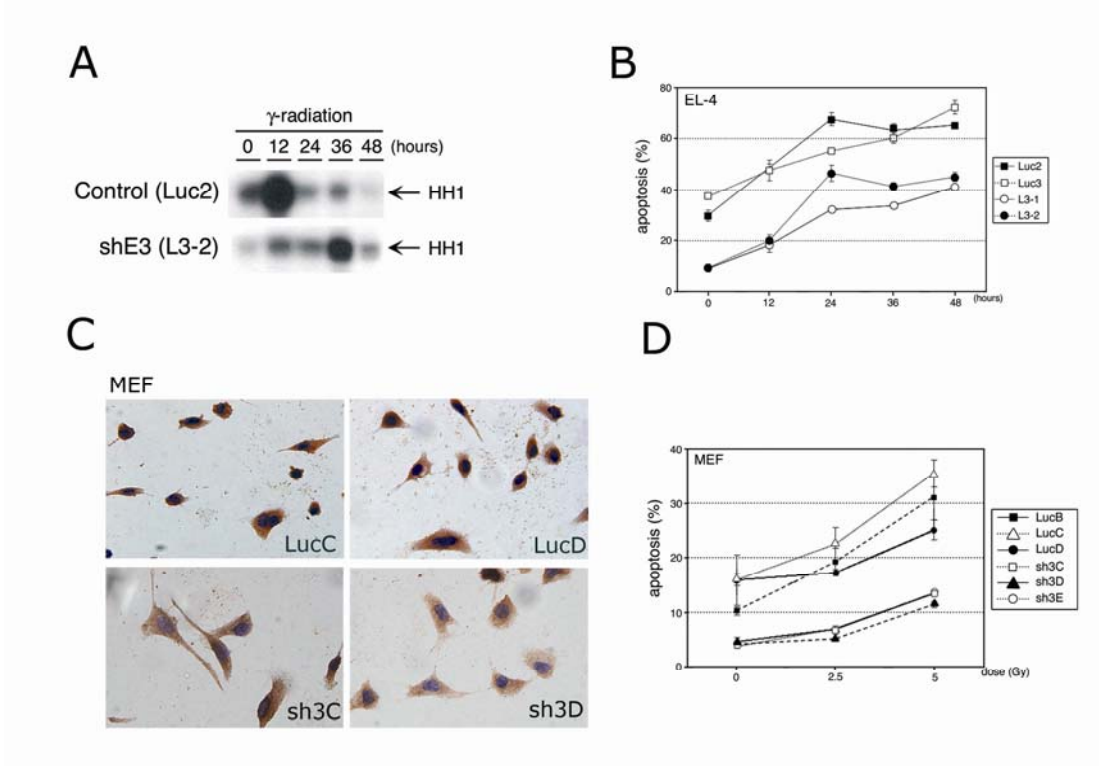


Figure 17. Cyclin O downregulation in mouse fibroblasts (MEF) and EL-4 lymphoid cells leads to resistance to DNA damaging stimuli. MEF and EL-4 cells transfected with a plasmid encoding the murine Cyclin O shRNA E3 or a shRNA targeting luciferase were selected with puromycine and single cell clones isolated. (A) Two representative EL-4 clones were selected to assess the effectiveness of the shRNA, one control (Luc2) and one shCyclin O (L3-2). Both clones were treated with 10Gy of γ-radiation and cells were collected at the indicated times. Cell extracts were obtained and Cyclin O associated kinase activity was measured by immunoprecipitation with the C2 antibody and using Histone H1 as exogenous substrate. (B) EL-4 clones expressing shRNA against Cyclin O (L3-1, L3-2) or against luciferase (L3-1, L3-2) were treated with 10Gy of γ-radiation and apoptosis was measured per triplicate at the indicated time points as described in Figure 12. Error bars show the standard deviation of the measure. (C) MEF clones stably expressing a shRNA against Cyclin O (sh3C, sh3D) or a shRNA against luciferase (LucC, LucD) were analyzed by immunohistochemistry for the expression of Cyclin O using N1 antibody.(D) MEF clones with shCyclin O (sh3C, sh3D, sh3E) or shLuciferase (LucB, LucC, LucD) were treated with 2,5 or 5Gy of γ-radiation and apoptosis was measured per triplicate after 48 hours as described. Error bars show the standard deviation of the measure.

8. Identification of Cyclin O-Cdk2 substrates

We hypothesized that if Cyclin O–Cdk2 complexes control apoptosis and Cdk2-CyclinA/E control cell cycle progression, then each complex should have different substrate specificities. In order to identify Cyclin O-Cdk2 specific substrates, we performed a yeast three hybrid screening using Cdk2-Cyclin O complex as bait. To achieve that we used a vector that allows the simultaneous expression of Cdk2 fused to Gal4 DNA binding domain and Cyclin O (Figure 18). The library we used for the screening was prepared from apoptotic thymocytes, in order to be sure that relevant proteins for our system were expressed. As a result of this screening we have identified several proteins (Table 1). Noticeably, we "fished" two proteins that were already known to interact with Cdk2, $p21^{Cip1}$ and p107; this fact validates the screening.

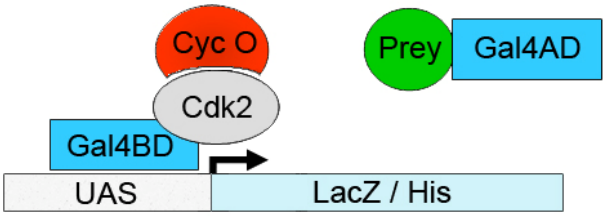


Figure 18. Scheme of the strategy used in the yeast three hybrid screening. Cdk2 was fused to the DNA binding domain of Gal4 (Gal4BD) and expressed in the yeast strain Y190 simultaneously with Cyclin O. This strain has a genetic construct in the genome containing the reporter genes LacZ and His under the control of a promoter with Upstream Activating Sequences (UAS), recognized by Gal4BD. For the screening, a library of cDNAs (Prey) fused to the transcriptional activation domain of Gal4 (Gal4AD) was transformed.

Among the proteins identified, AES (Amino-terminal Enhancer of Split) was the most prominent for the numerous times that appeared in independent clones. Septin 1, Kcnh2 and Son3 homolog were discarded because the fragment identified in the screening was very small. Then for further experiments we focused on c-Myb, AES, Baiap2, ATPi and Opa1. We analyzed by pull-down if those proteins were able to interact with Cyclin O and/or Cdk2. MBP fusion proteins were purified from bacteria and incubated with a cell extract of HEK293 cells transiently transfected with HA-tagged Cyclin O. We found that only $p21^{Cip1}$ was able to interact with Cdk2 and Cyclin O whereas p107 interacted with Cyclin O alone (data not shown). We thought that this could be due either to the fact that the interaction was very weak, as it is expected for kinase-substrate interactions or because the interaction was artifactual. We then checked if these proteins were substrates of Cdk2. To check that we used the purified MBP-fusion proteins as substrates for kinase assays using Cyclin O complexes immunoprecipitated from HA-tagged Cyclin O-transfected HEK293 cells. As shown in Figure 19, c-Myb and Baiap 2 (Brain-specific Angiogenesis

Inhibitor Associated Protein 2) are phosphorylated by Cyclin O associated kinases. Noticeably, AES did not interact with Cyclin O and/or Cdk2 and was neither a substrate, despite the abundant times that it appeared in the yeast two hybrid screening; then it can be considered as a false positive.

Protein	Number of clones identified
p107 (Rb-like)	2
$p21^{Cip1}$	3
Myeloblastosis oncogene (c-Myb)	1
AES	23
Baiap2	2
ATPase inhibitor	1
Septin 1	1
kcnh2 (Voltage-gated potassium channel	2
Son 3 homolog	2
Opa1	1

Table 1. Proteins indentified in the yeast three hybrid screening.

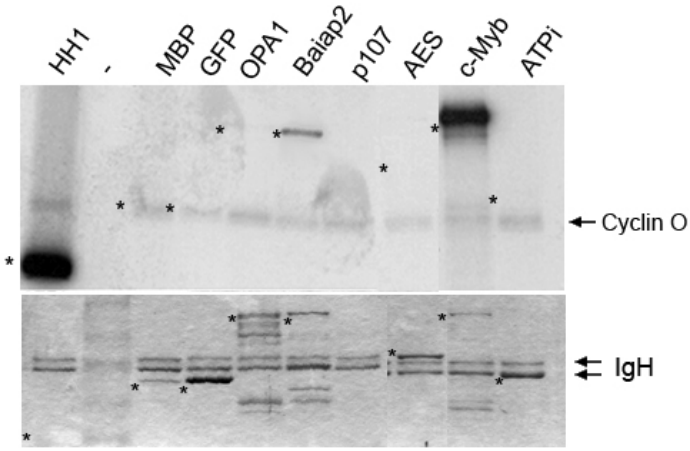


Figure 19. Cyclin O associated kinases phosphorylate c-Myb and Baiap2 in vitro. MBP, GFP and MBP fused with OPA1, Baiap2, p107, AES, c-Myb and ATPi were purified from bacteria and used as substrates for Cyclin O complexes immunprecipitated from transiently transfected HEK293 cells. As a control for the kinase assay, the generic kinase substrate Histone H1 (HH1) was used. Kinase reactions were loaded on a SDS-PAGE and the gel was stained with Coomassie to detect the proteins used (lower panel) and subjected to autoradiography (upper pannel). Asterisks denote the full length protein. Note that the amount of HH1 used was not detected by Coomassie staining and that the p107 protein was degraded and lost from the gel. Cyclin O autophosphorylation band and the bands corresponding to the immunoglobulin heavy chains are indicated.

9. Characterization of c-Myb as a Cyclin O target

For further experiments we decided to focus on c-Myb for many reasons. c-Myb is a transcription factor predominantly expressed in immature haematopoietic cells that regulates differentiation. It is highly expressed in immature haematopoietic progenitors and its expression comes down as cells differentiate (126). It has been shown that enforced c-Myb expression blocks differentiation and maintains an undifferentiated proliferating stage (127). On the other hand, c-myb knockout mice are not viable and embryos die due to haematopoietic failure (128). Interestingly, using conditional knockout mice, it has been shown that c-myb is essential for survival of double positive thymocytes (129), the predominant population of thymus. We then reasoned that during thymocyte apoptosis, c-myb would be downregulated. We analyzed the levels of c-myb mRNA after thymocyte apoptosis induction *in vivo*. As shown in Figure 20, c-myb mRNA is rapidly downregulated after irradiation. This result, showing that c-myb is regulated during apoptosis, encouraged us to further characterize the regulation of c-Myb by Cyclin O-Cdk2 complexes.

In our model of thymocyte apoptosis we have shown that gene transcription and protein translation is necessary after Cdk2 activation for apoptosis to proceed. This suggests that Cdk2-Cyclin O complexes are regulating the activity of a transcription factor necessary for apoptosis. Since c-Myb regulates survival in T-cells and its mRNA is downregulated after irradiation, we decided to further study its regulation by Cdk2-Cyclin O complexes.

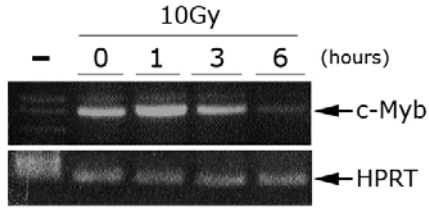


Figure 20. C-myb mRNA is downregulated during thymocyte apoptosis. Mice were treated with 10Gy of gamma radiation using a ^{137}Cs irradiator. At the indicated times, mice were sacrificed and thymuses were extracted for RNA isolation. The levels of c-Myb mRNA were analysed by semi-quantitative RT-PCR. As a loading control, the mRNA levels of HPRT were determined in the same samples.

First of all we tried to identify the region phosphorylated by Cyclin O complexes. C-Myb is a 75 KDa protein containing a highly conserved N-terminal DNA binding domain and a C-terminal regulatory domain (126); all putative Cdk phosphorylation sites (Ser of Thr followed by a Pro) are within the regulatory domain (Figure 21A). Since murine c-Myb contains 15 Cdk consensus phosphorylation sites, we made a general approach to map the phosphorylation site. We divided the protein in three different regions: the transcriptional activating domain (TA), the negative regulatory domain (NR) and the middle domain (MD) (Figure 21A) and checked if they could be phosphorylated by Cyclin O or Cyclin A complexes. As shown in figure 21B, both MD and NR fragments are efficiently phosphorylated by Cyclin O as well as by Cyclin A complexes. However, TA fragment, containing three Cdk consensus phosphorylation sites was not phosphorylated. Interestingly, those sites are not conserved between the mouse and the human c-Myb protein.

