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REGULATION OF E2F1 TRANSCRIPTION FACTOR BY GLYCOGEN SYNTHASE KINASE-3-BETA.

GISELA GARCIA ALVAREZ

Memoria presentada por GISELA GARCIA ALVAREZ, licenciada en Bioquímica por la Universidad de Barcelona, para optar al grado de Doctora por la Universidad de Barcelona.

Esta Tesis Doctoral ha sido realizada bajo la dirección del Dr. Albert Tauler i Girona, en el Departamento de Bioquímica y Biología Molecular-Farmacia de la Facultad de Farmacia de la Universidad de Barcelona.

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Dr. Albert Tauler i Girona

Gisela García Álvarez

A ti, Blanca

A todos aquellos que me quieren
y no tienen miedo a demostrarlo.

Only if we understand, can we care.
Only if we care, will we help.
Only if we help, shall all be saved.

- Jane Goodall-

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ABSTRACT

Whether a cell enters the cell cycle, undergoes apoptosis or survives is a consequence of the integration of several cellular and environmental signals. Growth factors, cell contact, and apoptotic inductors regulate a complex system of signal transduction pathways that trigger the activation of a large number of genes. One of the key proteins in the regulation of the cell cycle and the commitment to apoptosis is the transcription factor E2F1. It has been suggested that E2F1 activity levels could determine whether a cell enters the cell cycle, cell-cycle arrest or apoptosis. In these decisions, not only is transcriptional activity important, but also the synchronization of E2F1 activity with specific signal transduction pathways. In this context it has been suggested that activation of the PI 3-kinase pathway inhibits the apoptotic effect of E2F1 overexpression. Since GSK3β is a downstream effector of PI 3-kinase, we performed phosphorylation and binding analyses to examine the possible relationship between GSK3β and E2F1.

The results obtained in this thesis demonstrate that GSK3β phosphorylates human E2F1 *in vitro* at serine 403 and threonine 433. In earlier studies it has already been shown that these two residues are also phosphorylated by the TFIIH kinase, cdk7. We did not detect phosphorylation by GSK3β *in vivo*. However, immunoprecipitation experiments revealed *in vivo* binding of these proteins. By transient transfection experiments with GSK3β and E2F1 constructs, GSK3β-RNAi assays, and the use of PI 3-kinase and GSK3β–specific inhibitors, we demonstrate that GSK3β regulates E2F1 transcriptional activity through its interaction with E2F1 transactivation domain and that GSK3β kinase activity is not required for this regulation.

Our data obtained here integrates into a model in which translocation of GSK3β to the nucleus modulates E2F1 activity and, as a consequence, determines whether a cell enters the cell cycle or undergoes apoptosis.

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ABBREVIATIONS

AD	Active domain
APS	Ammonium persulfate
AP-1	Activator protein-1
APC	Adenomatous polyposis coli
ARF	Alternative reading frame
ATM	Ataxia telangiectasia mutated
ATP	Adenosin triphosphate
ATR	ATM and Rad3-related
CAK	cdk-activating kinase
Ci	Cubitus Interruptus
CBP	cAMP response element-binding protein
C/EBP	CCAAT/enhancer-binding protein
cdk	Cyclin-dependent kinases
CKIalpha	Casein kinase I alpha
CREB	Cyclic AMP response element binding protein
DAP	Death-associated protein
DHFR	Dihydrofolate reductase
DMSO	Dimethyl sulfoxide
DP	Differentiation regulated transcription factor (DRTF) protein
DRB	5,6-dichloro-1-β-D-ribofuranosylbenzimidazole
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid
eIF2B	Eukaryotic initiation factor 2B
ER	Endoplasmic reticulum
EtBr	Ethidium Bromide

FCS	Foetal calf serum
FBS	Foetal bovine serum
FRAT	Frequently rearranged in advanced T-cell lymphomas
GBP	GSK3 binding protein
GSK3α	Glycogen synthase kinase-3-alpha
GSK3β	Glycogen synthase kinase-3-beta
GST	Glutathione S-transferase
HDAC	Histone deacetylases
HeBS	HEPES-buffered saline
hnRNP	Heterogenous nuclear ribonucleoprotein D
HSF-1	Heat-shock factor-1
KRP	Kinase related protein
LZ	Leucine zipper
LC-MS	Liquid chromatography Electrospray mass spectrometry
MAP	Microtubule-associated protein
MB	Marked box
MITF	Microphthalmia-associated transcription factor
MS	Mass spectrometry
MS ^{3}	Triple mass spectrometry
MS/MS	Tandem mass spectrometry
MUC1/DF3	High molecular weight mucin-like glycoprotein
NCAM	Neural cell-adhesion molecule
NES	Nuclear export signal
NFAT	Nuclear factor of activated T cells
NF-κB	Nuclear factor kB
NGF	Nerve growth factor
NLS	Nuclear localization signal
nt	nucleotid

O.D.	Optical density at a wavelength of “n” nanometeters (nm)
P/CAF	p300/CREB-binding protein-associated factor
PBS	Phosphate buffer saline
PBS-T	Phosphate buffer saline + 0,1% Tween
PcG	Polycomb Group
PCR	Polymerase chain reaction
6-PF2K/Fru-2,6-BPase	6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase
PFU	Plaque formation units
PI	Phosphatidylinositol
PI 3-kinase	Phosphatidylinositol 3-kinase
PH	Pleckstrin homology
PKB	Protein kinase B
PMSF	Phenylmethylsulfonil fluoride
pRB	Retinoblastoma protein
PS1	Presenilin 1
PtdIns	Phosphatidylinositol
PVDF	Polyvinylidene difluoride
RISC	RNA induced silencing complex
RLU	Relative luciferase units
RNAi	RNA interference
rpm	Revolutions per minute
RYBP	Ring1 and YY1 binding protein
siRNA	small interfering RNA
SDS	Sodium dodecyl sulfate
SDS-PAGE	Polyacrylamide gel electrophoresis in SDS
TBS	Tris buffered saline
TBS-T	Tris buffered saline + 0,1% Tween
TEMED	N,N,N’,N’-Tetramethylethylendiamine

TNFR	Tumor necrosis factor receptor
TopBP1	Topoisomerase IIβ binding protein 1
Wnt	Wingless

I INTRODUCTION

CELL CYCLE AND CELL SIGNALING

The cell cycle is the process through which all living organisms propagate. In unicellular species each cell division gives rise to a new organism while in multicellular organisms many cell divisions can be required. In the later, the cell cycle is also necessary to generate new cells and replace others, lost either because of damage or apoptosis.

Therefore, the cell cycle is, by necessity, a very well controlled process in which cells proliferate, increase their mass and duplicate their DNA quantity. This obliges the machinery controlling the progress of the cell cycle to be in a tight communication with the cellular environment in order to interpret the correct stimulus and activate division only when favorable.

In most cases, cell proliferation is regulated through agents present in the external medium (serum in the case of multicellular organisms). Nearly all of these agents are proteins present in a very low concentration (nanomolar scale) and are classified under the general name of growth factors. Other cell cycle regulatory agents include hormones, neurotransmitters and stress. The majority of growth factors activate the cell cycle although some have the opposite effect.

The way cells interpret environmental conditions and gene expression is controlled through the stimulation of different receptors. Growth factors can stimulate proliferation through receptors that are, in general, protein kinases (enzymes capable of phosphorylating other proteins) and in particular receptors with tyrosine-kinase activity. The function of these and other types of receptors is to act as sensors for the presence of growth factors and to send a signal to the interior of the cell which ultimately has to reach the nucleus were the cell cycle machinery is. A common sequence of molecular events would include activation of the receptors, phosphorylation of various substrates and/or establishment of protein-protein interactions, activation of small G proteins and a protein-kinase cascade. As a result, transcription factors which control the expression of

genes responsible for growth, differentiation and others are phosphorylated and activated.

Taken together, the importance of the precise regulation of this machinery is evident. The integrity of the various cellular signaling pathways and precise control of the cell cycle is essential for cell viability and development.

CELL CYCLE

The events involved in the replication and partitioning of the chromosomes are common to all cell cycles. With a few exceptions, a newly divided cell needs to receive a full genome complement to survive. Chromosomes are present in low copy number and therefore special mechanisms are required to ensure their precise replication and partitioning.

The eukaryotic cell cycle is divided into two important phases: the **S phase** and the **M phase**. The S phase is a distinct time during interphase (period between one M phase and the following) in which DNA replication occurs. The M phase is the phase during which mitosis, the process of cell division, takes place. Between these two phases there are two other phases in which the cell grows and duplicates its protein mass and the organelles replicate DNA and divide. These intermediate phases are the **G1 phase** (before S phase) and the **G2 phase** (after S phase) which play a key role to initiate events such as the S phase and mitosis in the correct sequence in coordination with cellular growth and external conditions. There are cells that can stop cell cycle progression before S phase and remain in what is called the **G0 phase** for years without an increase in mass (Figure 1).

Checkpoints

In order to ensure that accurate progression of the cell cycle takes place there are three main checkpoints. The first one is the G1 checkpoint also known as the **restriction (R) point** which acts towards the end of the G1 phase. At this point, correct cell mass and adequate environmental conditions are examined before inititating DNA synthesis. A second checkpoint occurs before the M phase (G2-M checkpoint) which assures correct cell size, good environmental conditions and accurate and complete DNA replication before allowing the cell to enter mitosis. At the end of mitosis, another checkpoint, checkpoint M, ensures the correct alignment of the chromosomes before terminating cell division (Figure 1).

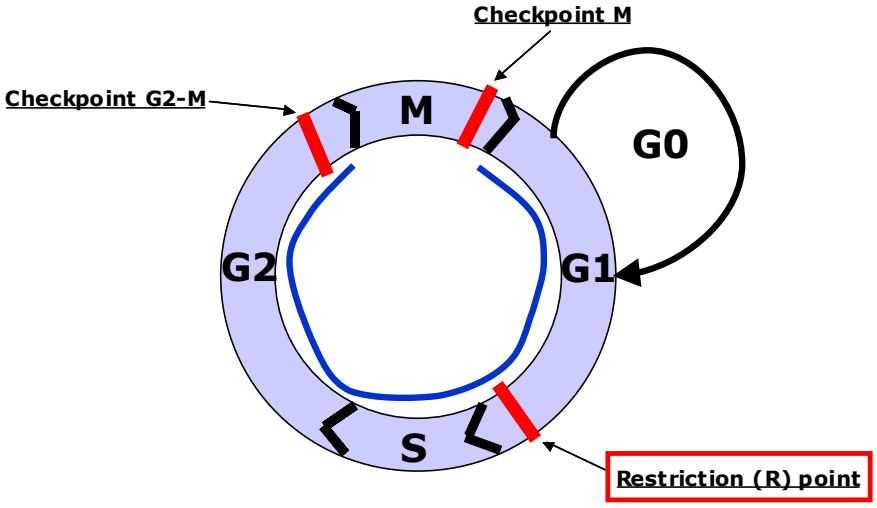


Figure 1. Cell cycle phases and checkpoints.
Inner solid blue line indicates the interphase.

Cell cycle regulation

The basic regulatory machinery of the cell cycle is composed of two main protein families: the cyclin-dependent protein kinases (cdk) and the cyclins. The cdks induce downstream processes by phosphorylating selected proteins on serines and threonines. The cyclins on the other hand bind to cdks and control their capacity to phosphorylate target proteins. Therefore the formation and destruction of cyclin-cdk complexes are central processes that control the cell cycle (Figure 2). In addition, the activity of these

two classes of proteins are also controlled by transcriptional regulation and stability. Modification of the cdks by phosphorylation and inhibition of their activity are also regulated processes. The subcellular localization of cyclins has also been shown to be important in their regulation. For example, Cyclins A and E are predominantly expressed in the nucleus due to their role in DNA replication and S phase progression [Pines and Hunter 1991; Ohtsubo et al 1995]. In contrast, cyclin B is found exclusively in the cytoplasm during the G1/S phase, but when the cell enters mitosis it is directed to the nucleus [Takizawa and Morgan 2000]. Additional control mechanisms of cyclin-cdk complex activity are activation through phosphorylation by the cdk-activating kinases (CAK) and inhibition through the binding of proteins of the CIP/KIP family and the INK4 family.

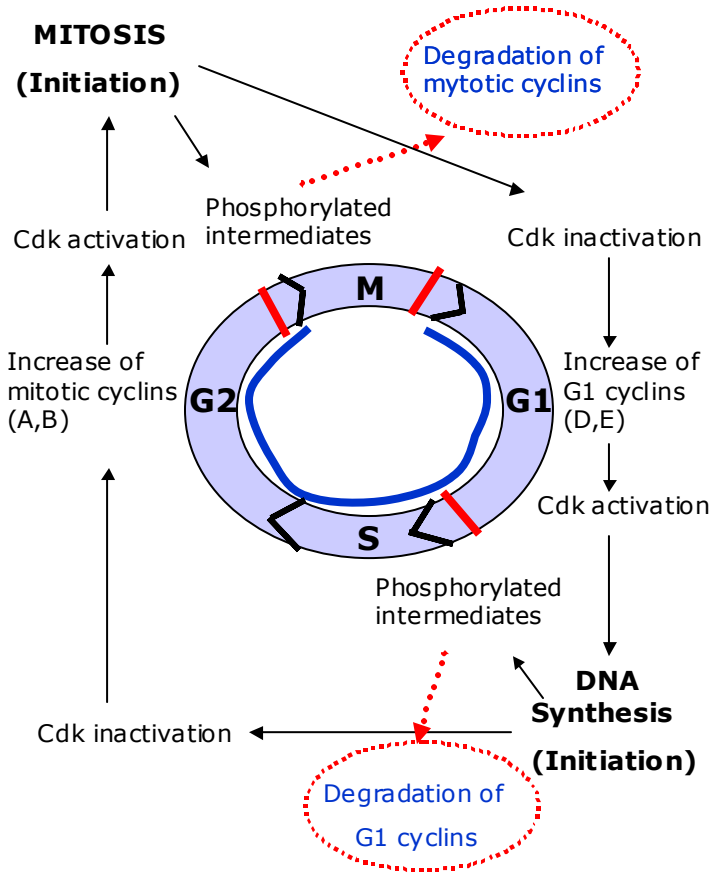


Figure 2. The fluctuation between synthesis and degradation of the different cyclins regulate the activity of cdks.

There are two main types of cyclins: G1 cyclins that bind to cdks during G1 phase and are important for the initiation of the S phase, and the mitotic cyclins which bind cdks during the G2 phase and are essential for entry into mitosis.

Cyclins important during the G1 phase are of the D or E type. They have a very short half-life and are regulated by transcriptional control. D type cyclins include D1, D2 and D3 and are implicated in the control of the restriction point 'R'. Their synthesis is induced by growth factors and suppressed by factors that reduce proliferation. Cyclin Ds can bind different cdks but mainly bind to cdk4 during the end of the G1 phase and the beginning of the S phase in a mitogenic signal dependent manner [Hitomi and Stacey 1999]. The main function of the cyclin D-cdk4 complex is to phosphorylate the retinoblastoma protein (pRB) and therefore activate the expression of many of the genes needed for entry into S phase. The pRB protein will remain hyperphosphorylated until the end of M phase. When it is hypophosphorylated it binds and inhibits distinct proteins including trancription factors, like E2F, which control the expression of critical genes involved in DNA synthesis. Cyclin E forms a complex with cdk2 and is involved in the replication of DNA. Its synthesis begins at the end of the G1 phase and continues until entry into S phase when it is rapidly degraded. pRB protein is also phosphorylated by cyclin E-cdk2 complexes.

The mitotic cyclins A and B regulate the G2-M checkpoint. They are stable during interphase but are quickly degraded during mitosis. During S phase, the cyclin A-cdk2 complex seems to have a role on the elongation of DNA synthesis. The cyclin B- cdk1 complex is important for the termination of mitosis.

There are also active cyclin complexes during the S phase. For example, the cyclin A-cdk2 complex plays a central role in regulating the accurate replication of DNA which should take place only once per cycle.

Restriction (R) point and E2F: the point of no return.

As mentioned before restriction point 'R' is one of the checkpoints cells have to ensure that the cell cycle progresses properly. It is mainly controlled by a family of proteins called the pocket proteins (pRB family) and members of the E2F family of transcription factors. The phosphorylation of pRB renders it incapable of binding to these factors resulting in their release and subsequent progression into S phase. This point of

transition between the G1 phase and the S phase is considered the 'point of no return', since from this moment the cell is committed to finish the cell cycle independent of external factors [Kondo et al., 2001].

There are three members of the pocket protein family: pRB, p107 and p130. They share many biological properties despite having unique functional roles. All of them bind to E2F through a conserved carboxy (C)-terminal domain called the 'pocket' domain, and mediate active repression of E2F-responsive genes (mechanism further detailed) [reviewed in Dyson 1998]. It is thought that pRB binds to E2F in both quiescent and actively dividing cells and that this association is responsible for pRB ability to block passage of cells past the G1 checkpoint [Sanchez and Dynlacht 1996].

The phosphorylation state of pRB is controlled by two opposing enzymatic reactions: phosphorylation by protein kinases and dephosphorylation, catalyzed by phosphatases which reconstitute active pRB. The phosphorylation state of pRB fluctuates as the cell passes through the division cycle (Figure 3). In cycling cells, pRB is found in its active form (hypophosphorylated) only during the early G1 phase. Phosphorylation in late G1 phase by cyclin D-ckd4/6 and later on by cyclin E-cdk2, and its dephosphorylation in late M phase are considered to be the two critical events regulating pRB growth-arresting activity.

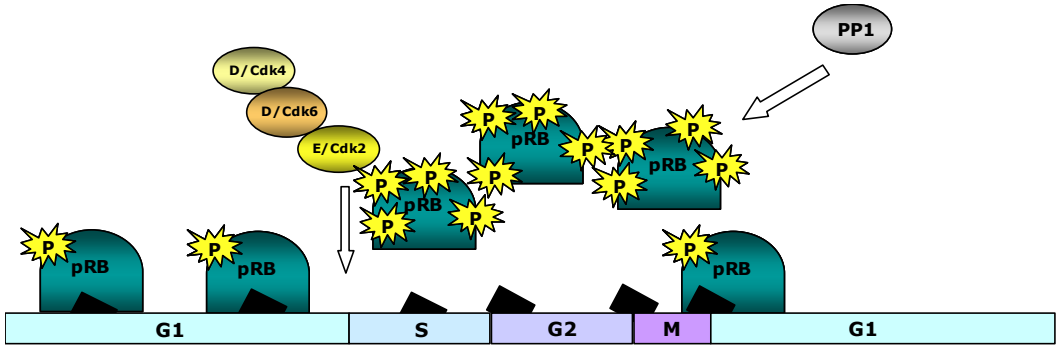


Figure 3. pRB phosphorylation state varies along the cell cycle due to kinase and phosphate activities. Adapted from [Mittnacht 1998]

There are other post-translational modifications, other than phosphorylation, that can regulate pRB activity such as acetylation. pRB acetylation is under cell-cycle control,

and acetylation hinders the phosphorylation of pRB by cyclin-dependent kinases. It has been proposed that acetylation may regulate protein-protein interactions in the pRB pathway [Chan et al., 2001].

THE E2F FAMILY

The E2F family of proteins consists of transcription factors which regulate the expression of key cell-cycle regulators. As mentioned before, activation of E2F is sufficient to irreversibly commit cells to undergo DNA replication. This places this family of transcription factors at a crucial position in the control of cellular proliferation in both normal and tumor cells. Because these proteins are crucial for the proper regulation of the cell cycle, understanding their possible functions and regulation is of vital importance. In fact, further studies of this transcription factor family implicates E2F regulation in mechanisms involved with DNA repair, replication, and G2/M checkpoints among others [Ren et al., 2002]

The E2F family encompasses two distantly related subfamilies, E2F and DP (differentiation regulated transcription factor (DRTF) protein). One subunit of E2F and one subunit of DP combine to form the E2F/DP heterodimeric complex that regulates the activity of promoters containing E2F binding sites. To date, eight members of the E2F subfamily of transcription factors, E2F1-E2F8, and two members of the DP subfamily, DP1-DP2, have been found in mammalian cells [Attwool et al., 2004 and references therein; Dyson 1998; Helin 1998] (Figure 4).

E2F and DP proteins contain highly conserved core domains that mediate DNA binding or dimerization with DP (leucine zipper (LZ) and marked box (MB) motifs). Sequences required for transcriptional activation and pocket-protein binding are only present in E2F1-E2F5. Moreover, E2F1, E2F2 and E2F3a+b share a canonical basic nuclear localization signal (NLS) that is not found in E2F4 and E2F5 which contain nuclear export signals (NES) instead. E2F6 diverges considerably and has almost no homology to the other E2F family members, apart from the core DNA-binding and dimerization domains. The C-terminal domain of E2F6 is required for the recruitment of Ring1 and

YY1 binding protein (RYBP) and is essential for the ability of E2F6 to repress transcription. The most notable feature of E2F7 is that it contains two DNA-binding domains and lacks the DP heterodimerization domain, suggesting that this protein recognizes the E2F consensus sites independently of a DP partner. E2F7, like E2F6, lacks the C-terminal domains necessary for transactivation and pocket protein binding, consistent with its inability to activate known E2F-responsive genes. It has been demonstrated that E2F7 function requires the integrity of its two DNA-binding domains and indicates that the interaction with DNA is as an E2F homodimer, instead of as an E2F-DP heterodimer [de Bruin et al., 2003; Logan et al., 2004].

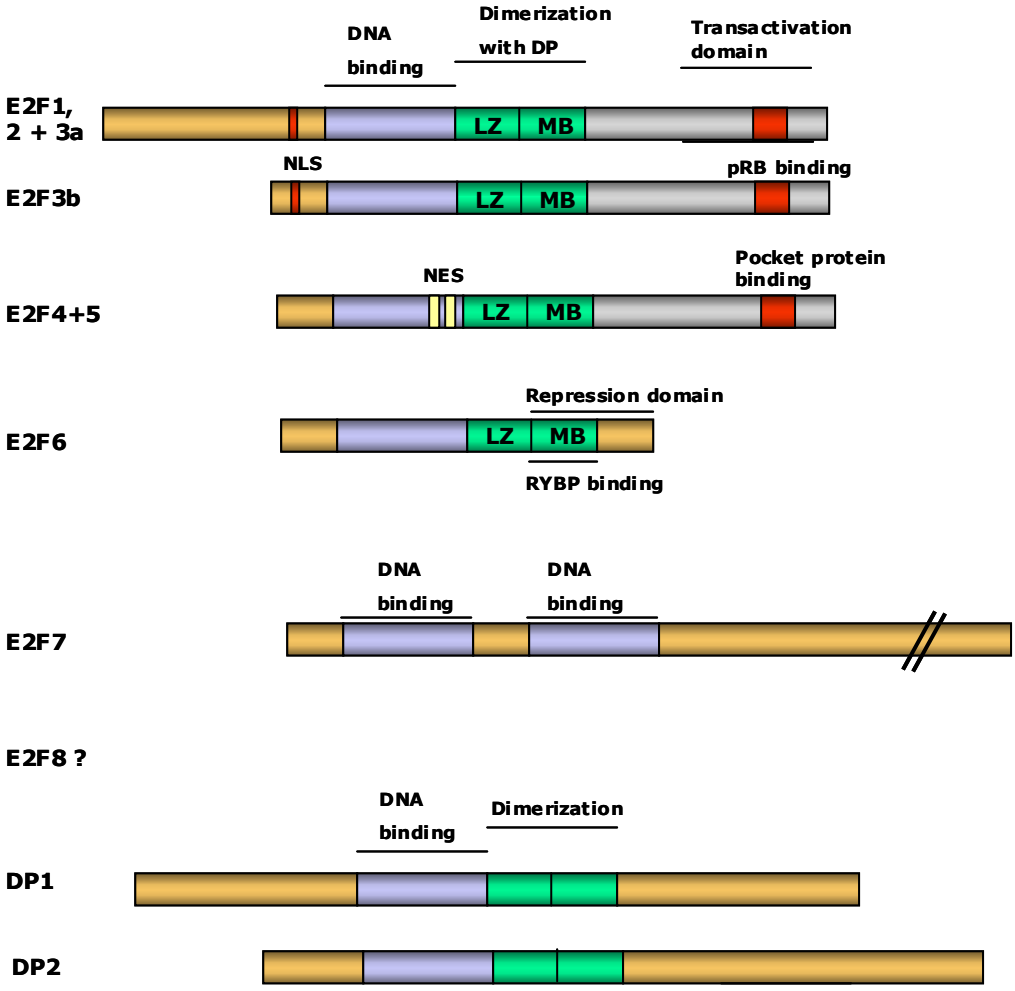


Figure 4. The organization of the eight E2F proteins and the two DP proteins. Partialy adapted from [Trimarchi and Lees 2002].

The E2F group can be divided into four subgroups based on their functional role and mechanism of action. The 'activating' E2Fs include E2F1, E2F2, and E2F3a and their

key role is to activate genes essential for correct progression through the cell cycle and for the induction of apoptosis. In contrast, E2Fs 4 and 5 are considered to be predominantly 'repressive' E2Fs. They are mainly nuclear in G0/G1 cells where they are bound to members of the pRB family [Müller et al., 1997; Verona et al., 1997]. Whereas the activating E2Fs described before are specifically regulated by pRB, E2F5 is mainly regulated by p130, and E2F4 associates with each of the pocket proteins at different points of the cell cycle. The major roles for E2F4 and 5 appear to be in the induction of cell cycle exit and differentiation [Lindeman et al., 1998; Gaubatz et al., 2000; Humbert et al., 2000; Rempel et al., 2000]. The other two subgroups consist of E2F6 and E2F7/E2F8 and are also considered to be transcriptional repressors [Morkel et al., 1997; Cartwright et al., 1998; Gaubatz et al., 1998; Trimarchi et al., 1998; de Bruin et al., 2003; Di Stefano et al., 2003]. The precise roles of these subgroups are as yet unclear although it seems as though E2F6 may play a role in quiescence and E2F7/8 is thought to regulate a subset of E2F target genes throughout the cell cycle.

Increasing numbers of genes bound by E2F in living human cells are being identified such as components of the DNA damage checkpoint and repair pathways, factors involved in chromatin assembly/condensation, chromosome segregation, and the mitotic spindle checkpoint. These data link cell cycle progression with DNA repair, replication, and G2/M checkpoints through E2F.

REGULATION OF E2F ACTIVITY

During the cell cycle, the activity and specificity of the E2F family members is tightly regulated and can be achieved via multiple mechanisms: through interaction with the pocket protein members (E2F1-5) or with the Polycomb Group (PcG) proteins (E2F6), phosphorylation or acetylation (E2F1-3).

Protein binding

While E2F1-3 bind preferentially to pRB, E2F4 is able to bind all the pocket proteins despite being regulated mainly by p107 and p130. When E2Fs are bound to pocket

proteins, their transactivating activity is inhibited. This inhibition is reversed when phosphorylation of the pocket proteins releases the E2Fs. This is the accepted 'classical' model for E2F function. In this model, E2F4-p130 is the predominant complex associated with target promoters in G0 and it inhibits the activation of E2F target genes involved in cell cycle progression. As the cell approaches the G1/S transition, E2F-pRB replaces the E2F4-p130 complex. pRB is phosphorylated by cyclin-cdk complexes, allowing target gene activation. In contradiction with this simple model, endogenous pRB has not been found on any human E2F target promoter during the normal cell cycle [Takahashi et al., 2000; Wells et al., 2000]. Indeed, E2F4-p107/p130 appears to be the complex predominant in the G1 phase and it is replaced by free activating E2Fs as the cell enters S phase.

E2Fs 6 and 7 do not bind to pocket protein and therefore are not regulated through the previously described mechanism [Morkel et al., 1997; Cartwright et al., 1998; Gaubatz et al., 1998; Trimarchi et al., 1998; de Bruin et al.; 2003; Di Stefano et al., 2003]. E2F6 interacts and forms complexes with the PcG proteins, but it is not yet clear if this interaction is regulatory [Trimarchi et al., 2001; Ogawa et al., 2002]. In addition to the fact that E2F7 does not require DP factors for DNA binding, interacting factors have yet to be discovered, suggesting another level of diversity [Di Stefano et al., 2003] (Figure 5).

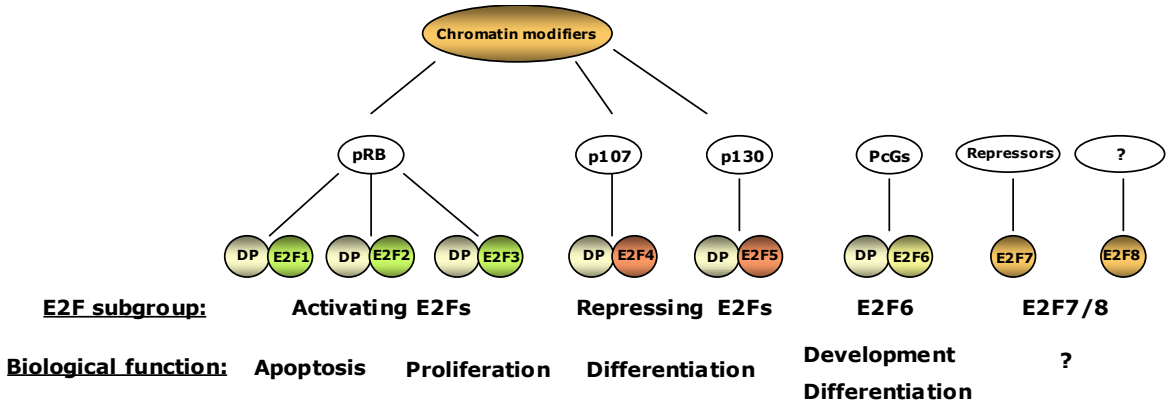


Figure 5. E2F family is divided into four subgroups according to their regulation (pocket proteins) and their physiological function (i.e. activation or repression). Adapted from [Attwool et al., 2004].

Phosphorylation

Phosphorylation of E2F1 protein has been described to control E2F1 protein half-life affecting total E2F1 activity in the cell. Stabilization of E2F1 by phosphorylation has been observed in response to DNA damage where proteins like Ataxia telangiectasia mutated (ATM), ATM and Rad3-related (ATR) or Chk2 are capable of phosphorylating E2F1 resulting in protein induction and stabilization, respectively [Lin et al., 2001; Stevens et al., 2003]. These two kinases are involved in DNA damage signaling to p53. The fact that both are capable of upregulating E2F1 emphasizes the interplay between the DNA damage signaling pathway and the pRB/E2F1 pathway. Furthermore, these results suggest that phosphorylation regulates E2F-dependent apoptosis independent of p53 (further described in this introduction).

On the other hand, phosphorylation of E2F has also been shown to trigger its degradation by the proteosome. This is the case for phosphorylation of E2F1 by cdk7 [Vandel and Kouzarides 1999] and the alternative reading frame (ARF) protein [Martelli et al., 2001; Mason et al., 2002]. In the first case cdk7, a kinase subunit of TFIIH, phosphorylates E2F1 at serine 403 and threonine 433 of its transactivation domain, localized at the pRB-binding site. Vandel and Kouzarides suggest that phosphorylation of E2F1 at these sites increases E2F1 ubiquitination and therefore triggers its degradation during S phase by the proteosome. Phosphorylation at these sites did not play a major role either in the transactivation capacity of the E2F1 transactivation domain, nor in the repression by pRB. On the other hand, Martelli et al. showed that p19ARF (mouse homologue of human p14ARF), interacts with E2F1 *in vivo* and targets E2F proteins for degradation dependent on the ubiquitin/proteasome system. Mason et al. further showed how p14ARF (human homologue of p19ARF) prompts E2F-dependent apoptosis. The interaction domain was mapped to the central DNA binding domain region of E2F1.