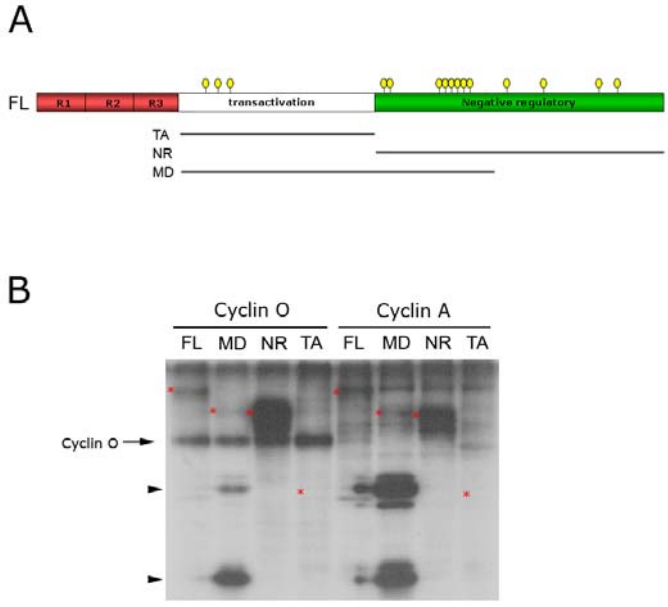


Figure 21. Cyclin O and Cyclin A associated kinases phosphorylate c-Myb on the negative regulatory domain. (A) Scheme of the murine c-Myb protein and the fragments used for kinase assays. The three different domains of the protein are showed. R1-3 denotes the three tandem repeats of the SANT-domain that conform the DNA-binding domain. The yellow circles indicate the presence of Cdk consensus phosphorylation sites. With a black line, it is represented the sequence that covers the diffents constructs used. (B) Full length c-Myb protein (FL) or different fragments (MD, NR and TA) were fused to MBP and purified from bacteria. The recombinant proteins were then used as substrates in kinase assays using either Cyclin O or Cyclin A complexes immunoprecipitated from HA-tagged Cyclin O-transfected HEK293 cells. Cyclin O autophosphorylation band is shown. Arrowheads indicate degradation bands of the recombinant protein. Asterisks indicate the band corresponding to the full length recombinant protein.

Then we checked if Cyclin O could induce a change in c-Myb transcriptional activity. For that purpose we used a reporter vector that contains three tandem repeats of c-Myb binding sites controlling the expression of the Luciferase gene. We transiently transfected this reporter vector together with c-Myb and Cyclin O or Cyclin A in HEK293 cells. Whereas Cyclin O induced an increase in c-Myb transcriptional activity, Cyclin A did not (Figure 22). This experiment not only suggests that Cyclin O is able to modulate c-Myb transcriptional activity but also that this effect is specific of Cyclin O since its closest homolog (Cyclin A) does not significatively modify c-Myb transcriptional activity.

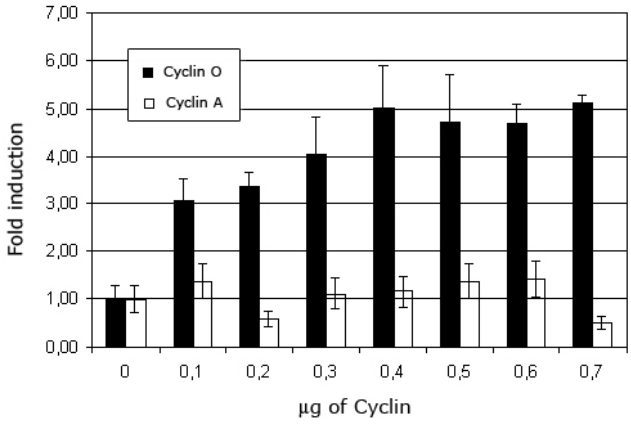


Figure 22. Cyclin O but not Cyclin A, increases c-Myb transcriptional activity in a reporter assay. HEK293 cells were transiently transfected with 0.75 µg of a reporter vector containing firefly luciferase under the control of c-Myb, 0.25 µg of a c-Myb expressing vector, 0.1µg of Renilla Luciferase to normalize transfection efficiencies, and variable amounts of a vector expression either Cyclin A or Cyclin O. The total amount of DNA transfected was adjusted to the same value using a mock vector. The transfections were done in triplicate and 48 hours after transfection, luciferase activity was measured using Dual Luciferase Assay System™. The level of firefly luciferase expression was normalized to Renilla activity and plotted the mean of the triplicates relative to the activity without Cyclin. Error bars correspond to the standard deviation of the mean.

To detect if Cyclin O is able to induce a specific phosphorylation on c-Myb *in vivo*, we took advantage of the fact that phosphate groups sometimes induce a change in the protein mobility in denaturing polyacrilamide gel electrophoresis. Moreover, electrophoretic mobility shifts due phosphorylation have been reported for c-Myb (130). We transiently transfected HEK293 cells with c-Myb alone or together with Cyclin O or A. As seen in Figure 23, c-Myb protein mobility is slower when Cyclin O is transfected, suggesting a phophorylation event. Interestingly, this shift is not induced with the transfection of Cyclin A. This data is in agreement of the reporter assays and together suggest that Cyclin O effect is specific.

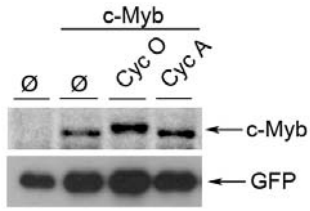


Figure 23. Cyclin O induces a shift in c-Myb protein. HEK293 cells were transiently transfected with a mock vector (Lane 1), with c-Myb (Lane 2) or with c-Myb in conjunction with Cylin O (Lane 3) or Cyclin A (Lane 3). To assess the trasfection efficiencies, the same amount of GFP expression vector was transfected in all conditions. 48 hours after transfection cells were analyzed by western blot for the presence of the c-Myb protein and GFP.

c-Myb protein has been reported extensively to be regulated by phosphorylation. Several stress-induced proline-directed kinases have been shown to phosphorylate c-Myb and regulate its transcriptional activity and/or its stability (130;131). Interestingly, proline-directed kinases have the same substrate requirements as Cdks. That is, both phosphorylate Serine and Threonine residues followed by a Proline. This fact made us speculate that the final result of phosphorylation could be the same independently of the kinase responsible of the phosphorylation. Since it has been described that those kinases mainly induce c-Myb degradation, we hypothesized that Cyclin O could mediate the same effect. To analyze that, we used the shRNA clones of the WEHI7.2 cell line we have previously described (Figure 11). If Cyclin O induces c-Myb degradation, then, in the clone that does not express Cyclin O c-Myb would have a higher half-life. We subjected one control (G1) and one shCycO (3.7) to 10Gy of γ-radiation in order to induce Cyclin O. As shown in Figure 24, only the clone that express Cyclin O downregulates c-Myb. Interestingly, c-Myb degradation becomes apparent 6 hours after stimulus, just at the same moment that we detect Cyclin O associated kinase activity (Figure 11B).

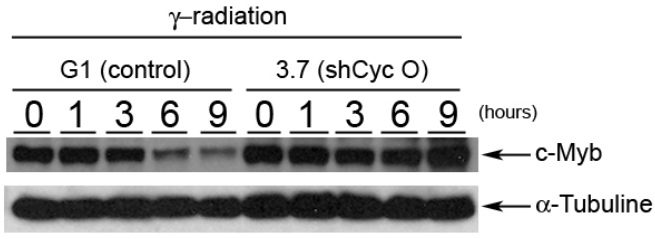


Figure 24. Cyclin O deficient cells do not downregulate c-Myb after genotoxic stress. WEHI7.2 cell clones expressing an shRNA agains Cyclin O (3.7) or against GFP (G1) were subjected to 10Gy of γ-radiation. At the indicated times, cells were collected and analyzed by western blot for the presence of c-Myb protein. As a loading control, the levels of α-tubuline were analyzed in the same samples.

V. DISCUSSION

1. Thymocytes as a model

The role of Cdks in cell cycle is robustly established based on compelling data from genetic and biochemical approaches. However their precise role in apoptosis has been less forthcoming. The main problem for the study of Cdks in apoptosis is the high activity present in cells due to their role in cell cycle that makes difficult to distinguish Cdk activity related to cell cycle regulation from additional activation consequence of apoptosis signalling. Moreover, this also limits the use of chemical Cdk inhibitors since blocking Cdks in proliferating cells induces apoptosis very rapidly due to cell cycle block and checkpoint activation (132). This apparent contradiction was addressed by Park and colleagues (133). They used the neuronal cell line PC12 and tested the effect of Olomoucine and Flavopiridol (two classical Cdk inhibitors) upon apoptosis when the cells were either differentiated or in proliferation. They reported that PC12 cells in proliferation were not protected from apoptosis by Cdk inhibitors, and in fact promoted apoptosis. By contrast, when cells were cultured in differentiation conditions and become post-mitotic, Cdk inhibitors protected cells from apoptosis. This fact pointed to quiescent cells as the best system for study the role of Cdks in apoptosis. The most often used quiescent systems are neuronal cells and thymocytes. By far those systems provided the clearest examples of the active role that Cdks play during apoptosis (134). The large number of papers reporting a role of Cdks in apoptosis, their role is continuously challenged because of the lack of a signalling pathway. Probably in neuronal tissues is where its participation is more accepted. This is due to the fact that the Cdk involved in this process is most likely Cdk5, a Cdk that does not participate in cell cycle and that is activated by a non-Cyclin protein(135).

Thymocyes are a naturally-occurring synchronized population since 90% of the cells are quiescent. In this system several independent laboratories reported that Cdk2 is activated during apoptosis (107;113;136). Previous results from our group 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 as expected for a quiescent tissue, and the Cdk2 kinase activity detected upon apoptosis induction is not associated with Cyclin A and E (107).

An *In silico* analysis of the whole genome was carried out in our laboratory in order to identify new Cyclins and lead to the identification of few candidates. Preliminary work from other members of the group showed that only Cyclin O mRNA was induced during thymocyte apoptosis. For that reason we have characterised Cyclin O as a candidate to be the so-called Apoptosis Related Cdk2 Activator.

2. Expression pattern of endogenous Cyclin O

Since Cyclin O has not been described before, we decided to analyze its expression in mouse tissues by RT-PCR. The first conclusion we got from this experiment is that the expression levels of Cyclin O mRNA in normal conditions are low in most tissues. 40 cycles of PCR are necessary to detect the mRNA of Cyclin O whereas for HRPT 30 cycles are enough. This means that Cyclin O is roughly 1000 times less abundant than HPRT. Despite the mRNA levels are low we can detect its expression in several tissues including brain, liver, intestine, testis, kidney, heart and ovary but not in thymus and spleen. What comes out at first glance is that the highest expression is detected in testis and brain. However, we have not further characterized the physiology of its expression in these tissues.

In order to detect the endogenous protein we have generated four different antibodies against Cyclin O. We have spent a lot of time and effort demonstrating the specificity of our antibodies. Nevertheless, this work has been hard since the levels of expression of the endogenous protein are extremely low, almost at the limit of detection both by immunohistochemistry and western blot.

By means of western blot we have been unable to detect the endogenous protein in extracts from mouse tissues even after cell fractioning (data not shown). However, we were able to detect the protein in the cytosolic fraction of immortalized cells only when using the highly sensitive western blot developing system SuperSignal. Remarkably, we can not detect endogenous Cyclin O in whole cell extracts of any cell line checked with any of the four antibodies we generated despite they all detect the transfected protein efficiently. This also suggests that the levels of expression are below the limit of detection.

We also analyzed the pattern of expression of Cyclin O in normal mouse tissues by immunohistochemistry. We analyzed most mouse tissues and the results we obtained were similar to those we got by RT-PCR. However, this technique, is more informative because gives you information about the cell types which express the protein and about its subcellular distribution. However, although Cyclin O is expressed in several tissues, we don't know if in those conditions forms active complexes with Cdk2. It is possible that although Cyclin O is expressed, it may have no activity due to additional regulatory events. In that sense, Cdks have several levels of regulation, including phosphorylations, association to specific inhibitory proteins, etc.