Acetylation

Recently, it has become clear that acetylation is a post-translational modification with a much wider functional role than the modification of histones to increase transcription. The diversity of substrates and regulation of different functions makes acetylation comparable to phosphorylation.

Acetylases are specific and modify very few lysines within a given protein. The transfer of an acetyl group from acetyl coenzyme A to the ε-amino group of a lysine residue, was initially discovered with histone proteins [Vidali et al., 1968] but it has been shown to occur in about 40 transcription factors (including E2F family members) and over 30 other proteins. This modification is reversible *in vivo*, with its specificity and level being controlled by signal-dependent association of substrates with acetyltransferases and deacetylases [for further details see Kouzarides 2000 and references therein].

N-ε-acetylation not only neutralizes the positive charge of the lysine side chain, but also impairs its ability to form hydrogen bonds [Yang 2004]. This translates into effects on protein-protein, protein-DNA and protein-RNA interactions, as well as on subcellular distribution and protein stability. Therefore acetylation exerts multifaceted effects through 'loss-of-function' and 'gain-of-function' mechanisms. One or the other consequence depends on where within the protein acetylation takes place. An example of 'loss-of-function' is the blocking of ubiquitination or disruption of DNA binding. Examples of 'gain-of-function' mechanisms are the increase in the DNA binding capacity and the generation of docking site for bromodomain-containing coactivators [Prives and Manley 2001; Brooks and Gu 2003]. A bromodomain is a protein module, that has recently been described to possess the ability to recognize (and bind to) acetyl-lysine motifs [Zeng and Zhou 2002]. Acetyllysine recognition by bromodomains reiterates the signaling principle that a modified residue serves as a ligand or generates a binding site for protein modules able to recognize the modification.

In the case of E2F family member, Marzio et al. demonstrated that E2F1, 2, and 3, but not E2F4, 5 and 6 associate with, and are acetylated by, p300 and cAMP response

element-binding protein (p300/CBP) acetyltransferases. Acetylation occurs at three conserved lysine residues (lysines 117, 120 and 125) located at the amino (N) -terminal boundary of their DNA binding domains, and results in enhanced DNA binding (further studied for E2F1) and stimulates its transcriptional activity. This acetylation can be reversed by histone deacetylase enzymes (HDAC-1) [Marzio et al., 2000].

Similar results were obtained by Tony Kouzarides and co-workers around the same time for E2F1 [Martínez-Balbás et al., 2000]. They described how the p300/CBP associated factor P/CAF, and to a lesser extent p300/CBP itself, can acetylate E2F1 *in vitro,* and that intracellular E2F1 is acetylated. Acetylation of E2F1 by P/CAF not only increased DNA-binding affinity and activation potential but also protein half-life. The residues that are acetylated are not directly involved in the DNA binding of E2F1 and therefore it is possible that acetylation may increase the DNA-binding capacity through a conformational change that allows better access to DNA. Increase in the activation potential may be due to an increase in the ability of E2F1 to recognize certain promoters while the mechanism through which the protein half-life is increased is still to be elucidated.

In addition, Pediconi et al. demonstrated the requirement of P/CAF to assemble an active E2F1 complex on the P1p73 promoter in order to induce apoptosis in response to DNA damage [Pediconi et al., 2003].

Others

E2F family protein activity is also regulated by gene expression and subcellular localization. Whereas the repressive E2Fs (E2F4 and 5) are expressed constitutively, the activating E2Fs (E2F1-3) are induced just before the G1/S transition point during the cell cycle. On the other hand the activating E2Fs are localized in the nucleus in cycling cells while the repressive E2Fs appear in the nucleus of non-cycling cells.

The converging body of evidence of the past few years suggests that the different mechanisms of control do not act isolated in time and space. For example, it has been

shown how histone-modifying enzymes may play a pivotal role in the regulation of E2F1 by the complex pRB-E2F1. First, pRB binds to an 18-amino-acid motif within the transactivation domain of E2F and blocks E2F ability to recruit the transcriptional machinery. Second, the pRB-E2F complex retains its ability to bind to the promoter of E2F-responsive genes and recruits various factors that influence the chromatin structure of these genes or E2F itself (in case of HDAC), leading to transcriptional repression. These factors include histone deacetylase enzymes (HDACs) and histone methyltransferase SUV39H1 (not discussed in this introduction). In parallel, cell cycle entry is dependent on the sequential activation of the cell-cycle-dependent kinases, cyclin D-cdk4/6 and cyclin E-cdk2, which phosphorylate pRB and cause it to release E2F (Figure 6).

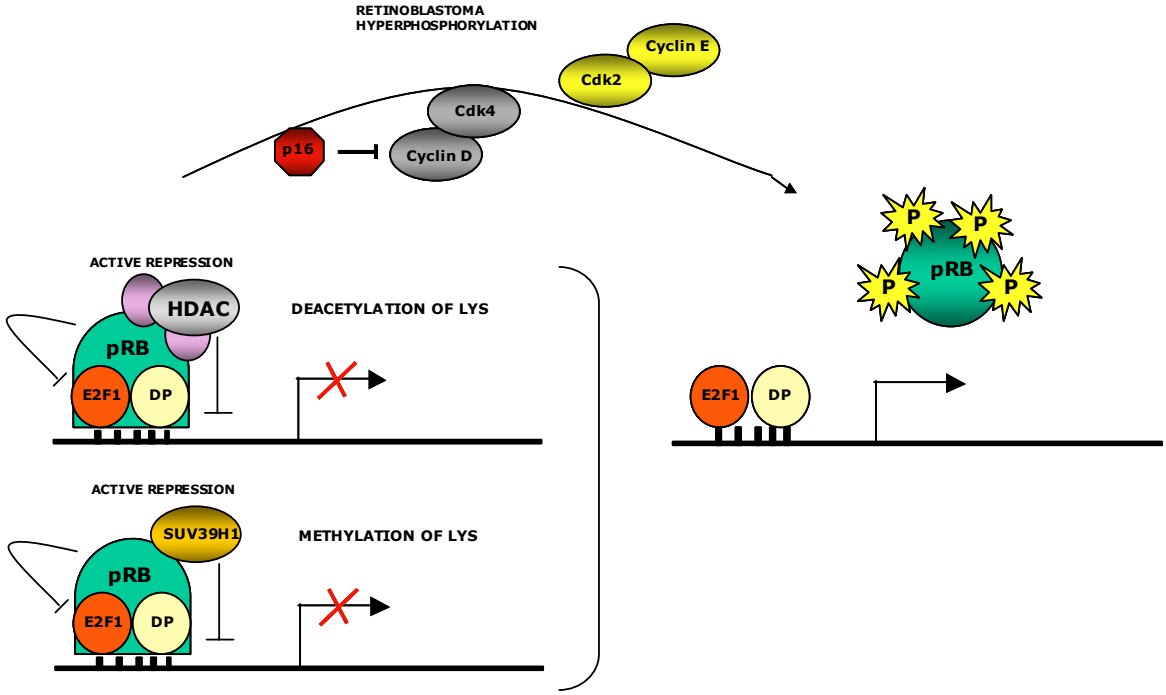


Figure 6. General mechanisms that regulate E2F activity. Adapted from [Trimarchi and Lees 2002].

E2F FUNCTIONS

Since its discovery, the knowledge of the functions of the E2F family of transcription factors have markedly expanded. What started as a simple model of positive regulation of genes required for DNA synthesis by E2F has become a complex network in which members of the E2F family play a pivotal role in mitosis, chromosome segregation, mitotic spindle checkpoints, DNA repair, chromatin assembly/condensation, apoptosis, differentiation, and development. Clearly the transcriptional regulation by E2Fs is more complex than previously thought and a simple explanation cannot be given. In addition redundancy of function exists between E2F members from the same subgroup and therefore the resulting phenotype is often a consequence of the balance between the different E2Fs [DeGregori 2002].

Among the E2F family members, E2F1, the founding member of the family, displays properties of both an oncogen (induction of proliferation) and tumor suppressor (induction of apoptosis). As a consequence, E2F1 has further been studied in order to explain the existence of these two antagonistic functions in which E2F1 is the key protein which decides if a cell must divide or initiate apoptosis [La Thangue 2003]. It appears that the level of expression and cellular environment influences whether E2F1 can function as an oncogene promoting the proliferation of cells beyond their normal constraints, or a tumor suppressor by inducing apoptosis (Figure 7) [Pierce et al., 1999].

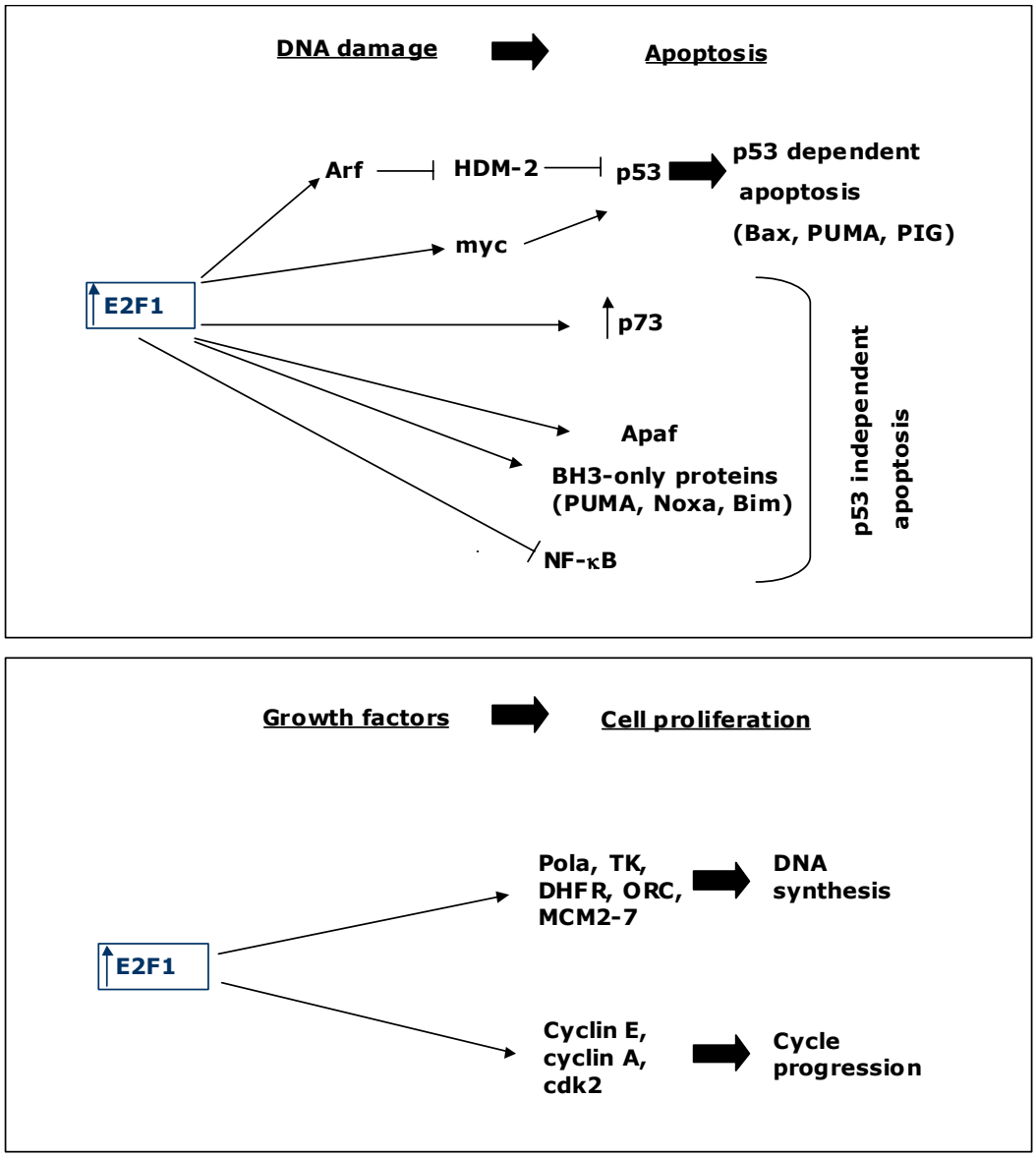


Figure 7. E2F1 double role: oncogene and tumor suppressor. E2F1 is activated as a result of pRB hyperphosphorylation and depending on the cell insult, activates genes involved in cell cycle progression or apoptosis.

Cell proliferation

E2F1 activity controls the transcription of a group of genes that encode proteins important for cell cycle progression including cyclin A, cyclin E, pRB, Cdc6, E2F1 itself, dihydrofolate reductase (DHFR), and a component of the pre-replication complex, among others [Wells et al., 2002; DeGregori et al., 1995; Araki et al., 2003; Lavia and Jansen-Durr 1999]. In cell proliferation control, the E2F1 transcription factor

is a critical target of the action of pRB as a growth suppressor. The interaction of pRB with E2F1 results in inhibition of E2F transcriptional activity and directly correlates with the ability of pRB to arrest cell growth in G1 [Hiebert 1993]. As mentioned before (see 'restriction (R) point'), during normal cell cycle progression, G1-cdks phosphorylate pRB (and p107 and p130), preventing pRB from binding to E2F and repressing E2F-dependent transcription.

Apoptosis

Some clarity has been shed on signalling pathways that regulate E2F1 apoptosis, supporting a function for DNA damage-dependent signal transduction. Different mechanisms have been described through which E2F1 can induce apoptosis (Figure 7) [Ginsberg 2002; La Thangue 2003; Stevens and La Thangue 2003; Bell and Ryan 2004; Knezevic and Brash 2004]:

-p53-dependent apoptosis

This pathway involves the transactivation of the p14ARF protein by E2F1. This can occur either directly through the transactivation of an E2F-binding site in the Arf promoter or indirectly through the activation of death-associated protein (DAP) kinase. Arf binds to the ubiquitin ligase HDM-2 (MDM2 in mice) to hinder the down-regulation of p53 and increase p53-dependent apoptosis. In this case E2F1 functions upstream of p53 leading to p53 stabilization and upregulation. Later on it has been discovered that E2F1 and myc could activate p53 even in the absence of Arf, indicating that there is an alternative pathway the cells can utilize [Sherr 1998]. p53 would then activate apoptosis by its strong pro-apoptotic function exerted through activation of Bax, PUMA or PIG [El-Deiry 1998].

Recently, Knezevic and Brash proposed that E2F1 is not only upstream of p53, but that both reinforce and control each other by an autoregulatory loop or network [Knezevic and Brash 2004]. Their observations of the role of E2F1 in apoptosis induced by ultraviolet light in mouse keratinocytes and fibroblasts can be explained because p53

lies functionally upstream of E2F1 and controls its activity through the activation of the p21-cdk4-pRB axis, displacement of DP1, or upregulation of MDM2 (Figure 8) [Wikonkal et al., 2003].

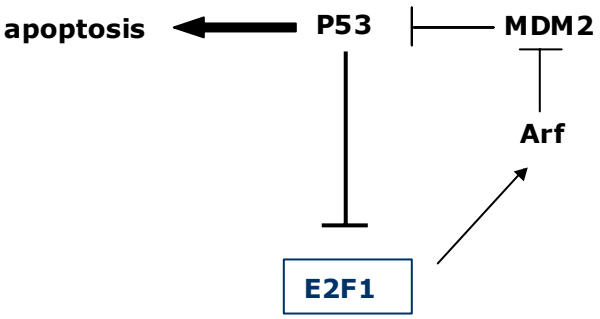


Figure 8. A p53 feedback loop regulates E2F1 pro- and antiapoptotic functions.