In most tissues and in cell lines Cyclin O expression is cytoplasmatic. This is in agreement with the subcellular fractioning data where we only detect the protein in the cytoplasmatic fraction. This observation was striking since most Cdk-Cyclin complexes develop their function in the nucleus and Cyclin O has a consensus Nuclear Localization Signal (NLS) at the N-terminal domain (results Figure 1). We though that maybe after treatment with an apoptotic stimulus,

Cyclin O could relocalize to the nucleus. The immunohistochemistry results in thymus and in WEHI7.2 cells clearly show that after apoptotic stimuli the protein is exclusively localized in the cytoplasm. This is very interesting since it suggests that Cyclin O may be doing something completely different to the other Cyclins described so far. Moreover, it may provide a direct link to the apoptotic cascade since the central players in the intrinsic apoptosis cascade, Mitochondria and Caspases, are localized in the cytoplasm. Additionally it also suggests that the consensus NLS of Cyclin O either is not an NLS or is somehow inhibited.

The pattern of expression in the cytoplasm is punctated in all the cells analyzed. This fact clearly suggests that the protein may be associated with a subcellular structure. However, its identity and functional relevance is currently under investigation.

We have also analyzed the expression of Cyclin O in developing embryos. The protein is expressed during mouse development and its expression does not correlate either with cell cycle markers such as Ki67 or phospho-Histone H3 or with apoptosis markers such as active Caspase-3 (data not shown). Together with the constitutive expression in some adult tissues, this suggests that its function is not linked to cell cycle and may not always be related to apoptosis regulation, at least in some tissues.

3. Cdk promiscuity

Although Cyclin O is able to bind and activate Cdk1, Cdk2 and Cdk5 (results Figure 8C), Cdk2 is the preferred partner in cells expressing all three kinases, like MEFs or HEK293 cells. Interestingly, in the absence of Cdk2, only Cdk1 binds and gets activated by Cyclin O. In the case of the mouse brain, where Cdk1 and Cdk2 are not expressed (results Figure 8B), Cdk5 takes over their role and forms complexes with Cyclin O. It is of note that in the co-immunoprecipitation experiments, we were never able to detect Cdk5-Cyclin O complexes (results Figure 7A), suggesting that the affinity of Cyclin O for Cdk5 is much lower than for Cdk1 and Cdk2. These results suggest that Cdk2 is the preferred partner of Cyclin O *in vivo* and additionally, that in the presence of Cdk1 and Cdk2, Cyclin O never binds Cdk5.

Thymocytes from Cdk2 knockout mice show defects neither in hematopoiesis nor in thymic apoptosis (136). This observation was striking since we have found that Cdk2 is necessary for thymocyte apoptosis. However, Cdk2 knockout mice are viable and only show defects in germ cell development and both male and female $Cdk2^{-/-}$ mice are sterile (137;138). Given the important role described for Cdk2 during cell cycle, this suggests that another protein is compensating for Cdk2 loss. This protein is likely to be Cdk1 for a couple of reasons. First, Cdk1 is the only Cdk necessary to drive a complete cycle of cell division (139) and second, Cdk1 can associate with Cyclin A and E leading to active complexes (140). Moreover, we have demonstrated that Cdk1 is also able to interact with Cyclin O and form active complexes (results Figure 8C). Then we propose that Cdk1 compensates Cdk2 loss in thymocyte apoptosis. Accordingly, apoptosis of $Cdk2^{-/-}$ thymocytes can be rescued by roscovitine as in Cdk2 wild type cells (136). At the concentrations used, roscovitine only inhibits Cdk1, 2, 5, 7 and 9 (123). Since Cyclin O does not form active complexes with Cdk7 and 9 and only interacts with Cdk5 if Cdk1 is absent (results Figure 8C) this strongly suggests that Cyclin O function is not altered in $Cdk2^{-/-}$ thymocytes because of its ability to interact with and activate Cdk1. In thymocytes from wild-type mice, Cdk1 is not activated during apoptosis induction. This fact can be explained because Cdk2 is the preferred partner of Cyclin O when all Cdks are present. However, in the Cdk2 knockout mice we would expect an activation of Cdk1 during thymocyte apoptosis due to Cyclin O induction. However, Cdk1 activation during apoptosis of $Cdk2^{-/-}$ thymocytes has not been described so far.

Another important finding is the fact that Cyclin O can associate with and activate Cdk5. However Cyclin O-Cdk5 interaction is only detected when Cdk1 and 2 are not present. For that reason we think that this interaction could be relevant in brain, were Cdk5 levels are high and Cdk1 and 2 are not expressed.

Although Cdk5 has been implicated in cellular processes in different cell types (141), it is in neuronal cells where it plays the major role. Probably the strongest evidences for that fact come from *cdk5* gene disruption in mice, which results in neuronal abnormalities and perinatal death (142).

Interestingly, Cdk5 has a dual role in brain physiology, either being neuroprotective or promoting cell death. This duality seems to be dependent on the activator subunit. The usual activator of Cdk5 is a non-Cyclin protein called p35. p35-Cdk5 complexes are abundantly found in adult brain and Cdk5 activity increases in neurons during development. When Cdk5 is activated by p35 it has a neuroprotective effect. However, p35 can undergo a proteolytic cleavage leading to p25. This product is more stable than p35 and binds tighter to Cdk5, leading to hyperactive p25-Cdk5 complex with a mislocation (135). Interestingly, the abnormal accumulation of p25 occurs as a result of the proteolytic cleavage of p35 by Calpain, a calcium-dependent protease which is activated in Alzheimer disease brains (143). Cdk5 hyperactivation contributes to Alzheimer disease at least by two mechanisms: first, direct phosphorylation of Tau that leads to the formation of intracellular aggregates (144), and second by activation of the Rb-pathway which in neurons is proapoptotic (106).

Given the fact that the highest levels of Cyclin O expression are found in brain, it is likely that Cyclin O may play a role in neurons. Since Cdk1 and 2 are not expressed and Cyclin O can activate Cdk5 *in vitro*, it is reasonable to think that in neuronal tissues Cyclin O mediates its function associated with Cdk5. However, it should be demonstrated that this association exists *in vivo* and that is productive (i.e. that leads to Cdk5 activation). A Cdk-independent function is improbable since all Cyclins mediate its function by association with Cdks and, *in vivo*, Cyclins are always found associated with its Cdk partner because otherwise are targeted for proteasome degradation.

Apart from its function in normal brain it would be interesting to analyze if Cyclin O participates in the pathology of neurodegenerative disorders. In that sense it would be interesting to analyze if Cyclin O participates in the hyperactivation of Cdk5 in patients of Alzheimer disease and if Cyclin O-Cdk5 complexes also phosphorylate Tau. Given the proapoptotic functions we reported for Cyclin O in lymphoid cells it is possible that could also mediate proapoptotic functions in brain and, thus, abnormal regulation of Cyclin O could account for neurodegenerative disorders.

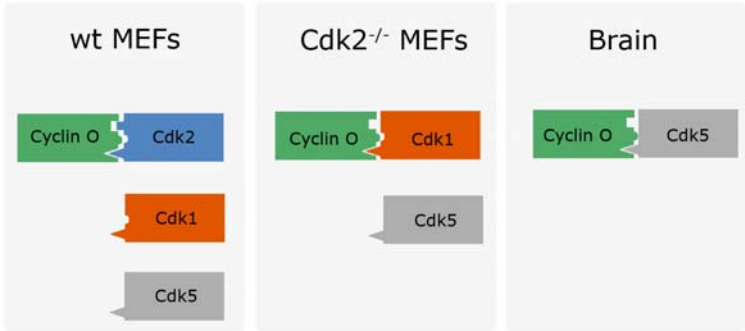


Figure 1. Cyclin O binding preferences. In wild type MEFs, where all Cdks are present, Cyclin O preferentially binds and activates Cdk2. In cells from Cdk2 knockout mice, Cyclin O binds to Cdk1 and in neuronal cells, where Cdk1 and Cdk2 are not expressed, Cyclin O binds to Cdk5.

4. Regulation of Cyclin O expression

The expression of Cyclin O is rapidly elevated after irradiation of thymocytes, splenocytes and mouse embryonic fibroblasts, reaching a peak at 3 hours (results Figure 9A and data not shown). Interestingly, this peak of expression is previous to apoptosis induction. The same conclusion is reached from the study of the kinetics of Cyclin O induction after etoposide or glucocorticoid-induced apoptosis in WEHI7.2 cells. Thus, the kinetics of Cyclin O expression after apoptosis induction are just what we would expect from a protein that has a causal role in apoptosis, a role that was confirmed with the use of the siRNA technology in WEHI7.2 cells, EL4 cells and MEFs.

Since Cyclin O is induced by glucocorticoids, it is possible that Cyclin O could be a direct target of the Glucocorticoid Receptor (GR). GR is a transcription factor that binds to Glucocorticoid Responsive Elements (GRE), a specific DNA sequence consisting of an inverted repeat separated by three nucleotides: GGTACANNNTGTTCT (145). The scanning of Cyclin O promoter revealed the presence of these boxes, thus suggesting that Cyclin O could be a direct target of GR.

In addition, Cyclin O is also induced after DNA-damage. Although the pathway activated by genotoxic agents is complex, the transcriptional response associated to DNA-damage is mediated basically by p53. p53 can act as a transcription factor binding to specific sequences (p53RE). p53RE consist in a tandem repeat of 10nt separated by a variable number of nucleotides ranging for 0 to 13 with a consensus sequence of 5'-RRRCWWGYYY N(1-13) RRRCWWGYYY ', where W can be A or T, and R and Y stand for purine and pyrimidine bases, respectively (146). The presence of these boxes in the Cyclin O promoter also suggests that Cyclin O could be a direct target of p53. In addition, experiments carried out by other members of the lab showed that MEFs from p53 knockout mice do not show Cyclin O induction after irradiation, thus suggesting that Cyclin O could be a direct target of p53.

4.1. Cyclin O expression is regulated by Bcl-2 and Bax

Previous work of our lab, positioned biochemically the activation of Cdk2 in DNA damage and glucocorticoid-induced thymocyte apoptosis as an early step preceding phosphatidylserine exposure, mitochondrial dysfunction and Caspase activation (117). The results presented in this memory show that Cyclin O downregulation leads to a complete apoptosis block that is characterized by a lack of phosphatidylserine exposure and mitochondrial dysfunction and a total absence of activation of the executioner Caspase, Caspase-3. In addition to this, the kinetics of induction of Cyclin O in the thymus after γ-irradiation of whole mice is initially not significantly affected by the transgenic expression of Bcl-2 or Bax (results Figure 9A), indicating that its action lies upstream of the mitochondrial action of the Bcl-2 family proteins. However, the magnitude of induction of the Cyclin O mRNA, measured by qRT-PCR, is increased by

transgenic expression of Bax and diminished by Bcl-2. This is in agreement with previous data showing that Bax overexpression in thymocytes leads to increased Cdk2 activity during apoptosis and Bcl-2 overexpression leads to the opposite effect. This effect was initially attributed to the levels of the Cdk inhibitor $p27^{Kip1}$. It was reported that Bax induced a rapid degradation of $p27^{Kip1}$, having Bcl-2 the opposite effect. Nevertheless, this can only explain that Cdk2 kinase activity appears earlier but not why it reaches higher intensity. In fact this can be explained because transgenic overexpression of Bax also induces a higher expression of Cyclin O mRNA.

The fact that Bcl-2 not only inhibits apoptosis but it has a role in cell cycle was shown by analyzing the phenotype of cells overexpressing the protein and in transgenic animals. From both systems, the same conclusions were reached: Bcl-2 overexpression delayed cell cycle entry whereas Bax speed it up (118). The effect was mapped at the level of regulation the stability of the Cdk inhibitor $p27^{Kip1}$(115;147). It was shown that Bcl-2 somehow stabilized the protein leading to a more pronounced Cdk inhibition and thus a delayed cell cycle entry. Despite those effects were identified more that 10 years ago the mechanism by which Bcl-2/Bax modulate $p27^{Kip1}$ levels are still not clear. It has been described that $p27^{Kip1}$ is a direct target of caspases (114), and caspase activation is regulated by the Bcl-2 family of proteins. Therefore, cells overexpressing Bax that are more prone to apoptosis, would have a higher and earlier caspase activity that will lead to $p27^{Kip1}$ degradation; Bcl-2 overexpression, on the other hand, would have the opposite effect, that is, a slower degradation of $p27^{Kip1}$ as a result of slower kinetics of apoptosis. However, since $p27^{Kip1}$ degradation is previous to mitochondrial permeabilization during thymocyte apoptosis, this mechanism could only explain a positive feedback loop but not the initial degradation. In addition, the effects of Bcl-2/Bax on cell cycle can not be explained by caspase activation.

In conclusion, the fact that Bcl-2/Bax modulate both Cdk2 activity and Cyclin O levels in the same direction, strongly supports the hypothesis that Cyclin O is the Apoptosis-Related Cdk2 Activator protein that we predicted for thymocytes to be necessary for apoptosis induced by intrinsic stimuli.

5. Cyclin O is only involved in the intrinsic apoptotic pathway

Previous results from our group have shown that Cdk2 does not participate in all apoptotic pathways. In thymocytes, Cdk2 has been show to participate in the intrinsic apoptotic pathway initiated by genotoxic agents and glucocorticoids. In addition, Cdk2 also mediates antigen-induced apoptosis during thymocyte negative selection (116). Despite the fact that relatively little is known about the specific apoptotic pathway involved in negative selection it is clear that it mediates apoptosis through the intrinsic apoptotic pathway. In contrast, Cdk2 is not activated by the extrinsic apoptotic pathway and Cdk inhibitors do not block apoptosis induced by Fas agonistic antibodies or treatment with FasL (117). In agreement with these data, activation of the Fas pathway in WEHI7.2 cells does not induce Cyclin O mRNA (data not shown). In addition to this, downregulation of Cyclin O in WEHI7.2 leads to a complete block of apoptosis induced by intrinsic apoptotic stimuli, whereas the extrinsic pathway mediated by Fas is intact. We demonstrate the integrity of this pathway in clones with dowregulated Cyclin O with two different approaches. First we analyzed biochemically the initial steps of the pathway and we showed that Caspase-8 is activated and its target Bid is cleaved similarly to the control clone. And second, we demonstrate that the final outcome, apoptosis, is also indistinguishable from control clones.

Fas-induced apoptosis is completely independent of protein synthesis. In fact, it is necessary to add a protein synthesis inhibitor (cycloheximide) in order to uncover the proapoptotic action of the anti-Fas antibody. FAS pathway also leads to the activation of the ERK, JNK, p38MAPK and NF-κB pathways (41), although the molecular connections are not completely understood. Since all these pro-survival pathways mediate their effects by gene transcription, cycloheximide completely inhibits all of them. It could be argued that, since Cyclin O is induced by proapoptotic stimuli, the addition of cycloheximide would avoid Cyclin O expression and this would explain why cells with or without Cyclin O behave indistinguishable in response to Fas activation. In other words, since we add cycloheximide, cells will not be able to induce Cyclin O. However, since Cdk2 protein is constitutively present and Cyclin O is not induced at the RNA level by this stimulus (process not affected by cycloheximide), we conclude that Cyclin O does not affect the extrinsic apoptotic pathway.

Probably the strongest evidence showing that Cyclin O has a major role in apoptosis came from the use of the shRNA technique in WEHI7.2 clones. The suitability of this cell line was assessed because it shows a similar behavior to thymocytes in response to apoptotic stimuli. In addition, the regulation of Cyclin O expression follows a similar pattern. In order to avoid unspecific effects we used two different shRNAs against Cyclin O and two different controls. It is of note that clones bearing empty vector or expressing an shRNA against GFP behaved indistinguishably of the parental cell line in

response to apoptotic stimuli. However, clones isolated with an shRNA against Cyclin O, independently of the construction used, were completely resistant to apoptosis induced by intrinsic stimuli. To assess the effectiveness of Cyclin O downregulation as a consequence of the shRNA expression, we have done two independent experiments: on one hand we have detected the expression of Cyclin O after induction by immunohistochemistry with the N1 antibody, and on the other hand we have analyzed the kinase activity of Cyclin O immunoprecipitates using the C2 antibody. With the use of two independent techniques and two independent antibodies, both experiments lead to the same conclusion: shRNA is effectively working. Unfortunately, despite the efforts, we were unable to detect Cyclin O by western blot in this cell line probably due to its low expression levels.

Given the importance of the findings derived from the WEHI7.2 shRNA clones we thought that it would be necessary to further characterize the lack of response of the clones to the apoptotic stimuli. First of all we characterized the response to DNA-damage. Cells respond to DNA damage with an integrated response called DNA-damage response (DDR) that includes activation of DNA-repair mechanisms and activation of cell cycle checkpoints. In the DDR contribute several proteins that can be divided in sensors, transducers and effectors. Given the huge number of proteins involved in this pathway we analyzed some of the most relevant proteins of each step. We analyzed the activation of the sensor protein ATM, the transducer protein Chk1 and several effector proteins (p53, Cdc25A, Cdc25C and $p21^{Cip1}$) (results Figure 14A). None of these steps were affected by downregulation of Cyclin O. This means that cells detected DNA breaks and activated the DDR effectively leading to checkpoint activation. In other words, Cyclin O is not in the pathway leading to checkpoint activation.

Another proof that checkpoints were not affected was found by measuring the DNA content by flow cytometry. Both control and shRNA clones show a complete cell cycle arrest in all the phases after γ-radiation (results Figure 14B). The only difference is the presence of a sub-diploid apoptotic peak in the control clone indicating that after checkpoint activation, cells activated apoptosis. Altogether those results suggest that we have not isolated a clone with defective DDR and that the effect of knocking down Cyclin O only abolishes the apoptotic branch of the response.

DNA-damage is thought to mediate apoptosis through the activation of p53 and subsequent transcription of proapoptotic genes. We show here that one target gene critical for the response to γ-radiation, $p21^{Cip1}$, is induced in both clones, suggesting that the p53 transcriptional activity is intact. However p53 has a dual function during checkpoint activation. On one hand it can induce cell cycle arrest genes like $p21^{Cip1}$, but on the other hand it can induce proapoptotic genes such as Noxa and Puma. How p53 is instructed to favor one response or the other is not well understood. It could be possible that Cyclin O was necessary for the activation of the proapoptotic response of p53. In that sense it would have been interesting to check if the major proapoptotic

targets of p53 are induced in cells with downregulated Cyclin O. However since those clones are also resistant to glucocorticoids and these hormones do not mediate their effect via p53, it is reasonable to think that Cyclin O does not modify p53 activity, and instead acts via a common step with the glucocorticoid pathway.

To rule out the possibility that the clones we obtained were intrinsically resistant to glucocorticoids due to a defect in the glucocorticoid receptor, we made a similar approach. Resistance to glucocorticoids is generally associated with defects in the receptor. In addition, several approaches that have been done in order to isolate cells resistant to glucocorticoids, revealed that the most common adaptation was a primary defect in the gucocorticoid receptor (148).
In order to demonstrate that the Glucocorticoid receptor is functional in the clones we isolated, we analyzed the expression of one of its best characterized target genes, Bim. Among the proapoptotic targets of the Glucocorticoid Receptor, Bim is the most broadly accepted since it has been described in several cell lines as well as in thymocytes (24). Bim is a proapoptotic BH3-only member of the Bcl-2 family of proteins. Together with Puma, they are the only proapoptotic proteins that are necessary, at least to some extend, for glucocorticoid-mediated apoptosis (91).

As it is shown in results Figure 15A, Bim is induced in both clones with a similar kinetics. However, activation of Caspase 3 and apoptosis only occurs in the control clone. These results indicate that Bim induction is not sufficient for glucocorticoid-induced apoptosis. Several reports including knockout in mice (91;149) and knockdown in cells (150) show that Bim is necessary for glucocorticoid-induced apoptosis. Altogether those results suggest that Bim has additional mechanisms of regulation. Interestingly, Bim is expressed in many cell types, but normally it is sequestered by the microtubular dynein motor complex by interaction with dynein-light chain LC8 (28). Apoptotic stimuli provoke release of Bim from LC8, allowing Bim to associate with Bcl-2-like proteins. The mechanism by which Bim is released is currently unknown. It is likely that in the shRNA clones Bim is not released from microtubules and thus can not mediate its proapoptotic effect.

6. Mapping Cdk2-Cyclin O effect in the apoptotic pathway

The biochemical events leading to apoptosis have been broadly studied in thymocytes and the post-mitochondrial biochemical steps leading to apoptosis are well characterized: formation of the apoptosome, Caspase-3 activation, etc. However, little is know about the upstream steps that lead to mitochondrial dysfunction as a consequence of signalling by intrinsic apoptotic stimuli. In fact, it is generally assumed that apoptotic stimuli induce BH3-only proteins and they are responsible for the mitochondrial permeabilization and subsequent activation of Caspase-3. In fact, DNA-damage induces the BH3-only proteins Puma and Noxa (151) and glucocorticoids induce Bim (24). However, this scheme has been challenged by several observations. First, induction of BH3-only molecules is not sufficient to induce apoptosis (as we have seen with Bim); second, knockout of the BH3-only proteins only leads to partial protection from apoptosis (29); and third, there is Caspase activity before mitochondrial permeabilization (117;152).

The detection of Caspase-8 activation before mitochondrial dysfunction can be monitored by two independent ways. First, Caspase-8 specific inhibitors block mitochondrial permeabilization, and second a time course analysis shows that Caspase-8 activation occur previously to mitochondrial permeabilization (117). Altogether these results suggest an scheme in which apical Caspase activation is an early event that contributes to mitochondrial dysfunction.

When we analyzed where was the positioning of Cdk2 in this scheme, we found that Cdk2 activity was necessary just upstream the mitochondrial events and before phosphatidylserine exposure. In addition, chemical inhibition of Cdk2 abolished Caspase-8 activation during thymocyte apoptosis induced by intrinsic stimuli. In WEHI7.2 cells where we have downregulated Cyclin O, the apoptosis signal is also blocked before mitochondrial dysfunction and phosphatidylserine exposure. In other words, blocking Cdk2 by means of drugs or by targeting its activator (Cyclin O) leads to the same effect.

An interesting question to address is identifying which are the direct targets of Cyclin O-Cdk2 complexes. In our scheme we propose that Cdk2-Cyclin O precedes Caspase-8 activation. However, the mechanisms leading to Caspase-8 activation can not be direct because Caspases are not activated by phosphorylation. Indeed, phosphorylations reported for Caspases impair their activation (153). Our hypothesis is that Cyclin O-Cdk2 complexes induce Caspase 8 activation through regulation of a Caspase-8 activating platform.

There are at least five different platforms for the activation of apical caspases (Figure 2) of whom the best charachacterized are apoptosome and DISC. The other platforms are inflamosome, PIDDosome and Hippisome. All these macromolecular complexes have a central scaffold protein that characteristically possesses three domains: a region involved in ligand sensing, a domain driving oligomerization and a domain involved in caspase recruitment. The domain involved in caspase recruitment is either a DED or a

Caspase-Activation and Recruitment Domain (CARD). The presence of either of these domains renders caspase specificity since CARD does not interact with DED and vice versa. The multimerization process is triggered by the presence of a 'ligand' which are Cytochome *c*, FasL, ligand-binded NOD-Like Recepors (NLR), p53-induced with DD (PIDD) and Hip-1 for apoptosome, DISC, inflamosome, PIDDosome and Hippisome, respectively. The regulation of each pathway is quite different in each case. The inflamosome is activated by the presence of pathogens recognized by NOD receptors. The PIDDosome is activated upon DNA damage as a result of p53-driven PIDD transcription. PIDD then mediates the oligomerization of RAIDD and the activation of Caspase-2. The Hippisome on the other hand, is activated when Hip-1 is released form Hungtingtin protein interaction.

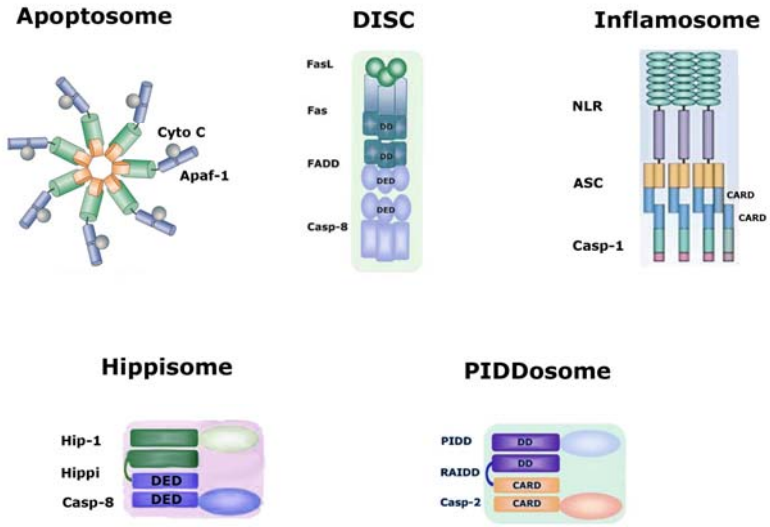


Figure 2. Apical caspase activating platforms. Five different apical caspase activating platforms have been described. Inflamosome mediates activation of inflamatory caspases like Caspase-1; it consists in a sensor protein of the NOD-Like Receptors (NLR) family and a central scaffolding protein, ASC, which recruits apical caspases through the CARD domain. Hippisome is formed by the central scaffolding protein Hippi that binds to Hip-1 and recruits Caspase-8 through DED-DED interactions. PIDDosome is assembled by RAIDD which with its Death Domain (DD) binds to the p53-induced protein with DD (PIDD) and with the CARD domain recruits Caspase-2. For apoptosome and DISC details see introduction section 1.3.