-p53-independent apoptosis

E2F1 can initiate apoptosis in a p53-independent manner by upregulating the pro-apoptotic proteins p73 [Irwin et al., 2000] or Apaf-1 [Moroni et al., 2001]. p73 is a p53 homolog and it has been demonstrated that E2F1 activates its transcription leading to the activation of targets genes and subsequent apoptosis. Some of the p73 target genes are shared with p53 [Yang and McKeon 2000]. When analyzing responses to DNA-damage it was observed that E2F1 is acetylated triggering the transcriptional activation of p73 [Pediconi et al., 2003]. The Apaf-1 gene encodes for apoptosis protease-activating factor 1 [Moroni et al., 2001; Furukawa et al., 2002]. When Apaf-1 is induced, it binds to cytochrome *c*, a mitochondrial protein released upon apoptotic signals, and activates procaspases 9. This leads to the activation of downstream effector caspases, including caspase-3, 6 and 7. Although E2F1 could activate transcription of Apaf-1 indirectly through p53, evidence suggests direct activation.

In NIH3T3 cells E2F1 up-regulates the expression of the proapoptotic BH3-only proteins PUMA, Noxa, Bim through a direct transcriptional mechanism [Hershko and Ginsberg 2004]. In B-amyloid-treated cortical neurons E2F1 has been reported to mediate death in a manner independent of p53 and dependent on Bax and caspase 3 [Giovanni et al., 2000].

Another mechanism by which E2F1 triggers apoptosis is by disrupting NF-kB signaling [Phillips et al., 1999; Tanaka et al., 2002]. NF-kB is a transcription factor that regulates cell survival in response to death-promoting stimuli by transcriptional activation of various apoptotic genes [reviewed in Karin and Lin, 2002].

E2F1 can disrupt NF-κB in several ways. E2F1 down-regulates TRAF2 (NF-κB activator) protein levels, and leads to impaired NF-κB activation. The mechanism through which E2F1 down-regulates TRAF2 is unknown but it does not require E2F1-dependent transactivation. Another way is by competing with p50 for RelA/p65 binding, which inhibits active dimer formation and therefore inhibits NF-κB DNA binding activity [Tanaka et al., 2002]. Finally E2F1 has been shown to impede NF-κB translocation to the nucleus by inhibiting phosphorylation of its cytoplasmic inhibitor protein IκB.

-implication of other E2F members in apoptosis

Although apoptosis induced by the E2F family is mainly related to E2F1, studies with knockout mice have begun to implicate the other E2F family members [Cam and Dynlacht 2003] (Figure 9). Loss of E2F1 in $pRB^{-/-}$ mice partially suppresses the incidence of pituitary and thyroid tumors [Yamasaki et al., 1998]. Whether E2F2 and E2F3 contribute to tumor suppression in a $pRB^{-/-}$ background is being studied. Ziebold et al. reported that E2F3 loss in $RB^{-/-}$ mice rescues most of the abnormal proliferation and apoptosis associated with $RB^{-/-}$ embryos [Ziebold et al., 2001]. This phenotype is reminiscent of the one obtained by Tsai and colleagues in which loss of E2F1 in $RB^{-/-}$ mouse embryos suppressed almost all of the p53-dependent apoptosis associated with $RB^{-/-}$ embryos [Tsai et al., 1998]. Therefore, it is easy to speculate that loss of E2F3 might contribute to a lower incidence of pituitary and thyroid tumors in mice also deficient for pRB. On other hand, E2F2 may also act as a tumor suppressor since E2F2 deficiency further predisposes $E2F1^{-/-}$ mice to additional tumor development [Zhu et al., 2001]. Finally, $E2F4^{-/-}$ and $E2F5^{-/-}$mice have been generated but died as neonates making it impossible to assess the compound loss of both E2F4 and E2F5 on tumorigenesis [Gaubatz et al., 2000]. Nevertheless, studies in cell lines show that both

E2F4 and E2F5 could induce apoptosis with an appropriate DP partner [Loughran and La Thangue 2000].

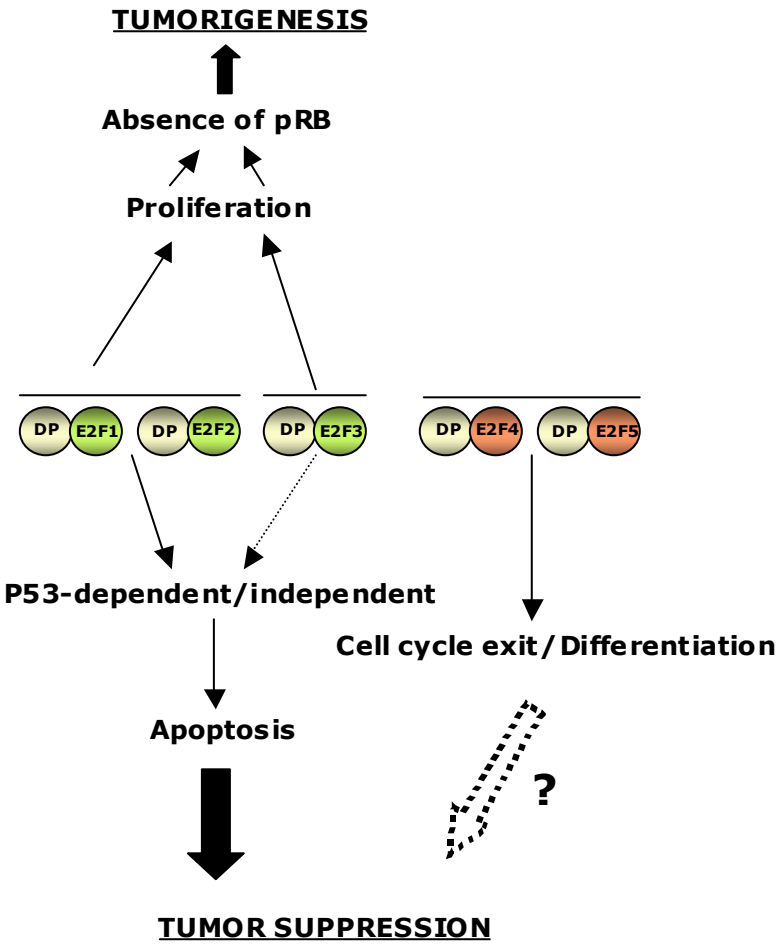


Figure 9. Other E2F members, apart from E2F1, contribute to tumorigenesis and tumor suppression. Adapted from [Cam and Dynlacht 2003].

REGULATION OF E2F THROUGH DIFFERENT SIGNAL TRANSDUCTION PATWHAYS.

As outlined before E2F is mainly regulated by its binding to pRB proteins. This interaction is regulated by the cyclin D-cdk4/6 complex which phosphorylates pRB, triggering E2F liberation from pRB repression. Many of the control mechanisms of E2F transcriptional activity affect cyclin D, cdk4/6, or both. Cdks are initially activated by their association with the cyclin subunit and by phosphorylation on a threonine residue that is located in a conserved amino acid sequence. Phosphorylation stabilizes the cdk, allowing for optimal ATP and substrate binding [Russo et al., 1996]. Thus

phosphorylation and dephosphorylation of crucial amino acids modulate cdks activity. The cdk activating kinase (CAK, complex cdk7-cyclin H) is involved in the phosphorylation of cdk while the Cdc25 phosphatase family is responsible for the dephosphorylation.

Increase in cyclin D protein levels is mediated both by ERK1/2-dependent and phosphatidylinositol 3-kinase (PI 3-kinase)-dependent mechanisms [Lukas et al., 1996a; Kuemmerle et al., 2004]. Extensive studies, reviewed by Roovers and Assoian [Roovers and Assoian 2000], show that the mitogen-induced amplitude and duration of ERK activity regulates transcription of cyclin D1. The phosphatidylinositol 3-kinase (PI 3-kinase)/protein kinase B (PKB) pathway regulates cyclin D translation through mTOR activation [Robin et al., 1998] and in a mTOR-independent way in some cell lines [Takuwa et al., 1999]. Cyclin D transcription is also promoted through the inactivation of forkhead transcription factors by the PI 3-kinase/PKB pathway. In addition, Hallstrom and Nevins demonstrated that suppression of E2F1 apoptotic activity during normal cellular proliferation requires the action of the Ras-phosphoinositide 3-kinase-Akt/PKB signalling pathway [Hallstrom and Nevins 2003]. Although the precise mechanism remains unknown, PKB was able to suppress E2F1-induced apoptosis. In many of the PKB effects on cellular processes, glycogen synthase kinase-3-beta (GSK3β) is the direct downstream target of PKB. GSK3β phosphorylates cyclin D1 and β-catenin (which increases cyclin D expression), stimulating the degradation of both proteins through the ubiquitin-proteasome pathway [Diehl et al., 1998; Aberle et al., 1997]. Inhibition of GSK3β through phosphorylation by PKB promotes cyclin D1 and β-catenin stabilization and therefore cell cycle progression.

Regulation of Cdc25 is also affected by different signal transduction pathways. TGF-β–mediated G1 growth arrest specifically targets inactivation of cyclin D-cdk4/6 complexes by down-regulation of Cdc25 and cdk4 protein levels [Ewen et al., 1993; Hannon and Beach 1994; Iavarone and Massague 1997]. Cdc25 is phosphorylated by the Chk1 protein kinase in response to DNA damage [Furnari et al., 1997; Furnari et al., 1999]. This phosphorylation implies Cdc25 functional inactivation and thus, cdk dephosphorylation and activation.

This and other data demonstrate that E2F1 and its effects are regulated by more than one signal transduction pathway. GSK3β regulates E2F1 indirectly, through the degradation of cyclin D and β-catenin, but this does not exclude direct control or even an autoregulatory loop between them.

GLYCOGEN SYNTHASE KINASE-3 (GSK3)

GSK3 was named for its ability to phosphorylate, and thereby inactivate, glycogen synthase. However new discoveries show that GSK3 plays an important role in many cellular processes including glycogen synthesis, differentiation, proliferation, and transformation [Ferkey and Kimelman 2000].

There are two isoforms of mammalian GSK3, encoded by distinct genes: GSK3α and GSK3β [Woodgett 1990], as well as a recently identified splice variant of GSK3β with substrate and subcellular differences (GSK3β2) [Mukai et al., 2002]. GSK3α has a mass of 51 KDa, whereas GSK3β is a protein of 47 KDa. The difference in size is due to a glycine-rich extension at the N-terminus of GSK3α. The two isoforms share nearly identical sequences in their kinases domains (98% identity), but differ in the last 76 C-terminal residues (only 36% identity). Homologues of GSK3 exist in all eukaryotes examined to date and display a high degree of homology [reviewed in Ali et al., 2001]. Although structurally similar and sharing many targets, GSK3α and GSK3β are not functionally identical and are differentially expressed in tissues.

More than 40 proteins have been reported to be phosphorylated by GSK3. Although there is not a strict consensus motif for substrate phosphorylation by GSK3, many GSK3β targets require prior phosphorylation by a priming kinase to form the motif -SXXXS(P)- before phosphorylation by GSK3 is possible. Considering the broad spectrum of possible GSK3 targets (not all of them are proven *in vivo* targets), precise control of GSK3 activity is necessary. This is achieved by a combination of phosphorylation, cellular localization, and interactions with GSK3-binding proteins.

REGULATION OF GSK3 ACTIVITY

GSK3 is subject to multiple mechanisms of regulation. Although phosphorylation of serine 9 in the case of GSK3β, or phosphorylation of serine 21 in the case of GSK3α, is the most widely studied mechanism of regulation, there are others. Protein complex formation, intracellular localization, and mood stabilizing drugs have important regulatory influences on GSK3β activity. These other control pathways and the signals that activate them are not fully understood. This is the case in its response to DNA-damage where there are differences in the GSK3β activation state, which could be explained by the activation of the pathway by different agents or a cell type-specific response. For example, when cells were exposed to Ionizing Radiation in human osteosarcoma cell line SAOS-2, GSK3β kinase activity was inhibited rather than enhanced [Turenne and Price 2001]. In contrast, in human SH-SY5Y and H1299 cells, DNA damage induced by camptothecin and etoposide activated GSK3β [Watcharasit 2002].

Regulation by phosphorylation

GSK3 activity is reduced by phosphorylation of an N-terminal serine, serine 9 in GSK3β and serine 21 in GSK3α. Several kinases can phosphorylate these serines, including PKB (PI 3-kinase pathway activated by insulin), p90RSK (MAPK pathway activated by growth factors) and p70S6K (p70 ribosomal S6 kinase activated by amino acids), among others. It must be kept in mind that each kinase probably affects only a specific pool of the GSK3 present in cells due to the subcellular distribution of GSK3 and each regulatory kinase. The phosphorylation of GSK3 at the indicated amino acids inhibits activity and therefore results in the dephosphorylation, and in most cases, activation, of their substrates. The molecular mechanism that explains how these residues when phosphorylated affect GSK3 activity is their ability to transform the N-terminus into a pseudosubstrate. As a pseudosubstrate the N-terminus blocks the binding site used by the GSK3 substrates which require a priming phosphorylation.

Activation of GSK3β by phosphorylation at tyrosine 216 of GSK3β was observed by Hughes et al. [Hughes et al., 1993]. Wang et al provided evidence for autophosphoryation of this site [Wang et al., 1994], while Murai and coworkers found that this phosphorylation, and thus GSK3β activity is regulated by intracellular signaling systems [Murai et al., 1996]. However, the physiological significance of phosphorylation at tyrosine 216 of GSK3β in mammalian cells is still unclear since the phosphorylation appears constitutive in resting cells [Hughes et al., 1993] and the kinase(s) responsible has not yet been found.

Regulation by intracellular localization

Mechanisms that regulate GSK3 cellular localization determine its access to substrates. Although GSK3 is predominantly cytosolic, it is also present in the nucleus and mitochondria, where it is highly activated (5 to 10 fold) compared to cytosolic GSK3 [Bijur and Jope 2003a]. The GSK3 nuclear pool is not static but changes in response to intracellular signals. Nuclear levels fluctuate during the cell cycle and are at the highest in the S phase and during the process of apoptosis. Cell cycle-dependent changes in the intracellular localization of GSK3β were detected by Diehl and co-workers [Diehl et al., 1998], who found increased nuclear GSK3β during the S phase in NIH3T3 cells [Alt et al., 2000]. Bijur and Jope observed that proapoptotic stimuli induce nuclear accumulation of GSK3β [Bijur and Jope 2001]. This accumulation is inhibited by PKB, an effector of the PI 3-kinase pathway, with antiapoptotic activity mentioned before. Activated PKB is imported into the mitochondria where it phosphorylates serine 9 of GSK3β to inhibit its activity, without changing the mitochondrial level of GSK3β [Bijur and Jope 2003b]. This indicates the GSK3 pools are independently regulated.

Regulation by binding proteins

Binding of GSK3 to GSK3 binding protein (GBP) family members and to Axin family members causes inhibition and activation of GSK3β, respectively. It has also been reported that the binding of GSK3 to *cubitus interruptus* (Ci) inhibits Ci activity [Price

and Kalderon 2002; Jia et al., 2002]. This suggests that a scaffolding protein might facilitate the process and ensure specificity but this has to be further studied.

Formation of specific protein complexes for a subcellular organelle has also been noted. This is the case of Frat-1 which binds GSK3 in the nucleus and facilitates its export [Franca-Koh et al., 2002]. Within the mitochondria, p53 binds GSK3β and it seems this interaction may facilitate apoptosis [Watcharasit et al., 2003].