In our model of thymocyte apoptosis we have shown that after Cdk2 activation and before Caspase-8 activity, transcription and translation of new genes are necessary. This fact suggests that a newly synthesized protein would mediate the activation of Caspase-8, in a model similar to that of PIDDosome for Caspase-2. However, it is unlikely that this complex could mediate Caspase-8 activation since it recruits CARD domain containing Caspases and Caspase-8 does not have a CARD domain (Introduction Figure 1). Then, the unique candidates for Caspase-8 activation are DISC and Hippisome. Several evidences show that DISC is not involved in apoptosis mediated by the intrinsic stimuli (154). Therefore, either the Hippi or and still unidentified DED-containing 'aggresome' is the responsible of Caspase-8 activation during thymocyte apoptosis. Interestingly, Hippi has been shown to activate Caspase-8 during neuronal apoptosis (155). It would be interesting to analyze if Hippi of any DED-containing proteins is induced during thymocyte apoptosis.

Nevertheless, although the mechanism is not clear, we propose that Cyclin O-Cdk2 complexes activate apical Caspases. This is relevant in thymocyte apoptosis but it also could provide an explanation for the function of Cyclin O in other tissues where its expression is constitutive: activation of Caspases in a non-apoptotic scenario. In fact, Caspases are known to mediate other functions apart from apoptosis, like proliferation and differentiation (156). Noticeably, knockout of Caspase-8 is prenatally lethal due to impaired cardiac muscle development (157) and specific knockout in endothelial cells or in bone marrow leads to cell degeneration and block of differentiation (158).

An example of Cyclin O associated with Caspase activation is found in epidermis. The epidermis is a stratified squamous epithelium in which keratinocytes progressively undergo terminal differentiation towards the skin surface. It has been shown that during this process there is a precise activation of Caspases, particularly Caspase 14. Caspase 14 activation is associated with terminal epidermal differentiation (159). Interestingly, the expression of Cyclin O in skin follows the same pattern: increased expression in the upper layers (*stratum spinosum*).

7. Can a Cyclin modulate Cdk substrate specificity?

An important aspect of our hypothesis is that Cyclin O would induce different substrate specificity than Cyclin A or E to Cdk2. That is, Cdk2 bound to Cyclin O would recognize and thus phosphorylate different substrates than when bound to other Cyclins. This would explain how the same protein can drive either cell cycle progression or apoptosis.

The properties of Cdk/Cyclin complexes are modulated by multiple mechanisms to promote the correct timing of Cdk substrate phosphorylation during cell cycle. Although Cyclins were shown to activate Cdks many years ago, their contribution to substrate specificity is still controversial. There are two viewpoints on the question of functional specialization of Cyclins: Cyclins are just Cdk activators and their precise regulation (induction, degradation, subcellular distribution, etc.) dictate their role during cell cycle or, alternatively, it is proposed that Cyclin identity is essential for interaction with an appropriate CDK and for selection of targets. The former hypothesis is based on the observation that in fission yeast there is only one Cdk and the cell cycle can be achieved with only one Cyclin, Cdc13 (160). In higher eukaryotes although cell cycle can be completed with only Cdk1 (139), the higher number of Cyclins makes difficult to assess if only one Cyclin would be enough. However, there is accumulating evidence that in higher organisms, Cyclins play a role in differential substrate recognition. Fore example, Cyclin A-Cdk2 complexes phosphorylate a p107-derived peptide only in one of the two consensus sites whereas CyclinB-Cdk2 phosphorylate both (161). Additionally, sequences responsible for substrate binding have been identified in almost all Cyclins (162). Conversely, it is probable that Cyclin O contains specific motifs responsible for specific substrate recognition. The fact that Cyclin O induces an effect on c-Myb that can not be mimicked by Cyclin A (see below) favors this hypothesis.

8. Cyclin O mediated phosphorylation of c-Myb

In a screening seeking for proteins that interacted with Cdk2-Cyclin O complexes, we have identified c-Myb. We have found that this interaction is relevant since Cdk2-Cyclin O complexes phosphorylate c-Myb. *c-myb* is a proto-oncogene highly expressed in immature hematopoietic cells (126). The product of this gene, c-Myb, is a transcription factor that has been involved in cell cycle control, lineage commitment in differentiation and blockage of apoptosis (163). The key to control the protein function is post-translational modifications including, phosphorylation (130;131;164), ubiquitinilation (165), sumoylation (166) and acetylation (167). Among all these modifications, phosphorylation was described to influence both transcriptional activity and stability of the c-Myb protein. Interestingly, most of the kinases that regulate c-Myb are proline-directed protein kinases which have the same sequence requirements as Cdks. In that sense, several kinases have been found to phosphorylate c-Myb at multiple [S/T]P sites and induce its degradation via proteasome; namely, p42MAPK (168), Nemo-Like Kinase (131) and p38MAPKδ (130). Accordingly, other groups found that C-terminal truncation, that eliminates multiple [S/T]P sites, results in increased protein stability (165). Collectively, all these reports suggest that it is not the phosphorylation of a single site what targets c-Myb for degradation. kanei-ishii and colleagues (131) reported that all residues were important and that the total level of phosphorylation determined the final outcome. Since we have found that c-Myb is also a subtrate of Cdk2-Cyclin O complexes, in light of these reports it seems that Cyclin O-Cdk2 will function just adding more phosphates to the c-Myb protein and perhaps leading to degradation.

It is important to stress out that c-Myb has a general antiapoptotic effect in lymphoid cells and interfering with its function with a dominant negative mutant causes apoptosis in T cells (169). This effect has been proposed to be mediated by direct transactivation of *bcl-2* (170;171). Altogether these results suggest that c-myb downregulation will be necessary for apoptosis. Interestingly, we have found that c-myb is downregulated not only at the level of protein that but also at the level of transcription (Results Figure 20) during thymocyte apoptosis, thus contributing to eliminate the protein. Altogether those results support a model were c-Myb is sensing the balance between apoptosis and cell survival. In response to stress, several stress-specific kinases are activated and phosphorylate c-Myb at several sites contributing to its degradation and thus decanting the balance to apoptosis.

An important issue to be addressed regarding the role of Cyclin O over c-Myb is the subcellular localization of both proteins. In most of the tissues analyzed so far, although not in all, Cyclin O shows a clear cytoplasmic localization pattern. Moreover, in thymocytes and in lymphoid cells where Cyclin O is clearly inducible and proapoptotic, it also seems that the protein localizes in the cytoplasm (Results Figure 9B, 11A). Then the question is how a cytoplasmatic protein can phosphorylate *in vivo* a transcription factor that resides in the nucleus? What we think is that Cyclin O-Cdk2 complexes shuttle between

nucleus and cytoplasm and the efficiency of the export is higher that the import, resulting in a net accumulation of the protein in the cytoplasm. In agreement with that, Cyclin O has conserved consensus sequences of nuclear localization and export signals. It would be interesting to analyze if the nuclear import and export pathways regulate the subcellular localization of Cyclin O.

Another interesting point is what could happen in tissues where Cyclin O expression is nuclear. In that sense we have observed nuclear expression of Cyclin O in colon epithelial cells (data not shown). Interestingly *c-myb* is also expressed in these cells and seems to play a role in the differentiation process (172). It would be interesting to analyze the function of Cyclin O in colon and its possible relationship with c-Myb.

9. Concluding remarks

Induction of apoptosis by unscheduled activation or expression of cell cycle regulators such as Cyclins or Cdks has been extensively described (134). Inappropriate activation of a Cdk such as Cdk1 perturbs the normal cell cycle progression leading to checkpoint activation and apoptosis induction (173). 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 (174). However, these cases are based on the forced expression 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 signaling of the intrinsic apoptotic stimuli. Its normal expression precedes apoptosis induction and if it is downregulated, apoptosis cannot take place. This is entirely different to the situation where overexpression of the Cyclin or Cdks in special conditions induced perturbations in otherwise normal processes such as the cell cycle. We propose Cyclin O to be a novel apoptosis-specific regulator of Cdk activity during thymocyte apoptosis. It is of note that all the effects reported by Cdk2 inhibition in thymocytes, are reproduced by downregulation of Cyclin O: pattern of induction, regulation by Bax and Bcl-2, positioning upstream mitochondria and finally, block of lymphoid-cell apoptosis.

We also provide some clues about the mechanistic function of Cyclin O. We show here that Cyclin O can induce substrate specificity to Cdk2 and thus it could explain the dualistic role of Cdk2: cell cycle control or apoptosis. Additionally we identify c-Myb as a substrate of Cdk2-Cyclin O complexes and although the work done on this regard is preliminary it suggests that Cyclin O-Cdk2 could induce its degradation thus leading to changes in the transcriptome that would contribute to apoptosis.

In addition, our observations in thymocytes suggested that Cdk2 induces the activation of Caspase-8. However, we do not know which is the precise mechanism but could be mediated by DED-containing proteins. Nevertheless,

we propose that Cyclin O-Cdk2 complexes mediate their effect by activation of apical caspases.

In conclusion, we propose Cyclin O-Cdk2 as an apical Caspase activating complex that induces apoptosis in lymphoid cells. The precise positioning within the apoptotic pathway is described in Figure 3 and based on data from this thesis together with the data published by Granés *et al*.(117).

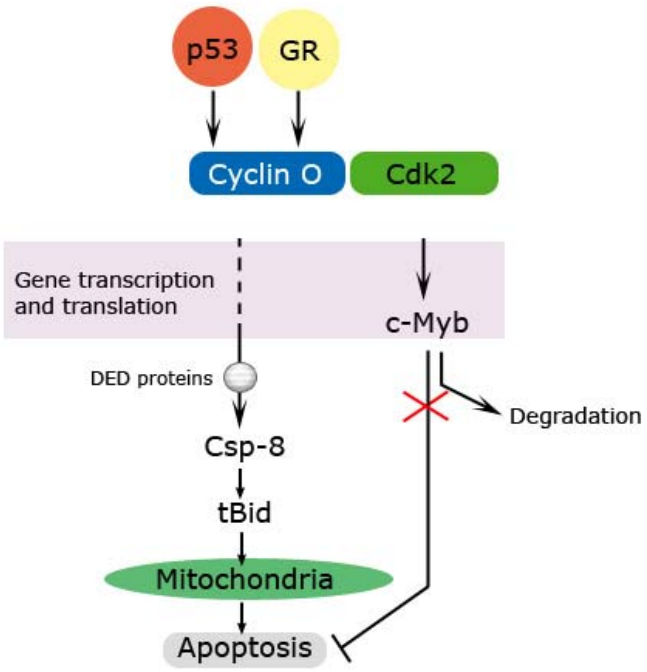


Figure 3. Model of Cyclin O action during thymocyte apoptosis. DNA-damage and glucocorticois induce Cyclin O expression through the p53 and GR transcription factors respectively. Cyclin O activates Cdk2 which is then able to phosphorylate the prosurvival protein c-Myb and thus, induce its degradation. Cdk2 activity is also required for Caspase-8 activation in a process that requires gene transcription and translation. Caspase-8, which is activated by DED-proteins, cleaves Bid and then activates the mitochondrial pathway that leads to apoptosis.

VI. CONCLUSIONS

With the results presented in this thesis we can conclude that:

1. Cyclin O is expressed constitutively in some tissues (brain, testis, gut, etc.) and in others (spleen and thymus) is induced by proapoptotic stilumi.

2. Cyclin O binds and activates Cdk2 and Cdk1.

3. Cyclin O is induced by proapoptotic stimuli in lymphoid cells and tissues.

4. Cyclin O downregulation abrogates caspase activation and apoptosis in lymphoid cells.

5. Cyclin O – Cdk2 complexes phosphorylate c-Myb protein.

6. c-Myb protein and RNA are dowregulated during apoptosis of lymphoid cells.

VII. ACKNOWLEDGEMENTS*

* The acknowledgements are written in Catalan, my mother tongue, in order to thank with more closeness all the people that helped me to finish this thesis.

Quan hom s'embranca en fer una tesi doctoral, es decideix a passar almenys quatre anys de la seva vida en un determinat laboratori i amb una determinada gent. En el meu cas això ha fet que es formessin uns lligams especials amb molta gent del laboratori i de l'entorn. He de dir que he viscut uns moments molt agradables i que el dia a dia ha estat excel·lent. Agraeixo sincerament l'amistat i l'ajuda que m'han lliurat tots els membres presents i passats de la ja desapareguda UBCM, començant pel seu ex-director i acabant per aquells estudiants que només hi són tres mesos. Gràcies a tots.

M'agradaria mencionar només les persones que han contribuït més directament en la consecució d'aquesta tesi ja que han participat directament en alguns experiments.

Primer de tot agraeixo al Doctor Gabriel Gil Gómez que m'hagi deixat gastar diners dels seus projectes per tal d'aprendre a investigar. També li agraeixo sincerament que m'hagi ensenyat ell mateix a pipetejar, a fer digestions i totes aquestes cosetes. Aquest aprenentatge ha estat fonamental per tal d'afrontar la tesi. Gràcies, doncs.

En segon lloc, agraeixo al senyor Maurici, un treballador infatigable, un bon amic i un bon mestre, per tot el que m'ha ensenyat i explicat.

Agraeixo també l'esforç de tots els membres del grup on plegats hem estat lluitant contra la Ciclina en qüestió. La Laia i els seus assajos de *pull-down*, l'Andrea i els seus clons, la Laura i les seves transfeccions i la Marta amb les seves immunos. La seva feina ha estat fonamental per entendre aquesta Ciclina.

També voldria agrair als companys de laboratori més propers que m'han ajudat a fer més suportables els divendres i més insuportables els dissabtes al matí. Doneu-vos per al·ludits Oriol, Elena, Raquel i tota la resta.

I per acabar, agraeixo a qui sigui que m'hagi donat la beca Javier Lamas. A fi de comptes sense aquesta beca aquesta tesi no hauria estat possible.

Gràcies, doncs a tots.

VIII. REFERENCES

(1) Kerr JF, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer* 1972;**26**(4):239-57.

(2) Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* 2000;**100**(1):57-70.

(3) Thompson CB. Apoptosis in the pathogenesis and treatment of disease. *Science* 1995;**267**(5203):1456-62.

(4) Youle RJ, Strasser A. The BCL-2 protein family: opposing activities that mediate cell death. *Nat Rev Mol Cell Biol* 2008;**9**(1):47-59.

(5) Soengas MS, Capodieci P, Polsky D *et al*. Inactivation of the apoptosis effector Apaf-1 in malignant melanoma. *Nature* 2001;**409**:207-11.

(6) Yuan J, Yankner BA. Apoptosis in the nervous system. *Nature* 2000;**407**(6805):802-9.

(7) Ellis HM, Horvitz HR. Genetic control of programmed cell death in the nematode C. elegans. *Cell* 1986;**44**(6):817-29.

(8) Hengartner MO, Horvitz HR. C. elegans cell survival gene ced-9 encodes a functional homolog of the mammalian proto-oncogene bcl-2. *Cell* 1994;**76**(4):665-76.

(9) Vaux DL, Weissman IL, Kim SK. Prevention of programmed cell death in Caenorhabditis elegans by human bcl-2. *Science* 1992;**258**(5090):1955-7.

(10) Nagata S, Nagase H, Kawane K *et al*. Degradation of chromosomal DNA during apoptosis. *Cell Death Differ* 2003;**10**(1):108-16.

(11) Leist M, Jaattela M. Four deaths and a funeral: from caspases to alternative mechanisms. *Nat Rev Mol Cell Biol* 2001;**2**(8):589-98.

(12) Kumar S. Caspase function in programmed cell death. *Cell Death Differ* 2007;**14**(1):32-43.

(13) Boatright KM, Renatus M, Scott FL *et al*. A unified model for apical caspase activation. *Mol Cell* 2003;**11**(2):529-41.

(14) Bump NJ, Hackett M, Hugunin M *et al*. Inhibition of ICE family proteases by baculovirus antiapoptotic protein p35. *Science* 1995;**269**(5232):1885-8.

(15) Eckelman BP, Salvesen GS, Scott FL. Human inhibitor of apoptosis proteins: why XIAP is the black sheep of the family. *EMBO Rep* 2006;**7**(10):988-94.

(16) Lens SM, Vader G, Medema RH. The case for Survivin as mitotic regulator. *Curr Opin Cell Biol* 2006;**18**(6):616-22.

(17) Shi Y. Mechanisms of caspase activation and inhibition during apoptosis. *Mol Cell* 2002;**9**(3):459-70.

(18) Vaux DL, Cory S, Adams JM. Bcl-2 gene promotes haemopoietic cell survival and cooperates with c- myc to immortalize pre-B cells. *Nature* 1988;**335**(6189):440-2.

(19) Zong WX, Lindsten T, Ross AJ *et al*. BH3-only proteins that bind pro-survival Bcl-2 family members fail to induce apoptosis in the absence of Bax and Bak. *Genes Dev* 2001;**15**(12):1481-6.

(20) Cheng EH, Wei MC, Weiler S *et al*. BCL-2, BCL-X(L) sequester BH3 domain-only molecules preventing BAX- and BAK-mediated mitochondrial apoptosis. *Mol Cell* 2001;**8**(3):705-11.

(21) Kim H, Rafiuddin-Shah M, Tu HC *et al*. Hierarchical regulation of mitochondrion-dependent apoptosis by BCL-2 subfamilies. *Nat Cell Biol* 2006;**8**(12):1348-58.

(22) Galonek HL, Hardwick JM. Upgrading the BCL-2 network. *Nat Cell Biol* 2006;**8**(12):1317-9.

(23) Yu J, Zhang L. The transcriptional targets of p53 in apoptosis control. *Biochemical and Biophysical Research Communications* 2005;**331**(3):851-8.

(24) Wang Z, Malone MH, He H *et al*. Microarray analysis uncovers the induction of the proapoptotic BH3-only protein Bim in multiple models of glucocorticoid-induced apoptosis. *J Biol Chem* 2003;**278**(26):23861-7.

(25) Zha J, Harada H, Yang E *et al*. Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-X(L) [see comments]. *Cell* 1996;**87**(4):619-28.

(26) Luo X, Budihardjo I, Zou H *et al*. Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. *Cell* 1998;**94**(4):481-90.

(27) Puthalakath H, Villunger A, O'Reilly LA *et al*. Bmf: a proapoptotic BH3-only protein regulated by interaction with the myosin V actin motor complex, activated by anoikis. *Science* 2001;**293**(5536):1829-32.

(28) Puthalakath H, Huang DC, O'Reilly LA *et al*. The proapoptotic activity of the Bcl-2 family member Bim is regulated by interaction with the dynein motor complex. *Mol Cell* 1999;**3**(3):287-96.

(29) Roset R, Ortet L, Gil-Gomez G. Role of Bcl-2 family members on apoptosis: what we have learned from knock-out mice. *Front Biosci* 2007;**12**:4722-30.

(30) Muchmore SW, Sattler M, Liang H *et al*. X-ray and NMR structure of human Bcl-xL, an inhibitor of programmed cell death. *Nature* 1996;**381**(6580):335-41.

(31) Antignani A, Youle RJ. How do Bax and Bak lead to permeabilization of the outer mitochondrial membrane? *Current Opinion in Cell Biology* 2006;**18**(6):685-9.

(32) Cheng EHY, Sheiko TV, Fisher JK *et al*. VDAC2 inhibits BAK activation and mitochondrial apoptosis. *Science* 2003;**301**(5632):513-7.

(33) Frezza C, Cipolat S, Martins dB *et al*. OPA1 controls apoptotic cristae remodeling independently from mitochondrial fusion. *Cell* 2006;**126**(1):177-89.

(34) Dorstyn L, Read S, Cakouros D *et al*. The role of cytochrome c in caspase activation in Drosophila melanogaster cells. *J Cell Biol* 2002;**156**(6):1089-98.

(35) Riedl SJ, Salvesen GS. The apoptosome: signalling platform of cell death. *Nat Rev Mol Cell Biol* 2007;**8**(5):405-13.

(36) Stennicke HR, Deveraux QL, Humke EW *et al*. Caspase-9 can be activated without proteolytic processing. *J Biol Chem* 1999;**274**(13):8359-62.

(37) Scaffidi C, Fulda S, Srinivasan A *et al*. Two CD95 (APO-1/Fas) signaling pathways. *EMBO J* 1998;**17**(6):1675-87.

(38) Kataoka T. The caspase-8 modulator c-FLIP. *Crit Rev Immunol* 2005;**25**(1):31-58.

(39) Chang DW, Xing Z, Pan Y *et al*. c-FLIP(L) is a dual function regulator for caspase-8 activation and CD95-mediated apoptosis. *EMBO J* 2002;**21**(14):3704-14.

(40) Yeh WC, Itie A, Elia AJ *et al*. Requirement for Casper (c-FLIP) in regulation of death receptor-induced apoptosis and embryonic development. *Immunity* 2000;**12**(6):633-42.

(41) Peter ME, Budd RC, Desbarats J *et al*. The CD95 receptor: apoptosis revisited. *Cell* 2007;**129**(3):447-50.

(42) Burke B. Lamins and apoptosis: a two-way street? *J Cell Biol* 2001;**153**(3):F5-F7.

(43) Sebbagh M, Renvoize C, Hamelin J *et al*. Caspase-3-mediated cleavage of ROCK I induces MLC phosphorylation and apoptotic membrane blebbing. *Nat Cell Biol* 2001;**3**(4):346-52.

(44) Fischer U, Janicke RU, Schulze-Osthoff K. Many cuts to ruin: a comprehensive update of caspase substrates. *Cell Death Differ* 2003;**10**(1):76-100.

(45) Enari M, Sakahira H, Yokoyama H *et al*. A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD [see comments] [published erratum appears in Nature 1998 May 28;393(6683):396]. *Nature* 1998;**391**(6662):43-50.

(46) Halenbeck R, MacDonald H, Roulston A *et al*. CPAN, a human nuclease regulated by the caspase-sensitive inhibitor DFF45. *Curr Biol* 1998;**8**(9):537-40.

(47) Zhang J, Liu X, Scherer DC *et al*. Resistance to DNA fragmentation and chromatin condensation in mice lacking the DNA fragmentation factor 45. *Proc Natl Acad Sci U S A* 1998;**95**(21):12480-5.

(48) Samejima K, Tone S, Earnshaw WC. CAD/DFF40 nuclease is dispensable for high molecular weight DNA cleavage and stage I chromatin condensation in apoptosis. *J Biol Chem* 2001;**276**(48):45427-32.

(49) Li LY, Luo X, Wang X. Endonuclease G is an apoptotic DNase when released from mitochondria. *Nature* 2001;**412**(6842):95-9.

(50) Susin SA, Lorenzo HK, Zamzami N *et al*. Molecular characterization of mitochondrial apoptosis-inducing factor [see comments]. *Nature* 1999;**397**(6718):441-6.

(51) Sahara S, Aoto M, Eguchi Y *et al*. Acinus is a caspase-3-activated protein required for apoptotic chromatin condensation [see comments]. *Nature* 1999;**401**(6749):168-73.

(52) Kovacsovics M, Martinon F, Micheau O *et al*. Overexpression of Helicard, a CARD-containing helicase cleaved during apoptosis, accelerates DNA degradation. *Curr Biol* 2002;**12**(10):838-43.

(53) Rosen A, Casciola-Rosen L. Autoantigens as substrates for apoptotic proteases: implications for the pathogenesis of systemic autoimmune disease. *Cell Death Differ* 1999;**6**(1):6-12.

(54) Lauber K, Blumenthal SG, Waibel M *et al*. Clearance of apoptotic cells: getting rid of the corpses. *Mol Cell* 2004;**14**(3):277-87.

(55) Brown S, Heinisch I, Ross E *et al*. Apoptosis disables CD31-mediated cell detachment from phagocytes promoting binding and engulfment. *Nature* 2002;**418**(6894):200-3.