GSK3 FUNCTIONS

GSK3 is a fascinating enzyme with an astoundingly diverse number of actions in intracellular signaling systems. GSK3 has been shown to phosphorylate *in vitro* and possibly *in vivo* a large number of proteins, the outcome of which is to regulate the stability of the target protein [Frame and Cohen 2001 and references therein; Doble and Woodgett 2003; Jope and Johnson 2004], enhance transcriptional activity [Turenne and Price 2001] and regulate subcellular localization [Zhou et al., 2004]. GSK3β is thought to phosphorylate cyclin D1 at threonine 286 and the transcription factor c-myc at threonine 58, which targets these proteins for ubiquitylation and subsequent proteolytic destruction [Alt et al., 2000; Diehl et al., 1998; and Sears et al., 2000]. In the case of GSK3β phosphorylation of p53 at serine 33 has been described to increase p53 transcriptional activity while phosphorylation of Snail controls its subcellular localization. Snail is a zinc-finger transcription factor controlling motility and invasiveness of cancer cells. GSK3β binds to and phosphorylates Snail at two consensus motifs to regulate the function of this protein in two ways: phosphorylation of the first motif regulates its beta-Trcp-mediated ubiquitination, whereas phosphorylation of the second motif controls its subcellular localization [Zhou et al., 2004]. These various effects add more complexity to further understanding this kinase. Putative proteins phosphorylated and modulated by GSK3β are listed in Table 1.

Metabolic and signaling proteins	Structural proteins	Transcription factors
AcetylCoA carboxylase	Dynamin-like protein	AP-1 (Jun family)
Amyloid precursor	Kinesin light chain	β-Catenin
APC	MAP1B	C/EBPα
ATP-citrate lyase	MAP2	C/EBPβ
Axin	MUC1/DF3	CREB
Cyclic AMP-dependent protein kinase	NCAM	Glucocorticoid receptor (rat)
Cyclin D1	Neurofilaments	HSF-1
eIF2B	Ninein	MITF
Glycogen synthase	Tau	Myc
IRS-1		NFAT
Myelin basic protein		NFκB
NGF receptor		Notch
Nucleoporin p62		p53
Protein Kinase A (RII subunit)		
Protein phosphatase 1		
Protein phosphatase inhibitor-2		
Pyruvate dehydrogenase		
Presenilin-1		

Table 1. Proteins reported to be phosphorylated by GSK3β.

GSK3 putative substrates are numerous and the effect of phosphorylation by GSK3β varies: inhibition of activity and/or binding, increase of activity, target for degradation or determination of cell localization between others.

APC, adenomatous polyposis coli gene product; C/EBP, CCAAT/enhancer-binding protein, CREB, cyclic AMP response element-binding protein; MUC1/DF3, high molecular weight mucin-like glycoprotein; eIF2B, Eukaryotic initiation factor 2B; HSF-1, heat shock factor-1; KRP, kinase related protein; IRS-1, Insulin receptor substrate-1; MAP, microtubule-associated protein; MITF, microphthalmia-associated transcription factor; NCAM, Neural cell-adhesion protein; NFAT, nuclear factor of activated T cells; NF-κB, nuclear factor kB; NGF, nerve growth factor.

The substrates listed can be found in the following references: Frame and Cohen 2001 and references therein; Grimes and Jope 2001 and references therein; Doble and Woodgett 2003 and references therein; Jope and Johnson 2004 and references therein.

Considering all the putative targets listed above and the distinct effect of GSK3β on them we can imagine GSK3β affects in one way or another the following cellular aspects: metabolism (glycogen synthase, eIF2B), cellular architecture and motility (MAP1B, Tau), and cell survival/apoptosis (eIF2B, p53). In metabolism, GSK3β phosphorylates glycogen synthase, inhibiting glycogen syntheses, and eukaryotic initiation factor 2B (eIF2B), inhibiting protein synthesis. Phosphorylation of eIF2B not only inhibits protein synthesis but also facilitates apoptosis and release of cytochrome *c* from the mitochondria [Pap and Cooper 2002]. Binding to p53 also facilitates apoptosis through induction of cytochrome *c* release from the mitochondria. GSK3β seems to act on several targets during apoptosis, but these might vary with cell type and insult. The exact mechanisms are not clear yet. GSK3β controls motility and cellular architecture of neurites inhibiting growth cone extension [Eickholt et al. 2002]. MAP1B and Tau, microtubule-associated proteins (MAPs) that play a central role in regulating microtubule function, are phosphorylated by GSK3β. GSK3β regulates microtubule dynamics.

II OBJECTIVES

OBJECTIVES

Historically, E2F1 has been associated exclusively with its function as a transcription factor that is essential for the G1/S transition. Recent results have changed this view, however, and E2F1 has now been implicated in other cellular processes such as cell-cycle arrest and apoptosis. It has been suggested that the levels of this protein could be the biological sensor which determines the process to be performed. In addition, other signal transduction effectors are needed to define cell processes.

The activation of the PI 3-kinase pathway is determinant in the cell decisions in which E2F1 participates. Activation of this pathway inhibits the apoptotic effect of E2F1 overexpression. GSK3β is an important physiological substrate of this pathway and plays a role in several apoptotic processes. Our working hypothesis is that phosphorylation or binding of GSK3β to E2F1 are important signals that regulate E2F1 activity and, as a consequence, cell fate.

Therefore the objectives of this thesis were defined as follows:

-1. Analysis of the phosphorylation of E2F1 by GSK3β *in vitro* and *in vivo.*

-2. Study of the interaction between GSK3β and E2F1 *in vitro* and *in vivo*.

-3. Study of the regulation of E2F1 transcription factor by GSK3β.

III RESULTS

Results1. Phosphorylation of human E2F1 *in vitro* and *in vivo*

PHOSPHORYLATION OF HUMAN E2F1 *IN VITRO* AND *IN VIVO*

INTRODUCTION

Previous results of our group have demonstrated the involvement of the PI 3-kinase pathway in the regulation of E2F1 activity. These results were obtained by studying the regulation of the proliferative-specific F-type isoform of the 6-PF2K/Fru-2,6-BPase [Fernandez de Mattos et al., 2002]. PI 3-kinase contributes positively to the activation of the F-type 6-PF2K/Fru-2,6-BPase transcription by EGF. Activation of PI 3-kinase is necessary and sufficient for this activation, which is inhibited or completely abrogated in the presence of the PI 3-kinase inhibitor LY 294002 and the kinase dead catalytic subunit p110, respectively. We later showed that the transcription factor E2F is a target of the PI 3-kinase/PKB pathway involved in the control of 6PF2K/Fru-2, 6-BPase expression [Fernandez de Mattos et al., 2002]. In this study, the nuclear protein binding to the E2F-binding site localized in the F-type promoter of the 6PF2K/Fru-2, 6-Bpase was analyzed under a range of experimental conditions.

Although no data have been reported to indicate that the E2F transcription factor is a direct target of PKB, there is strong evidence that PKB regulates the transcriptional activity of E2F indirectly through one of its targets [Brennan et al., 1997]. One of the PKB targets described is GSK3β. For many years GSK3β was considered as a protein kinase whose function was confined to glycogen metabolism. However, study of GSK3β kinase has taken center stage again since it was found to be involved in several of the biological processes regulated by PKB such as regulation of metabolism [Cross et al., 1995], protein synthesis [Kandel and Hay 1999 and references therein] and the cell cycle [Diehl et al., 1998]. During the cell cycle, GSK3β was found to indirectly regulate E2F activity through phosphorylation of cyclin D1, resulting in an increase of cyclin D1 degradation [Diehl et al., 1998]. Nevertheless, GSK3β also seems to be a potential candidate as a PKB downstream effector on E2F, particularly given that in *Drosophila*, CycD-Cdk4 does not act as a direct G_1/S-phase regulator [Datar et al., 2000].

Here we examine whether E2F is directly controlled by GSK3β–mediated phosphorylation. From all the members of the E2F family, we chose E2F1 because of its role in the activation of genes that are essential for cellular proliferation and induction of apoptosis. These functions make it a good candidate as a GSK3β downstream effector. Unlike E2F2 and E2F3, E2F1 contains GSK3β phosphorylatable sequences (further discussed below).

RESULTS

1.1 Computational analysis of GSK3β phosphorylation sites in E2F1 protein with Scansite 2.0.

In order to evaluate the possible phosphorylation sites of GSK3β in human E2F1 amino acid sequence we used Scansite (http://scansite.mit.edu). This search method is built on experimental binding and/or substrate information from oriented peptide library screening and phage display experiments together with detailed biochemical characterization, and aims to predict protein-protein interactions and sites of phosphorylation [Obenauer et al., 2003].

One can search for motifs recognized by commonly occurring domains within a protein sequence of interest, or search an entire protein sequence database for optimal motif matches. Threshold values are given in order to decide which scores are likely to be real interactions. The lower the score, the higher the chance of a hit. Moreover, three levels of stringencies can be chosen to control the restriction of the analysis. The high stringency setting is the most restrictive and reports a "hit" only if the score falls within the top 0.2% of scores when the motif matrix of interest is applied to the vertebrate subset of SWISS-PROT. The medium and low stringencies are set at 1% and 5%, respectively.

We searched the Scansite database for GSK3 phosphorylation motifs in human E2F1 protein (SwissProt Accession Number Q01094) applying all three stringencies. Figure

1.1 shows the information predicted by Scansite. Up to ten possible phosphorylation sites were predicted when the predictions from all stringency searches were included (Panel A and Table B). The query protein (in this case human E2F1) is represented schematically as a line, with colored rectangle marking the known E2F-TDP domain. The E2F-TDP domain is the E2F/DP family winged-helix DNA-binding domain. Labels above the protein indicate where the motifs were found and identify the motif family (acidophilic serine/threonine kinase group in our case). The prediction for surface accessibility is also plotted and the scale at the bottom marks numbered intervals along the protein sequence.

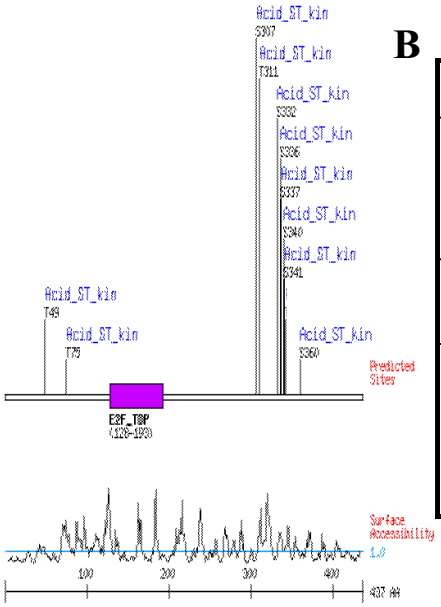
The table under Panel B of Figure 1.1 summarizes the Motif Scan output results. For each GSK3 site on the graphical output (Panel A), the stringency, position of the site, score, percentile and surface accessibility are indicated. As explained earlier, a higher stringency value indicates a more restrictive screening condition. Therefore, hits predicted under high stringency conditions (S341, S336 and S337 in our case) are most likely to be phosphorylated *in vivo*.

On Panel C of Figure 1.1, the human E2F1 amino acid sequence is displayed with the predicted phosphorylation sites indicated in the colors that correspond to the respective stringencies indicated in the table under Panel B.

If one searches for other kinases motifs (e.g. Akt, PKC, PKA, Erk1, cdk5) on human E2F1 using Scansite, many of them have putative phosphorylation sites which are a serine or threonine residue located four residues carboxy-terminal to the site of the predicted GSK3 phosphorylation. This would be consistent with the hierarchal phosphorylation behavior described for GSK3. This mechanism gives GSK3 unique substrate specificity in cases in which, substrates must first be phosphorylated by another protein kinase. After this prime phosphorylation GSK3 can phosphorylate more than one residue, as long as it follows the motif **-SXXXS(P)-,** creating a cluster [Roach 1990, Frame et al., 2001]. In this motif, **X** stands for any amino acid and **S(P)** stands for a previously phosphorylated serine localized four residues C-terminal to the site of GSK3 phosphorylation. For example, residues S336, S337, S360, S332 and S307 have

one or more Scansite-predicted kinase/s that could potentially phosphorylate position n+4. These are residues S340 (GSK3α), S341 (GSK3α), S364 (Akt, PKC$_{\alpha/\beta/\gamma}$, PKA, PKC$_{\mu}$, Calmodulin dependent kinase 2), S336 (GSK3α, Erk1 kinase) and T311 (GSK3α,cdk5, cdc2, Erk1 kinase).

A



B

Stringency	Site	Score	Percentile	Surface Accesibility
High	S341 S336 S337	0.4265 0.4283 0.4391	0.069% 0.071% 0.089%	1.093 2.353 2.039
Medium	S340 T311	0.5215 0.5544	0.435% 0.738%	1.171 2.349
Low	S360 T75 T49 S332 S307	0.5731 0.5951 0.6035 0.6035 0.6153	1.118% 1.611% 1.846% 1.846% 2.275%	0.401 2.097 0.799 0.591 0.652

C

1 malagapagg pcapaleall gagalrllds sqiviisaaq dasappaptg paapaagpcd
61 pdlllfatpq aprptpsapr palgrppvkr rldletdhgy laessgparg rgrhpgkgvk
121 spgeksryet slnlttkrfl ellshsadgv vdknwaaevl kvqkrriydi tnvlegiqli
181 akksknhiqw lgshttvgvg grlegltqdl rqlqeseqql dhlmnicttq lrllsedtds
241 qrlayvtcqd lrsiadpaeq mvmvikappe tqlqavdsse nfqislkskq gpidvflcpe
301 etvggispgk tpsqevtsee enratdsati vsppspspps slttdpsqsl lsleqeplls
361 rmgslrapvd edrlsplvaa dsllehvred fsgllpeefi slspphealđ yhfgleegeg
421 irdlfdcdfg dltpldf

Figure 1.1. Motif Scan graphical output for human E2F1.

Analysis of the GSK3β kinase motif on human E2F1 protein. (A) Schematic representation of human E2F1 protein. Known E2F-TDP domain is indicated as a purple box and GSK3 motifs found labeled above the protein. Below the protein scheme, a plot of the predicted surface accessibility at each residue is shown. (B) Table that summarizes Motif Scan output. For each predicted site on the graph (A), the stringency, score, percentile and amino acid of the site are indicated, as is the calculated surface accessibility from that site. (C) Human E2F1 amino acid sequence (Swiss Prot Q01094). Predicted GSK3 hits are indicated in different colors according to their stringency as in Table (B). *In vivo* phosphorylated sites according to Phosphosite ^{TM} database are underlined.

The results of this computational analysis presented here are strictly valid only for GSK3α because the hyperlink of the Scansite output to its GeneCard reference domains with an entry in the Weizman Institute´s GeneCard database (http://bioinformatics.weizmann.ac.il/cards) lists only GSK3α but not GSK3β as the best matching domains for GSK3 phosphorylation. Since our interest is centered on GSK3β phosphorylation motifs due to its participation in the PI 3-kinase signaling pathway, we wanted to assess whether our Scansite results were also valid for the GSK3β isoform. We compared GSK3α and GSK3β amino acid sequences in order to determine the homology of the catalytic domains. There is 76% of identity which rises to 84% if the positive homologies are also considered. If the comparison is restricted to the kinase domain, homology between the two isoforms increases to 98%. In addition, amino acids with a key role in the activation loop of GSK3β are fully conserved in GSK3α. Residues R96, R180 and K205 of GSK3β, which interact with the "prime phosphate", correspond to R159, R243 and K268 on GSK3α. Moreover, Y216 of GSK3β, acting as a gate to the substrate-binding groove, corresponds to Y279 of GSK3α. Furthermore, a similar substrate specificity *in vivo* and a parallel regulation in response to growth factors has been described for the two isoforms [Kim and Kimmel 2000; Xavier et al., 2000; Eldar-Finkelman et al., 1996; Cross et al., 1995]. If all these results are considered together, the comparison of the two isoforms strongly suggests an identical activation mechanism for GSK3α and GSK3β, and that the predicted domains to be phosphorylated are equally valid for both isoforms.

Scansite also allows a link to PhosphoSite™, which is a sequence-oriented protein database that collects the published data about known *in vivo* phosphorylation sites. The *in vivo* phosphorylation sites given for E2F1 are (underlined in Figure 1.1 Panel C): S31 [Lin et al., 2001], S332 and S337 [Fagan et al., 1994], S364 [Stevens et al., 2003] and S403 and T433 [Vandel and Kouzarides 1999]. ATM phosphorylates serine 31 in response to DNA damage and induces E2F1. p34cdc2 kinase phosphorylates E2F1 at serine 332 and serine 337, modulating its interaction with the retinoblastoma gene product and the adenoviral E4 19kDa protein. On the other hand, Chk2 activates E2F1 in response to DNA damage by phosphorylation of serine 364 and cdk7 phosphorylates E2F1 in residues 403 and 433, increasing its degradation by the proteosome. Two of these experimentally described phosphorylation sites, S332 and S337, coincide with

predicted GSK3 kinase sites for Cdc2 kinase at medium stringency, which reinforces the value of Scansite as a motif prediction tool. Another site, S364, phosphorylated by Chk2 is localized at n+4 from a predicted site for GSK3 therefore it may be a 'priming' phosphorylation site.

Considering Scansite GSK3 motif predictions at all the three stringencies, we can assign 10 putative GSK3 phosphorylation sites to E2F1. This is consistent with the hypothesis that E2F1 is a direct target of GSK3.

1.2 Kinase assay of GST fusion proteins with purified GSK3β *in vitro*.

In order to experimentally test the previously predicted *in silico* phosphorylation sites of human E2F1 by GSK3β, we performed an *in vitro* kinase assay using glutathione S-transferase (GST)-fusion proteins.

The Glutathione S-Transferase Gene Fusion System is an integrated system for the expression, purification and detection of fusion proteins produced in *E.coli* (further described in Material and Methods). Fusion proteins are easily purified from bacterial lysates by affinity chromatography using Glutathione Sepharose 4B. Cleavage of the desired protein from GST can then be achieved, when required, by using a site-specific protease whose recognition sequence is located immediately upstream of the multiple cloning site on the pGEX plasmids. Furthermore, fusion proteins can be detected using a colorimetric assay or an immunoassay. All these qualities makes this system useful for studies involving protein-protein interactions.

Here, we used the GST-Fusion System in a preliminary step to check whether commercially purified rabbit recombinant histidine-tagged GSK3β can phosphorylate human E2F1 *in vitro.*

We first assayed E2F1 *in vitro* phosphorylation using purified GSK3β (60mU) together with 3μg of GST-hE2F1 (fused full human E2F1 protein) protein as substrate (kind gift

from Dr. Eric Lam). GST protein alone served as a negative control (Figure 1.2, Panel A). The results show a unique phosphorylated band of 94 KDa, which corresponds to the purified GST-hE2F1. In contrast, no signal was detected for purified GST protein alone. This clearly indicates that GST-hE2F1 is phosphorylated by purified GSK3β *in vitro* under our experimental conditions. Absence of GST phosphorylation confirms that the fusion protein GST-hE2F1 is phosphorylated on E2F1 and not on GST. Protein loading was checked by Coomassie staining (Figure 1.2 Panel B).

In order to assess the specificity of this *in vitro* phosphorylation we repeated the assay and included GST-DP1 (the fused full human DP1 protein) which binds to E2F1 to form the functional heterodimer [Trimarchi and Lees 2001], GST-CycD1 (fused full human cyclin D1 protein), a described substrate of GSK3β [Diehl et al., 1998] and GST-$P53_{1\text{-}70}$ (fused amino acids 1-70 of human p53 protein) for which phosphorylation at serine 33 by GSK3β in a non-DNA damage mechanism has been described [Turenne and Price 2001]. GST protein alone served as a negative control. GST-hDP1 was a kind gift from Dr. Eric Lam, GST-CycD1 and GST-$p53_{1\text{-}70}$ were kindly provided by the cloning service of the Department of Molecular Biology of the Faculty of Medicine, from *Universitat de Barcelona.*

GST-hE2F1 phosphorylation by GSK3β *in vitro* is specific and does not require priming phosphorylation (Figure 1.2, Panels C and D). We obtained a signal for GST-hE2F1 (94 KDa) which appeared to be stronger than the signals of the other GSK3β substrates assayed, p53 and cyclin D1. GST protein alone served as negative control and Coomassie staining of the gel (Figure 1.2, Panel D) shows equal protein loading. The experiment was performed in triplicate.

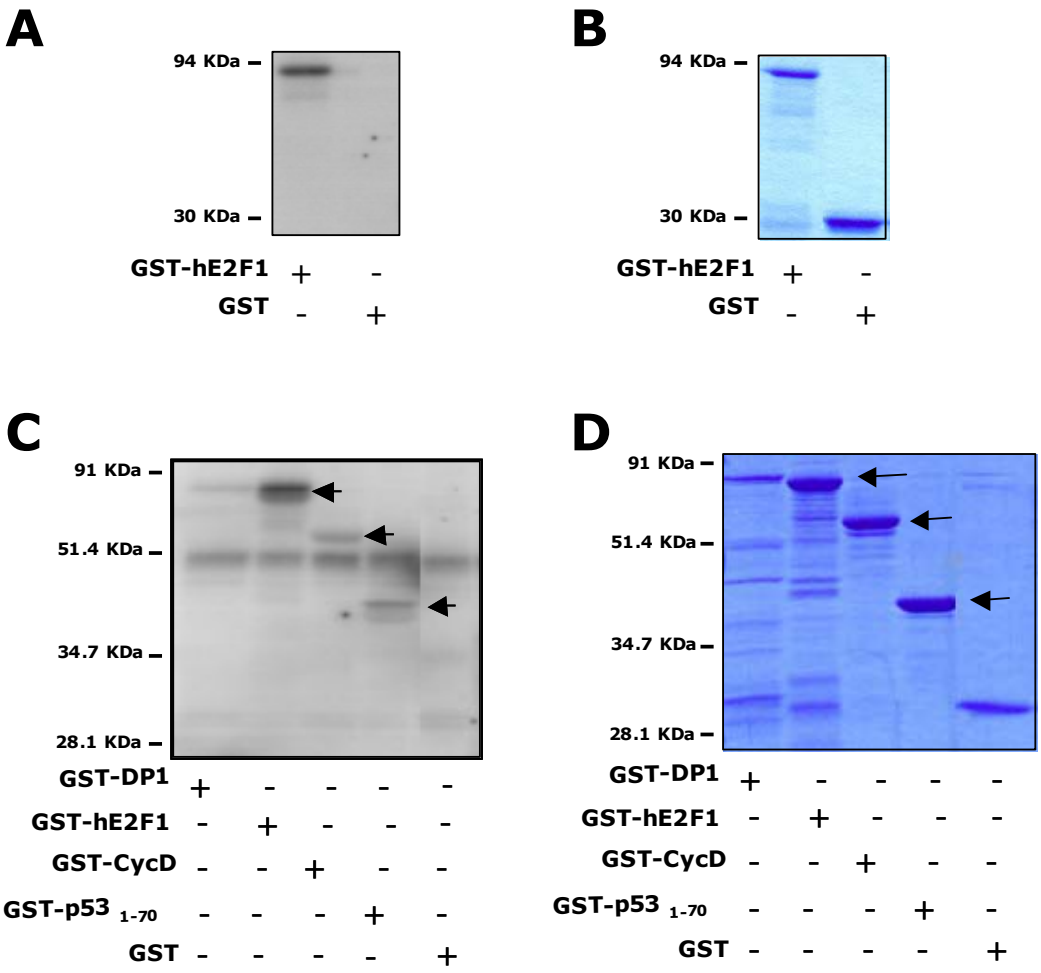


Figure 1.2. Kinase assay with purified GSK3β and GST-hE2F1 *in vitro*.

The same amounts (3μg) of different GST fusion proteins were used in an *in vitro* kinase assay in the presence of purified GSK3β (60mU). The reaction products were resolved on a 15% SDS-polyacrylamide gel and the phosphorylated proteins were visualized by autoradiography. (A) GSK3β phosphorylates GST-hE2F1 fusion protein and not GST protein alone. (B) Coomassie staining of gel (A). (C) Comparison of phosphorylation levels of known GSK3b substrates, GST-CyCD1 and GST-p53$_{1-70}$, with GST-DP1 and GST-hE2F1 phosphorylation. The latter appears to be a better *in vitro* substrate. (D) Coomassie staining of gel (B).

Under our *in vitro* kinase assay conditions, phosphorylation of all substrates (CycD1, p53$_{1-70}$ and E2F1) took place without priming phosphorylation of other kinases. Nevertheless, to assess whether priming phosphorylation increases GSK3β phosphorylation activity in a synergistic or additive way, the *in vitro* phosphorylation

assay of human E2F1 described in Figure 1.2 was repeated including a preincubation with p38. p38 was chosen because it phosphorylates the GSK3β substrate Tau (microtubule-associated protein Tau) in a sequential manner [Kyriakis and Avruch 2001] and may phosphorylate human E2F1 according to Scansite predictions.

A kinase assay was performed *in vitro* in the presence of purified GSK3β (60mU) and in the presence or absence of GST-hE2F1 (0,5 μg) as substrate. Purified p38 (20 mU) was either preincubated for 5 hours (5 h) with the substrate or added together with GSK3β. Phosphorylation of GST-hE2F1 and GSK3β autophosphorylation were evaluated.

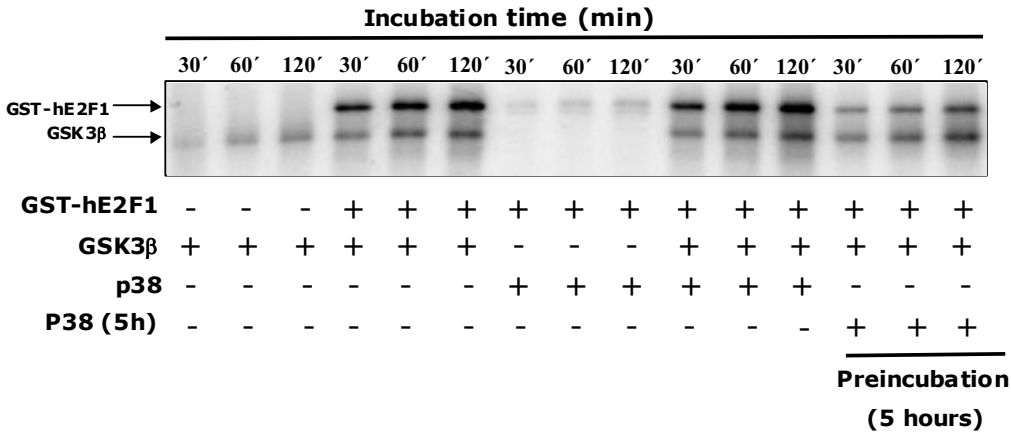


Figure 1.3. Effect of preincubation of p38 on GST-hE2F1 *in vitro* phosphorylation by GSK3β.

The same amount of substrate (0.5 μg GST-hE2F1) was used under the different conditions assayed: phosphorylation by purified GS3β (60mU), p38 (20mU) alone, or GSK3β after a 5-hour pre-incubation with p38 and cold ATP. Each of these conditions was tested at incubation times of 30, 60 and 120 minutes. The reaction products were resolved on a 15% SDS-polyacrylamide gel and the phosphorylated proteins were visualized by autoradiography.

GSK3β phosphorylates GST-hE2F1 in a time-dependent manner. p38 also phosphorylates GST-hE2F1, but to a lesser extent in all the three incubation times tested (Figure 1.3). Although we did not examine which E2F1 amino acid/s was phosphorylated by p38, the result supports the Scansite predictions. Furthermore, we

detected GSK3β autophosphorylation. The differential phosphorylation rates of GSK3β alone or together with p38 do not clarify whether the effect is additive or synergistic. They do, however, indicate that priming phosphorylation is not needed for the phosphorylation of human E2F1 by GSK3β *in vitro.*

1.3 Mapping of the phosphorylated sites of GST-hE2F1.

Since GST-hE2F1 appears to be specifically phosphorylated by GSK3β, we attempted to map the phosphorylated sites by liquid chromatography and mass spectrometry (LC-MS).

GST-hE2F1 is phophorylated *in vitro* by GSK3β (Figure 1.2). GST-hE2F1 band was excised from the gel, reduced with DTT, alkylated with iodoacetamide and cleaved with trypsin (Promega, sequencing grade), as described by Shevchenko and colleagues [Shevchenko et al., 1996]. An AspN cleavage followed (Boehringer).

Fig 1.4 shows the results of an LC-MS of tryptic and Asp-N digested GST-hE2F1. Amounts of radioactivity incorporated were quantified. Results are expressed in cherenkov counts of the fraction. There were two main peaks of radioactivity: fraction 13/14 with 27000 and 16000 cpm and fraction 24 with 20000 cpm.

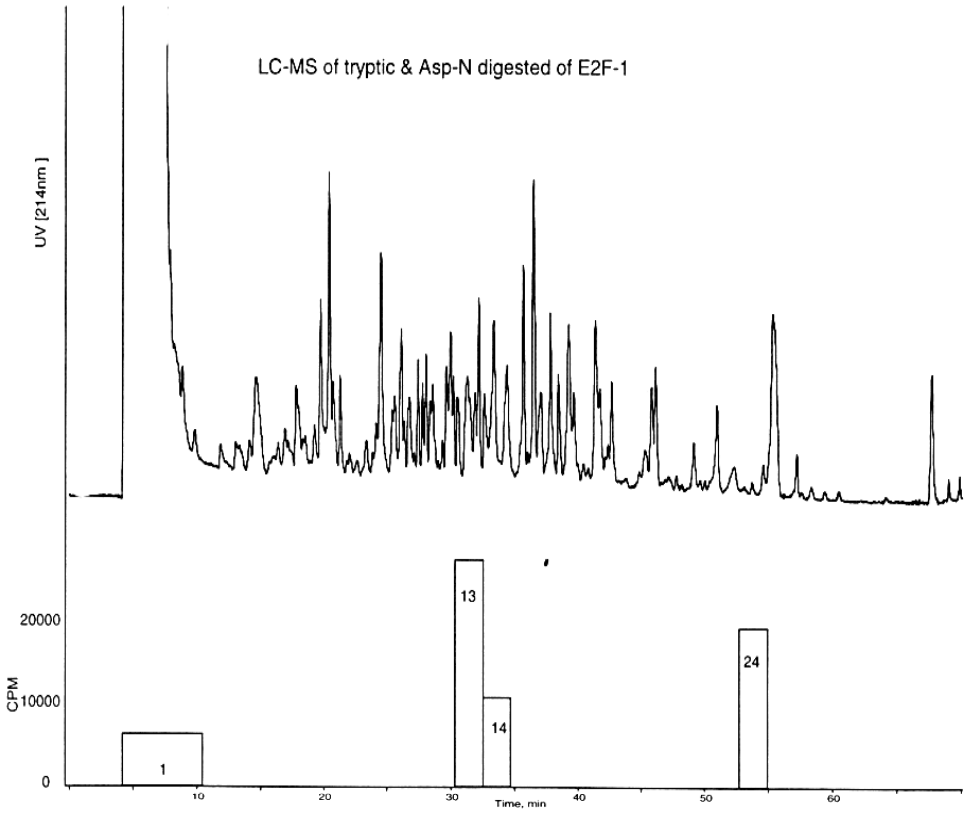


Figure 1.4. LC-MS of tryptic and Asp-N digested of GST-hE2F1.

Peptides were fractionated and analyzed by liquid chromatography interfaced with electrospray mass spectrometry (LC-MS) for the detection of phosphopeptides. There are two main peaks of radioactivity corresponding to fractions 13/14 and fraction 24.