(56) Lauber K, Bohn E, Krober SM *et al*. Apoptotic cells induce migration of phagocytes via caspase-3-mediated release of a lipid attraction signal. *Cell* 2003;**113**(6):717-30.

(57) Mueller RB, Sheriff A, Gaipl US *et al*. Attraction of phagocytes by apoptotic cells is mediated by lysophosphatidylcholine. *Autoimmunity* 2007;**40**(4):342-4.

(58) Ellis RE, Jacobson DM, Horvitz HR. Genes required for the engulfment of cell corpses during programmed cell death in Caenorhabditis elegans. *Genetics* 1991;**129**(1):79-94.

(59) Zhou Z, Hartwieg E, Horvitz HR. CED-1 is a transmembrane receptor that mediates cell corpse engulfment in C. elegans. *Cell* 2001;**104**(1):43-56.

(60) Niida H, Nakanishi M. DNA damage checkpoints in mammals. *Mutagenesis* 2006;**21**(1):3-9.

(61) Parrilla-Castellar ER, Arlander SJH, Kamitz L. Dial 9-1-1 for DNA damage: the RaO-Hus1-Rad1 (9-1-1) clamp complex. *Dna Repair* 2004;**3**(8-9):1009-14.

(62) Bartek J, Lukas J. DNA damage checkpoints: from initiation to recovery or adaptation. *Curr Opin Cell Biol* 2007;**19**(2):238-45.

(63) Moreno-Herrero F, de Jager M, Dekker NH *et al*. Mesoscale conformational changes in the DNA-repair complex Rad50/Mre11/Nbs1 upon binding DNA. *Nature* 2005;**437**(7057):440-3.

(64) You Z, Chahwan C, Bailis J *et al*. ATM activation and its recruitment to damaged DNA require binding to the C terminus of Nbs1. *Mol Cell Biol* 2005;**25**(13):5363-79.

(65) Shiloh Y. ATM and related protein kinases: safeguarding genome integrity. *Nat Rev Cancer* 2003;**3**(3):155-68.

(66) Rothkamm K, Kruger I, Thompson LH *et al*. Pathways of DNA double-strand break repair during the mammalian cell cycle. *Mol Cell Biol* 2003;**23**(16):5706-15.

(67) Allen C, Kurimasa A, Brenneman MA *et al*. DNA-dependent protein kinase suppresses double-strand break-induced and spontaneous homologous recombination. *Proc Natl Acad Sci U S A* 2002;**99**(6):3758-63.

(68) Bekker-Jensen S, Lukas C, Kitagawa R *et al*. Spatial organization of the mammalian genome surveillance machinery in response to DNA strand breaks. *J Cell Biol* 2006;**173**(2):195-206.

(69) Lavin MF. ATM and the Mre11 complex combine to recognize and signal DNA double-strand breaks. *Oncogene* 2007;**26**(56):7749-58.

(70) Lavin MF, Kozlov S. ATM activation and DNA damage response. *Cell Cycle* 2007;**6**(8):931-42.

(71) Pellegrini M, Celeste A, Difilippantonio S *et al*. Autophosphorylation at serine 1987 is dispensable for murine Atm activation in vivo. *Nature* 2006;**443**(7108):222-5.

(72) Stucki M, Clapperton JA, Mohammad D *et al*. MDC1 directly binds phosphorylated histone H2AX to regulate cellular responses to DNA double-strand breaks. *Cell* 2005;**123**(7):1213-26.

(73) Kumagai A, Lee J, Yoo HY *et al*. TopBP1 activates the ATR-ATRIP complex. *Cell* 2006;**124**(5):943-55.

(74) Bartek J, Lukas J. Chk1 and Chk2 kinases in checkpoint control and cancer. *Cancer Cell* 2003;**3**(5):421-9.

(75) Busino L, Chiesa M, Draetta GF *et al*. Cdc25A phosphatase: combinatorial phosphorylation, ubiquitylation and proteolysis. *Oncogene* 2004;**23**(11):2050-6.

(76) Falck J, Mailand N, Syljuasen RG *et al*. The ATM-Chk2-Cdc25A checkpoint pathway guards against radioresistant DNA synthesis. *Nature* 2001;**410**(6830):842-7.

(77) Peng CY, Graves PR, Thoma RS *et al*. Mitotic and G2 checkpoint control: regulation of 14-3-3 protein binding by phosphorylation of Cdc25C on serine-216. *Science* 1997;**277**(5331):1501-5.

(78) Uto K, Inoue D, Shimuta K *et al*. Chk1, but not Chk2, inhibits Cdc25 phosphatases by a novel common mechanism. *EMBO J* 2004;**23**(16):3386-96.

(79) Lavin MF, Gueven N. The complexity of p53 stabilization and activation. *Cell Death Differ* 2006;**13**(6):941-50.

(80) Maya R, Balass M, Kim ST *et al*. ATM-dependent phosphorylation of Mdm2 on serine 395: role in p53 activation by DNA damage. *Genes Dev* 2001;**15**(9):1067-77.

(81) Moll UM, Wolff S, Speidel D *et al*. Transcription-independent pro-apoptotic functions of p53. *Curr Opin Cell Biol* 2005;**17**(6):631-6.

(82) Das S, Raj L, Zhao B *et al*. Hzf Determines cell survival upon genotoxic stress by modulating p53 transactivation. *Cell* 2007;**130**(4):624-37.

(83) Homer C, Knight DA, Hananeia L *et al*. Y-box factor YB1 controls p53 apoptotic function. *Oncogene* 2005;**24**(56):8314-25.

(84) Shmueli A, Oren M. Mdm2: p53's lifesaver? *Mol Cell* 2007;**25**(6):794-6.

(85) Smets LA, Salomons G, van den BJ. Glucocorticoid induced apoptosis in leukemia. *Adv Exp Med Biol* 1999;**457**:607-14.

(86) Pratt WB, Toft DO. Steroid receptor interactions with heat shock protein and immunophilin chaperones. *Endocr Rev* 1997;**18**(3):306-60.

(87) Kassel O, Herrlich P. Crosstalk between the glucocorticoid receptor and other transcription factors: molecular aspects. *Mol Cell Endocrinol* 2007;**275**(1-2):13-29.

(88) Herold MJ, McPherson KG, Reichardt HM. Glucocorticoids in T cell apoptosis and function. *Cell Mol Life Sci* 2006;**63**(1):60-72.

(89) Reichardt HM, Kaestner KH, Tuckermann J *et al*. DNA binding of the glucocorticoid receptor is not essential for survival [see comments]. *Cell* 1998;**93**(4):531-41.

(90) Schmidt S, Rainer J, Ploner C *et al*. Glucocorticoid-induced apoptosis and glucocorticoid resistance: molecular mechanisms and clinical relevance. *Cell Death Differ* 2004;**11 Suppl 1**:S45-S55.

(91) Erlacher M, Michalak EM, Kelly PN *et al*. BH3-only proteins Puma and Bim are rate-limiting for gamma-radiation- and glucocorticoid-induced apoptosis of lymphoid cells in vivo. *Blood* 2005;**106**(13):4131-8.

(92) Yoshida H, Kong YY, Yoshida R *et al*. Apaf1 is required for mitochondrial pathways of apoptosis and brain development. *Cell* 1998;**94**(6):739-50.

(93) Cecconi F, Alvarez-Bolado G, Meyer BI *et al*. Apaf1 (CED-4 homolog) regulates programmed cell death in mammalian development. *Cell* 1998;**94**(6):727-37.

(94) Kuida K, Haydar TF, Kuan CY *et al*. Reduced apoptosis and cytochrome c-mediated caspase activation in mice lacking caspase 9. *Cell* 1998;**94**(3):325-37.

(95) Lindsten T, Ross AJ, King A *et al*. The combined functions of proapoptotic Bcl-2 family members bak and bax are essential for normal development of multiple tissues. *Mol Cell* 2000;**6**(6):1389-99.

(96) Botz J, Zerfass-Thome K, Spitkovsky D *et al*. Cell cycle regulation of the murine cyclin E gene depends on an E2F binding site in the promoter. *Mol Cell Biol* 1996;**16**(7):3401-9.

(97) Morgan DO. Principles of CDK regulation. *Nature* 1995;**374**(6518):131-4.

(98) Kraft C, Herzog F, Gieffers C *et al*. Mitotic regulation of the human anaphase-promoting complex by phosphorylation. *EMBO J* 2003;**22**(24):6598-609.

(99) Pines J, Lindon C. Proteolysis: anytime, any place, anywhere? *Nat Cell Biol* 2005;**7**(8):731-5.

(100) Fisher RP. Secrets of a double agent: CDK7 in cell-cycle control and transcription. *J Cell Sci* 2005;**118**(Pt 22):5171-80.

(101) Dessev G, Iovcheva-Dessev C, Bischoff JR *et al*. A complex containing p34cdc2 and cyclin B phosphorylates the nuclear lamin and disassembles nuclei of clam oocytes in vitro. *J Cell Biol* 1991;**112**(4):523-33.

(102) Kihlmark M, Imreh G, Hallberg E. Sequential degradation of proteins from the nuclear envelope during apoptosis. *J Cell Sci* 2001;**114**(Pt 20):3643-53.

(103) Shi L, Nishioka WK, Th'ng J *et al*. Premature p34cdc2 activation required for apoptosis [see comments]. *Science* 1994;**263**(5150):1143-5.

(104) Biswas SC, Liu DX, Greene LA. Bim is a direct target of a neuronal E2F-dependent apoptotic pathway. *Journal of Neuroscience* 2005;**25**(37):8349-58.

(105) Greene LA, Liu DX, Troy CM *et al*. Cell cycle molecules define a pathway required for neuron death in development and disease. *Biochim Biophys Acta* 2007;**1772**(4):392-401.

(106) Hamdane M, Bretteville A, Sambo AV *et al*. p25/Cdk5-mediated retinoblastoma phosphorylation is an early event in neuronal cell death. *J Cell Sci* 2005;**118**(Pt 6):1291-8.

(107) Gil-Gomez G, Berns A, Brady HJ. A link between cell cycle and cell death: Bax and Bcl-2 modulate Cdk2 activation during thymocyte apoptosis. *EMBO J* 1998;**17**(24):7209-18.

(108) Shen SC, Huang TS, Jee SH *et al*. Taxol-induced p34cdc2 kinase activation and apoptosis inhibited by 12-O-tetradecanoylphorbol-13-acetate in human breast MCF-7 carcinoma cells. *Cell Growth Differ* 1998;**9**(1):23-9.

(109) Choi KS, Eom YW, Kang Y *et al*. Cdc2 and Cdk2 kinase activated by transforming growth factor-beta1 trigger apoptosis through the phosphorylation of retinoblastoma protein in FaO hepatoma cells. *J Biol Chem* 1999;**274**(45):31775-83.

(110) Kim SG, Kim SN, Jong HS *et al*. Caspase-mediated Cdk2 activation is a critical step to execute transforming growth factor-beta1-induced apoptosis in human gastric cancer cells. *Oncogene* 2001;**20**(10):1254-65.

(111) Liao CB, Chang YC, Kao CW *et al*. Deregulation of cyclin-dependent kinase 2 activity correlates with UVC-induced apoptosis in Chinese hamster ovary cells. *J Cell Biochem* 2006;**97**(4):824-35.

(112) Choi JS, Shin S, Jin YH *et al*. Cyclin-dependent protein kinase 2 activity is required for mitochondrial translocation of Bax and disruption of mitochondrial transmembrane potential during etoposide-induced apoptosis. *Apoptosis* 2007;**12**(7):1229-41.

(113) Hakem A, Sasaki T, Kozieradzki I *et al*. The cyclin-dependent kinase Cdk2 regulates thymocyte apoptosis. *J Exp Med* 1999;**189**(6):957-68.

(114) Levkau B, Koyama H, Raines EW *et al*. Cleavage of p21Cip1/Waf1 and p27Kip1 mediates apoptosis in endothelial cells through activation of Cdk2: role of a caspase cascade. *Mol Cell* 1998;**1**(4):553-63.

(115) Brady HJ, Gil-Gomez G, Kirberg J *et al*. Bax alpha perturbs T cell development and affects cell cycle entry of T cells. *EMBO J* 1996;**15**(24):6991-7001.

(116) Williams O, Gil-Gomez G, Norton T *et al*. Activation of Cdk2 is a requirement for antigen-mediated thymic negative selection. *Eur J Immunol* 2000;**30**(2):709-13.