To further identify the mass of the phosphopeptides, a precursor scan of m/z 79 $(PO_3)^-$ was performed (Figures 1.5 and 1.6). Fractions 13 and 24 gave a signal of 636 and 1202 cpm, respectively, while fraction 14 gave no signal for a phosphopeptide for reasons unknown.

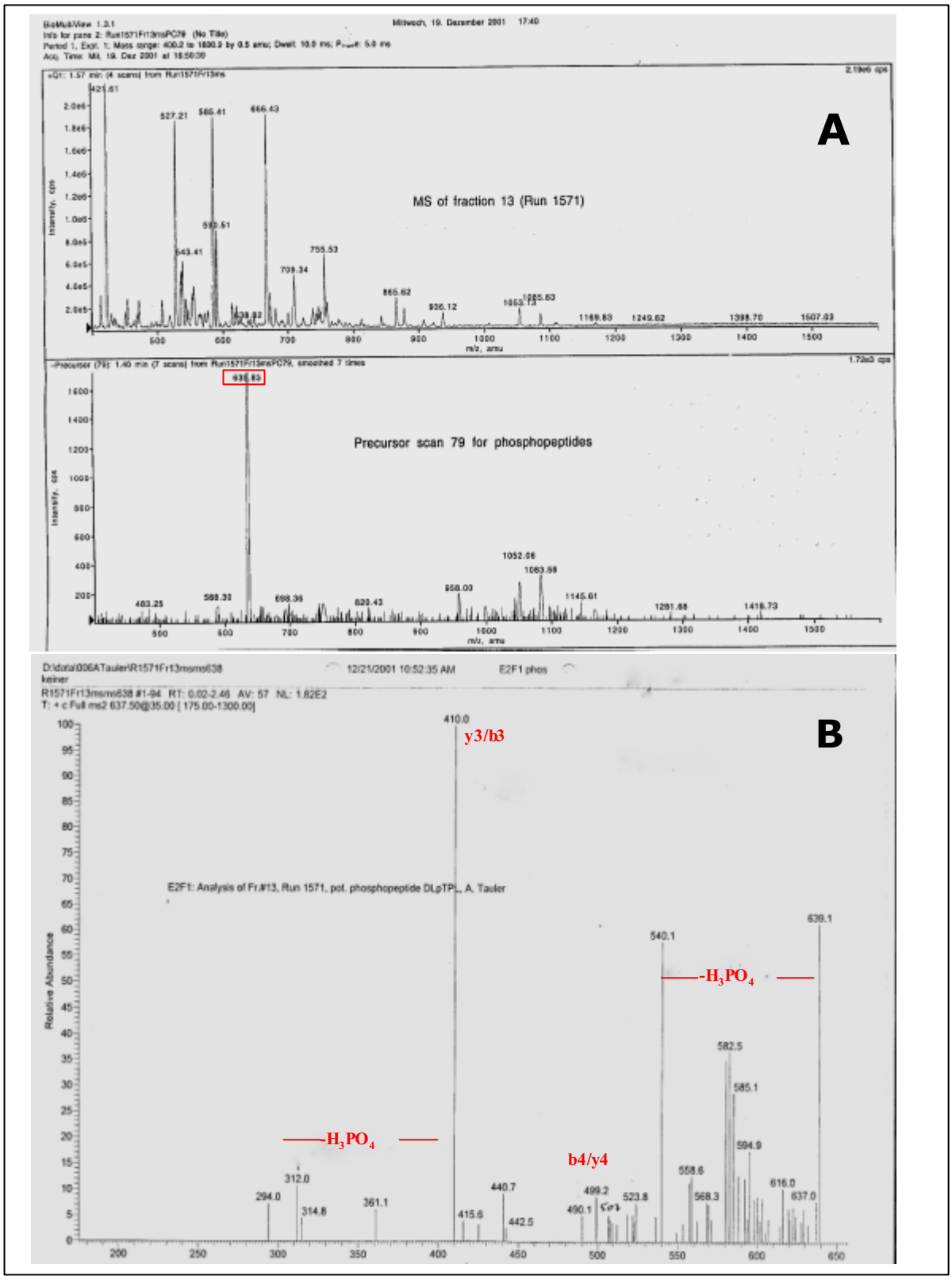


Figure 1.5. Analysis of fraction 13.

Precursor scan m/z 79 was performed on fraction 13. (A) Fraction 13 gave a signal of a m/z of about 636, single charged The only tryptic, AspN phosphopeptide of GST-hE2F1 with this mass is DLTPL. (B) The MS/MS spectra shows the y3/b3, y4 as well as H_3PO_4 loss of the precursor ion and the y3/b3 ion. This is consistent with the peptide $DLpT_{433}PL$.

As shown in Figure 1.5, the only tryptic AspN phosphopeptide of GST-hE2F1 (fraction 13) with a mass (m/z) of 637 is **DLTPL**. The tandem mass spectrometry (MS/MS) spectra showed the y3/b3, y4 ions, as well as H_3PO_4 loss of the precursor ion and the y3/b3 ion (Figure 1.5 Panel B). This is consistent with the peptide $DLpT_{433}PL$.

On the other hand, phosphopeptide from fraction 24 (see Figure 1.6 Panel A) has a mass (m/z) of 2406 and appears double negatively charged. The only tryptic, AspN phosphopeptide of GST-hE2F1 with this mass is **EDFSGLLPEEFISLS$_{403}$PPHEAL**. The MS/MS spectra showed y6, y7, y8, y9, y14 ions as well as b7, b12 and b14 ions (Figure 1.6 Panel B). Y7 ion (mass 830) is the fragment SPPHEAL still phosphorylated. Therefore serine is phosphorylated. To confirm that the ion 830 is y7, triple mass spectrometry (MS^3) was done on this fragment (Figure 1.6 Panel C). H_3PO_4 loss of the precursor as well as ions y6, y5 and b6 confirmed the fragment **S$_{403}$PPHEAL** phosphorylated on the serine.

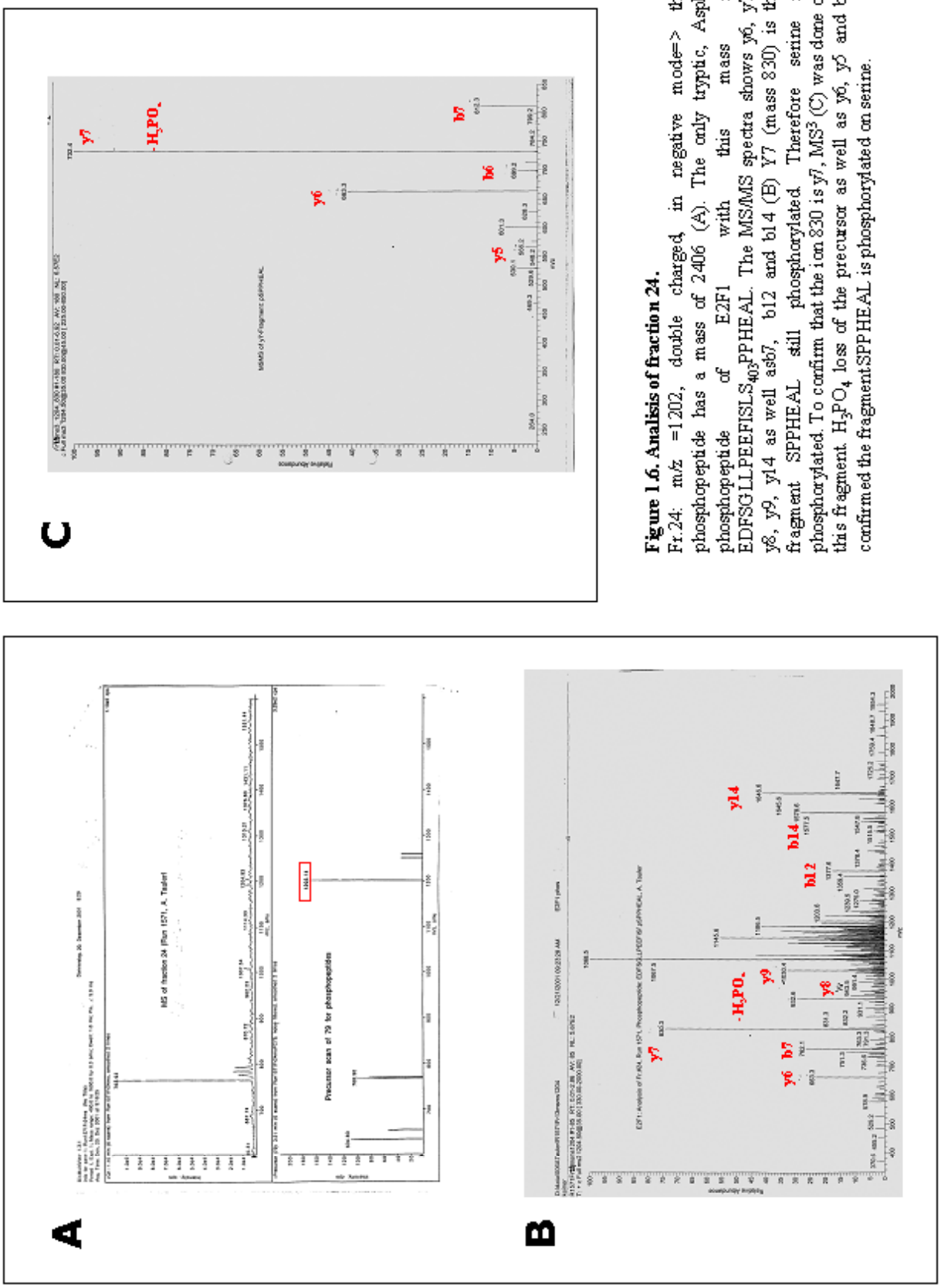


Figure 1.6. Analisis of fraction 24.
Fr.24: m/z =1202, double charged in negative mode=> the phosphopeptide has a mass of 2406 (A). The only tryptic, AspN phosphopeptide of E2F1 with this mass is EDFSGLLPEEFISLS$_{403}$PPHEAL. The MS/MS spectra shows y6, y7, y8, y9, y14 as well as b7, b12 and b14 (B) Y7 (mass 830) is the fragment SPPHEAL still phosphorylated. Therefore serine is phosphorylated. To confirm that the ion 830 is y7, MS^3 (C) was done on this fragment. H_3PO_4 loss of the precursor as well as y6, y5 and b6 confirmed the fragment SPPHEAL is phosphorylated on serine.

Taking all these results together, we have identified three fractions that gave a high phosphorylation signal. In two cases we could not identify the phosphopeptide down to the particular amino acid phosphorylated in it. In fraction 24, serine 403 (S403) of human E2F1 was the amino acid identified as being phosphorylated *in vitro* by GSK3β, while threonine 433 (T433) was the amino acid identified in fraction 13. For the third identified fraction (fraction 14), we were not able to depict any phosphopeptide. Nevertheless this fraction may be expected to contain at least one of the amino acids predicted by Scansite.

Laurence Vandel and Tony Kouzarides [Vandel and Kouzarides 1999] identified cdk7 as the component of the TFIIH that phosphorylated E2F1 on S403 and T433 (two putative phosphorylation sites for cdk7). TFIIH is a factor involved in both transcription and DNA repair which phosphorylates RNA pol II and nuclear receptors. Their results suggested that this TFIIH-mediated phosphorylation of E2F1 plays a role in triggering E2F1 degradation during S phase. However, kinase activity towards the E2F1 active domain (were S403 and T433 are localized) is not in agreement with cdk7 activity along the cell cycle phases. While cdk7 activity has been shown not to change during the G1, S and G2 phases of the cycle [Tassan et al., 1994], E2F1 degradation appears high in S phase and low in G1 phase. One explanation for this is that the binding of pRB to E2F1 could inhibit the binding of TFIIH and thus the phosphorylation of E2F1 [Nevins 1998]. As an alternative explanation, we propose that GSK3β could also play a key role in the regulation of E2F1 phosphorylation since its activity correlates with the E2F1 degradation cycle.

1.4 Analysis of phosphorylation of serine 403 and threonine 433 of human E2F1 *in vitro*.

To test whether GSK3β was phosphorylating human E2F1 at the two residues identified by LC-MS we performed *in vitro* kinase assays as described in section 1.2. and compared human E2F1 phosphorylation by GSK3β in GST-human E2F1 fusion proteins: wild-type or with the amino acids of interest (S403 and T433) mutated.

Five GST-E2F1 fusion proteins were available since positions S403 and T433 were studied as target of cdk7 phosphorylation [Vandel and Kouzarides 1999]. One of them contains the whole human E2F1 sequence while the other four contain the active domain (amino acids 380-437) with none, one, or two point mutations. A schematic representation of these constructs is shown in figure 1.7.

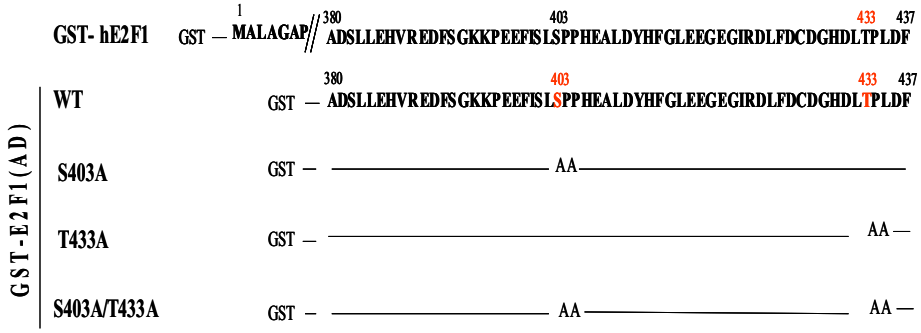


Figure 1.7. Schematic representation of the different GST fusion proteins.

GST-hE2F1: GST fusion protein containing whole human E2F1 sequence. GST-hE2F1 (AD): GST fusion protein containing E2F1 active domain.

AD stands for E2F1 active domain which comprises amino acids 380 to 437. WT: wild-type , S403A: point mutation of serine 403 and proline 404 to two alanines, T433A: point mutation of threonine 433 and proline 434 to two alanines. S403A/T433A: doble point mutation

We analyzed GSK3β phosphorylation of all these GST fusion proteins *in vitro* in order to assess if mutation of amino acids 403 and/or 433 implied reversibility of the phosphorylation previously observed in the wild-type form (see Figure 1.2).

The results obtained are shown in Figure 1.8 and revealed that when both, serine 403 (S403) and threonine 433 (T433), are mutated (together with prolines 404 and 434), purified GSK3β does not phosphorylate the human E2F1 active domain. When only one of the two putative target amino acids (S403 or T433) is mutated phosphorylation is reduced. The effect appears stronger in case of the S403A mutation. The integrity of the fusion proteins was checked by Western blot technique. Anti-GST blotting confirmed that the expression and purification of the recombinant proteins were successful for each construct (Figure 1.8 Panel B).

Taken together, these results support the data obtained in section 1.3, which identified amino acids 403 and 433 as the ones being phosphorylated by GSK3β *in vitro*.