(117) Granes F, Roig MB, Brady HJM *et al*. Cdk2 activation acts upstream of the mitochondrion during glucocorticoid induced thymocyte apoptosis. *European Journal of Immunology* 2004;**34**(10):2781-90.

(118) O'Reilly LA, Huang DC, Strasser A. The cell death inhibitor Bcl-2 and its homologues influence control of cell cycle entry. *EMBO J* 1996;**15**(24):6979-90.

(119) Brummelkamp TR, Bernards R, Agami R. A system for stable expression of short interfering RNAs in mammalian cells. *Science* 2002;**296**(5567):550-3.

(120) Murray AW, Marks D. Can sequencing shed light on cell cycling? *Nature* 2001;**409**(6822):844-6.

(121) van den HS, Harlow E. Distinct roles for cyclin-dependent kinases in cell cycle control. *Science* 1993;**262**(5142):2050-4.

(122) Pinhero R, Liaw P, Bertens K *et al*. Three cyclin-dependent kinases preferentially phosphorylate different parts of the C-terminal domain of the large subunit of RNA polymerase II. *European Journal of Biochemistry* 2004;**271**(5):1004-14.

(123) Bach S, Knockaert M, Reinhardt J *et al*. Roscovitine targets, protein kinases and pyridoxal kinase. *Journal of Biological Chemistry* 2005;**280**(35):31208-19.

(124) Askew DJ, Kuscuoglu U, Brunner T *et al*. Characterization of Apt- cell lines exhibiting cross-resistance to glucocorticoid- and Fas-mediated apoptosis. *Cell Death Differ* 1999;**6**(8):796-804.

(125) Bladh LG, Liden J, Pazirandeh A *et al*. Identification of target genes involved in the antiproliferative effect of glucocorticoids reveals a role

for nuclear factor-kappa B repression. *Molecular Endocrinology* 2005;**19**(3):632-43.

(126) Oh IH, Reddy EP. The myb gene family in cell growth, differentiation and apoptosis. *Oncogene* 1999;**18**(19):3017-33.

(127) Kumar A, Lee CM, Reddy EP. c-Myc is essential but not sufficient for c-Myb-mediated block of granulocytic differentiation. *J Biol Chem* 2003;**278**(13):11480-8.

(128) Mucenski ML, McLain K, Kier AB *et al*. A functional c-myb gene is required for normal murine fetal hepatic hematopoiesis. *Cell* 1991;**65**(4):677-89.

(129) Bender TP, Kremer CS, Kraus M *et al*. Critical functions for c-Myb at three checkpoints during thymocyte development. *Nat Immunol* 2004;**5**(7):721-9.

(130) Pani E, Ferrari S. p38MAPKdelta controls c-Myb degradation in response to stress. *Blood Cells Mol Dis* 2007.

(131) Kanei-Ishii C, Ninomiya-Tsuji J, Tanikawa J *et al*. Wnt-1 signal induces phosphorylation and degradation of c-Myb protein via TAK1, HIPK2, and NLK. *Genes Dev* 2004;**18**(7):816-29.

(132) Senderowicz AM. Targeting cell cycle and apoptosis for the treatment of human malignancies. *Current Opinion in Cell Biology* 2004;**16**(6):670-8.

(133) Park DS, Farinelli SE, Greene LA. Inhibitors of cyclin-dependent kinases promote survival of post-mitotic neuronally differentiated PC12 cells and sympathetic neurons. *J Biol Chem* 1996;**271**(14):8161-9.

(134) Golsteyn RM. Cdk1 and Cdk2 complexes (cyclin dependent kinases) in apoptosis: a role beyond the cell cycle. *Cancer Letters* 2005;**217**(2):129-38.

(135) Hamdane M, Buee L. The complex p25/Cdk5 kinase in neurofibrillary degeneration and neuronal death: the missing link to cell cycle. *Biotechnol J* 2007;**2**(8):967-77.

(136) Berthet C, Rodriguez-Galan MC, Hodge DL *et al*. Hematopoiesis and thymic apoptosis are not affected by the loss of Cdk2. *Mol Cell Biol* 2007;**27**(14):5079-89.

(137) Ortega S, Prieto I, Odajima J *et al*. Cyclin-dependent kinase 2 is essential for meiosis but not for mitotic cell division in mice. *Nat Genet* 2003;**35**(1):25-31.

(138) Berthet C, Aleem E, Coppola V *et al*. Cdk2 knockout mice are viable. *Curr Biol* 2003;**13**(20):1775-85.

(139) Santamaria D, Barriere C, Cerqueira A *et al*. Cdk1 is sufficient to drive the mammalian cell cycle. *Nature* 2007;**448**(7155):811-5.

(140) Aleem E, Kiyokawa H, Kaldis P. Cdc2-cyclin E complexes regulate the G1/S phase transition. *Nat Cell Biol* 2005;**7**(8):831-6.

(141) Rosales JL, Lee KY. Extraneuronal roles of cyclin-dependent kinase 5. *Bioessays* 2006;**28**(10):1023-34.

(142) Ohshima T, Ward JM, Huh CG *et al*. Targeted disruption of the cyclin-dependent kinase 5 gene results in abnormal corticogenesis, neuronal pathology and perinatal death. *Proc Natl Acad Sci U S A* 1996;**93**(20):11173-8.

(143) Lee MS, Kwon YT, Li M *et al*. Neurotoxicity induces cleavage of p35 to p25 by calpain. *Nature* 2000;**405**(6784):360-4.

(144) Cruz JC, Tseng HC, Goldman JA *et al*. Aberrant Cdk5 activation by p25 triggers pathological events leading to neurodegeneration and neurofibrillary tangles. *Neuron* 2003;**40**(3):471-83.

(145) Beato M, Chalepakis G, Schauer M *et al*. DNA regulatory elements for steroid hormones. *J Steroid Biochem* 1989;**32**(5):737-47.

(146) Ma B, Pan Y, Zheng J *et al*. Sequence analysis of p53 response-elements suggests multiple binding modes of the p53 tetramer to DNA targets. *Nucleic Acids Res* 2007;**35**(9):2986-3001.

(147) Greider C, Chattopadhyay A, Parkhurst C *et al*. BCL-x(L) and BCL2 delay Myc-induced cell cycle entry through elevation of p27 and inhibition of G1 cyclin-dependent kinases. *Oncogene* 2002;**21**(51):7765-75.

(148) Cook PW, Swanson KT, Edwards CP *et al*. Glucocorticoid receptor-dependent inhibition of cellular proliferation in dexamethasone-resistant and hypersensitive rat hepatoma cell variants. *Mol Cell Biol* 1988;**8**(4):1449-59.

(149) Bouillet P, Metcalf D, Huang DC *et al*. Proapoptotic Bcl-2 relative Bim required for certain apoptotic responses, leukocyte homeostasis, and to preclude autoimmunity. *Science* 1999;**286**(5445):1735-8.

(150) Abrams MT, Robertson NM, Yoon K *et al*. Inhibition of glucocorticoid-induced apoptosis by targeting the major splice variants of BIM mRNA with small interfering RNA and short hairpin RNA. *J Biol Chem* 2004;**279**(53):55809-17.

(151) Villunger A, Michalak EM, Coultas L *et al*. p53- and drug-induced apoptotic responses mediated by BH3-only proteins puma and noxa. *Science* 2003;**302**(5647):1036-8.

(152) Marchetti MC, Di Marco B, Cifone G *et al*. Dexamethasone-induced apoptosis of thymocytes: role of glucocorticoid receptor-associated Src kinase and caspase-8 activation. *Blood* 2003;**101**(2):585-93.

(153) Allan LA, Clarke PR. Phosphorylation of caspase-9 by CDK1/cyclin B1 protects mitotic cells against apoptosis. *Mol Cell* 2007;**26**(2):301-10.

(154) Peter ME, Krammer PH. The CD95(APO-1/Fas) DISC and beyond. *Cell Death Differ* 2003;**10**(1):26-35.

(155) Gervais FG, Singaraja R, Xanthoudakis S *et al*. Recruitment and activation of caspase-8 by the Huntingtin-interacting protein Hip-1 and a novel partner Hippi. *Nat Cell Biol* 2002;**4**(2):95-105.

(156) Lamkanfi M, Festjens N, Declercq W *et al*. Caspases in cell survival, proliferation and differentiation. *Cell Death Differ* 2007;**14**(1):44-55.

(157) Varfolomeev EE, Schuchmann M, Luria V *et al*. Targeted disruption of the mouse Caspase 8 gene ablates cell death induction by the TNF receptors, Fas/Apo1, and DR3 and is lethal prenatally. *Immunity* 1998;**9**(2):267-76.

(158) Kang TB, Ben Moshe T, Varfolomeev EE *et al*. Caspase-8 serves both apoptotic and nonapoptotic roles. *J Immunol* 2004;**173**(5):2976-84.

(159) Lippens S, Kockx M, Knaapen M *et al*. Epidermal differentiation does not involve the pro-apoptotic executioner caspases, but is associated with caspase-14 induction and processing. *Cell Death Differ* 2000;**7**(12):1218-24.

(160) Fisher DL, Nurse P. A single fission yeast mitotic cyclin B p34cdc2 kinase promotes both S-phase and mitosis in the absence of G1 cyclins. *EMBO J* 1996;**15**(4):850-60.

(161) Brown NR, Lowe ED, Petri E *et al*. Cyclin B and cyclin A confer different substrate recognition properties on CDK2. *Cell Cycle* 2007;**6**(11):1350-9.

(162) Miller ME, Cross FR. Cyclin specificity: how many wheels do you need on a unicycle? *J Cell Sci* 2001;**114**(Pt 10):1811-20.

(163) Ness SA. Myb binding proteins: regulators and cohorts in transformation. *Oncogene* 1999;**18**(19):3039-46.

(164) Bies J, Feikova S, Bottaro DP *et al*. Hyperphosphorylation and increased proteolytic breakdown of c-Myb induced by the inhibition of Ser/Thr protein phosphatases. *Oncogene* 2000;**19**(24):2846-54.

(165) Bies J, Wolff L. Oncogenic activation of c-Myb by carboxyl-terminal truncation leads to decreased proteolysis by the ubiquitin-26S proteasome pathway. *Oncogene* 1997;**14**(2):203-12.

(166) Sramko M, Markus J, Kabat J *et al*. Stress-induced inactivation of the c-Myb transcription factor through conjugation of SUMO-2/3 proteins. *J Biol Chem* 2006;**281**(52):40065-75.

(167) Sano Y, Ishii S. Increased affinity of c-Myb for CREB-binding protein (CBP) after CBP-induced acetylation. *J Biol Chem* 2001;**276**(5):3674-82.

(168) Aziz N, Miglarese MR, Hendrickson RC *et al*. Modulation of c-Myb-induced transcription activation by a phosphorylation site near the negative regulatory domain. *Proc Natl Acad Sci U S A* 1995;**92**(14):6429-33.

(169) Taylor D, Badiani P, Weston K. A dominant interfering Myb mutant causes apoptosis in T cells. *Genes Dev* 1996;**10**(21):2732-44.

(170) Frampton J, Ramqvist T, Graf T. v-Myb of E26 leukemia virus up-regulates bcl-2 and suppresses apoptosis in myeloid cells. *Genes Dev* 1996;**10**(21):2720-31.

(171) Salomoni P, Perrotti D, Martinez R *et al*. Resistance to apoptosis in CTLL-2 cells constitutively expressing c-Myb is associated with induction of BCL-2 expression and Myb-dependent regulation of bcl-2 promoter activity. *Proc Natl Acad Sci U S A* 1997;**94**(7):3296-301.

(172) Zorbas M, Sicurella C, Bertoncello I *et al*. c-Myb is critical for murine colon development. *Oncogene* 1999;**18**(42):5821-30.

(173) Castedo M, Perfettini JL, Roumier T *et al*. Cyclin-dependent kinase-1: linking apoptosis to cell cycle and mitotic catastrophe. *Cell Death Differ* 2002;**9**(12):1287-93.

(174) Kranenburg O, vanderEb A, Zantema A. Cyclin D1 is an essential mediator of apoptotic neuronal cell death. *Embo Journal* 1996;**15**(1):46-54.

IX. ANNEX

Roset R, Ortet L, Gil-Gomez G.
Role of Bcl-2 family members on apoptosis: what we have learned from knock-out mice.
Front Biosci. 2007 May 1;12:4722-30.