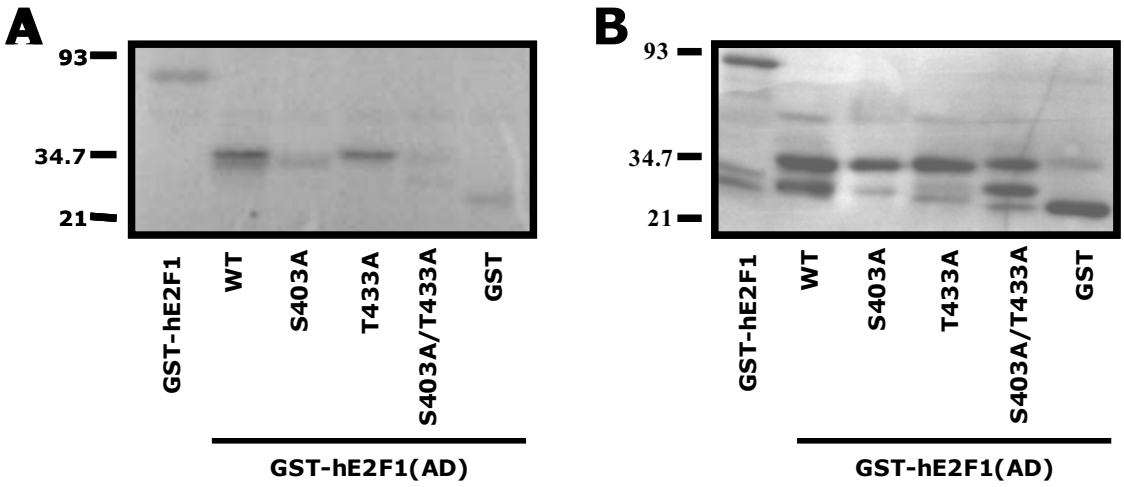


Figure 1.8. Kinase assay with purified GSK3β and GST-hE2F1, GST-E2F1(AD) WT, S403A, T433A and S403A/T433A *in vitro*.

(A) Kinase assay with the same amount (3μg) of GST-hE2F1, GST-E2F1(AD) WT, S403A, T433A and S403/T433A, or GST alone was performed *in vitro* in the presence of purified GSK3β (60mU). Reaction products were resolved on a 15% SDS-polyacrylamide gel and phosphorylated proteins were visualized by autoradiography. (B) Equal amounts of fusion proteins were subjected to SDS-gel electrophoresis followed by Western blotting as described in Material and Methods. Recombinant proteins were detected with a GST antibody.

1.5. Study of phosphorylation of endogenous human E2F1 *in vivo*.

In order to study phosphorylation of human E2F1 *in vivo* we performed pull-down assays from HeLa cells transfected with either wild-type GST-hE2F1(AD) or mutated GST-hE2F1(AD)S403A/T433A (Figure 1.7). GST alone served as negative control. A similar approach was used to demonstrate cdk7 binding to human E2F1 and its phosphorylation on serine 403 and threonine 433 [Vandel and Kouzarides 1999]. Whole HeLa cell extracts were incubated with equal amounts of the different GST-hE2F1(AD) fusion proteins in a pull-down assay (for more details see Materials and Methods). After extensive washes of the pull-down products the remainder was analyzed in a kinase assay. As shown in Figure 1.9., unspecific binding of GSK3β to GST protein alone was obtained under our experimental conditions. Additional experiments using different protocols failed to reduce this unspecific binding. This technique was no longer used to study E2F1 *in vivo* phosphorylation.

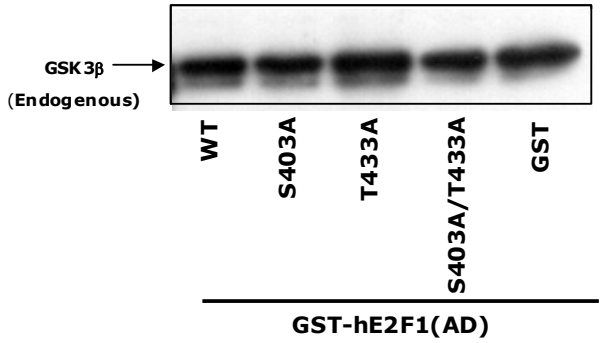


Figure 1.9. Unspecific binding of GSK3β on pull-down assay.

The same amounts (500ng) of wild-type and the mutated GST-E2F1(AD) fusion proteins, as indicated in Figure 1.7, were used in pull-downs from HeLa cell extracts. Pull-down products were resolved on a 12% SDS-polyacrylamide gel and the binding of endogenous GSK3β was checked by blotting against GSK3β protein.

On a second attempt to investigate the phosphorylation of E2F1 by GSK3β *in vivo* we performed a kinase assay overexpressing GSK3β protein in HeLa cells. Although other physical DNA transformation techniques such as $Ca_3(PO_4)_2$ co-precipitation, lipofection, or electroporation could have been used for this type of cell line, recombinant adenovirus were used since the amount of protein overexpressed by this

technique is higher. In addition, adenovirus infection provides a 60-100% efficiency of gene transfer versus the 10-20% obtained with the mentioned physical techniques.

Viruses were amplified, particle concentration was determined and expression of the GSK3β constructs was verified by Western blot of dilutions of whole transduced HeLa cell extracts. Furthermore, adenovirus constructs were sequenced to confirm the presence of point mutations (see Material and Methods for further details).

Adenovirus infection was normalized according to protein expression and not to the number of active particles, since we observed that a same value of plaque formation units (PFU) could give different levels of GSK3β, depending on the construct. Figure 1.10 shows immunoblotting of HeLa cells transduced with the adenovirus constructs, in which the amount of adenovirus used was normalized to ensure equal expression of the GSK3β proteins. The constructs were: wild-type GSK3β (Ad-WT-GSK3β), a catalytically inactive form containing two point mutations in the kinase domain (Ad-KM-GSK3β) and an adenovirus form in which the serine 9 of GSK3β regulatory site has been mutated to alanine (Ad-S9A-GSK3β) [Summers et al., 1999]. Four amplifications of the viruses were performed and the infective quantities determined: 40μl of Ad-S9A-GSK3β and 60μl of Ad-WT-GSK3β and Ad-KM-GSK3β per 150-mm culture dish of HeLa cells. Viruses were divided into aliquots for a single use.

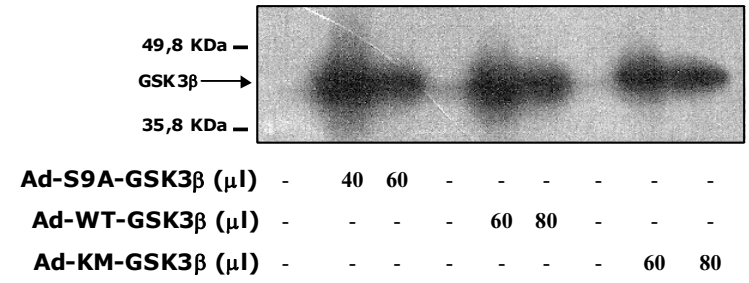


Figure 1.10. Normalization of Ad-S9A-GSK3β, Ad-WT-GSK3β and Ad-KM-GSK3β protein expression.

HeLa cells were infected in 150-mm culture dishes with the indicated amounts of Ad-S9A-GSK3β, Ad-WT-GSK3β or Ad-KM-GSK3β and incubated for 48 hours. Extracts were obtained and run on 10% SDS-PAGE gel, transferred to PVDF membranes and analyzed for the presence of GSK3β protein by Western blot with anti-GSK3β antibody, as described in Materials and Methods.

We measured the kinase activity of the infected cells to check the three different GSK3β adenovirus constructs used. HeLa cells were transduced with the infective quantities of each construct determined in Figure 1.10. Cells were incubated for 12 hours in the absence of foetal calf serum, the condition under which GSK3β is active. GSK3β was immunoprecipitated and a kinase assay was performed using Phospho-Glycogen Synthase Peptide-2 as substrate. Figure 1.11. shows the expected result in which transduction with Ad-WT-GSK3β and Ad-S9A-GSK3β shows higher GSK3β activity than the endogenous GSK3β alone or together with Ad-KM-GSK3β.

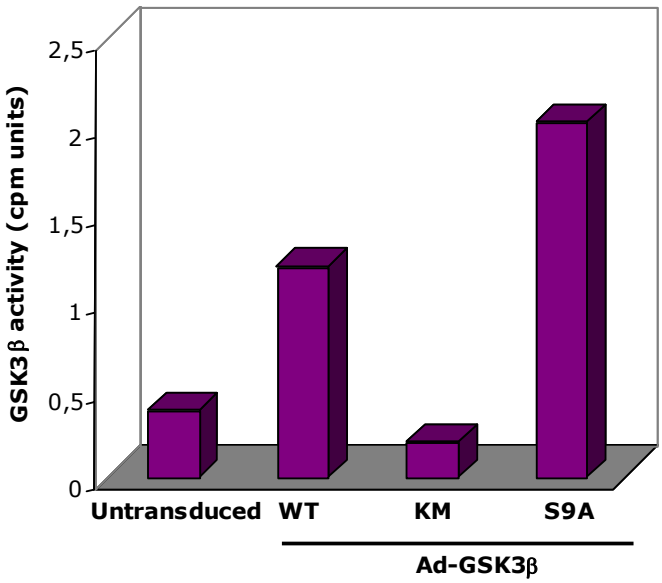


Figure 1.11. Analysis of kinase activities of Ad-GSK3β constructs.

Kinase assay were performed *in vitro* with immunoprecipitated GSK3β from transduced HeLa cells and Phospho-Glycogen Synthase Peptide-2 as substrate. Incorporation of [γ^{32} P] ATP was measured. GSK3β kinase activity is expressed as cpm units. For further details see Materials and Methods.

Phosphorylation *in vivo* was evaluated by radioactive labeling with ^{32}P-ortophosphate and further immunoprecipitation of the target in order to measure the incorporated phosphate. HeLa cells were incubated with the corresponding volumes of adenovirus for 36 hours in the absence of foetal calf serum (- FCS). Next, radioactive labeling with orthophosphate was performed and immediately after, immunoprecipitation of endogenous E2F1 was carried out. Radioactive labeling was determined by autoradiography (Figure 1.12, upper panel). GSK3β overexpression as well as

immunoprecipitation of endogenous E2F1 were tested (see Figure 1.12, lower panel and middle panel, respectively).

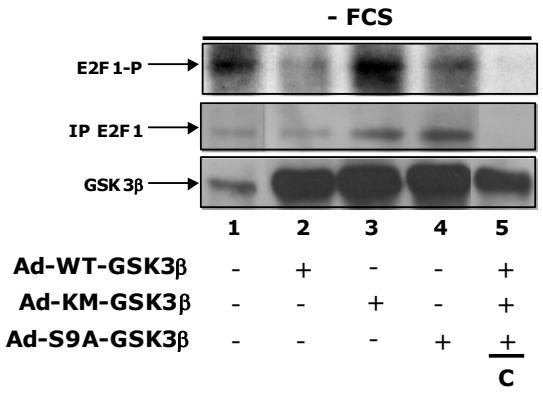


Figure 1.12. Phosphorylation of endogenous E2F1 *in vivo*.

HeLa cells (250000 cells/p35 well) were transduced with the adenoviral GSK3β constructs: 12µl of Ad-WT-GSK3β form, 11µl of Ad-KM-GSK3β and 10µl of Ad-S9A-GSK3β forms. Kinase assay was performed in the absence of foetal calf serum (- FCS) as described in Material and Methods. Reaction products were resolved on a 12% SDS-polyacrylamide gel and phosphorylated proteins were visualized by autoradiography (upper panel). E2F1 immunoprecipitation as well as GSK3β overexpression was evaluated by blotting against E2F1 protein (middle panel) and GSK3β protein (lower panel), respectively. 'C' stands for control were the immunoprecipitation was done from a mixture of all other extracts up to the same amount as the other points using an antibody against His tagged. Quantities were normalized by volume since a Bradford assay was difficult to perform due to problems with radioactive contamination.

E2F1 is phosphorylated *in vivo* even in the absence of GSK3β overexpression (Figure 1.12 upper panel, compare Lane 1 upper panel with Lanes 2-4). The increase of GSK3β activity did not alter the phosphorylation rate. The wild-type construct (Ad-WT-GSK3β) phosphorylated E2F1, but to a lesser extent. Surprisingly, cells infected with the catalytic inactive GSK3β construct (Ad-KM-GSK3β) showed higher phosphorylation of endogenous E2F1. The assay was repeated three times, obtaining the same result in all cases. This result indicates that other kinases might be phosphorylating E2F1 under our conditions or that endogenous GSK3β levels saturate the system.

In order to rule out the possibility that E2F1 phosphorylation generates a conformational change that reduces its immunoprecipitation under our conditions, we measured the capacity of anti-E2F1 antibody to immunoprecipitate the unphosphorylated and phosphorylated forms of E2F1. GST-hE2F1 was phosphorylated *in vitro* by purified GSK3β. The recovery of this form by immunoprecipitation was determined and compared to that obtained for the non-phosphorylated form.

Briefly, GST-hE2F1 (5 μg) and cold ATP (10 μM final concentration) were incubated at 30ºC for 30 minutes in the presence or absence of purified GSK3β (60 mU) and [γ^{32}P] ATP. Next, BSA (200 μg) was added and the total volume was made up to 500 μl in order to facilitate the following immunoprecipitation step. GST-hE2F1 protein was immunoprecipitated and resolved on a 10% SDS-polyacrylamide gel. Phosphorylation was visualized by autoradiography and the presence of E2F1 and GSK3β was determined by Western Blot.

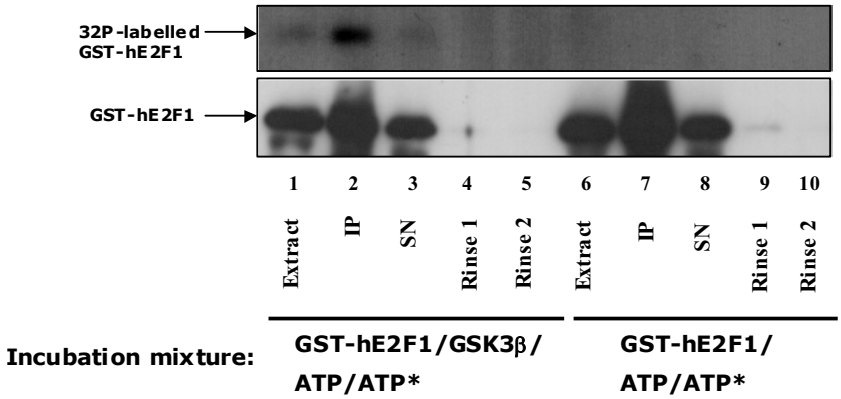
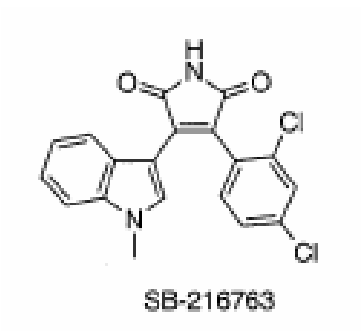


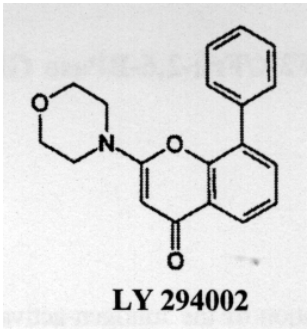
Figure 1.13 Immunoprecipitation *in vitro* of purified GST-hE2F1.

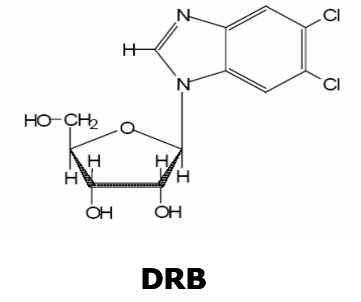
A kinase assay was performed *in vitro* with GST-hE2F1 (5 μg) as a substrate and a final concentration of ATP of 10 μM, in the presence or absence of purified GSK3β (60mU) and [γ^{32}P]-labelled ATP. Components were incubated for 30 minutes at 30ºC. Immediately thereafter GST-hE2F1 protein was immunoprecipitated using an antibody against E2F1. Extensive rinses of the immunoprecipitated products were done and these were resolved on a 10% SDS-polyacrylamide gel. Phosphorylation was visualized by autoradiography (upper panel), while immunoprecipitation of E2F1 was determined by immunoblotting against E2F1 proteins (lower panel). IP: immunoprecipitation product; SN: supernatant fraction; GSK3β: glycogen synthase kinase-3-beta.

GST-hE2F1 was immunoprecipitated independently of its phosphorylation state (Fig 1.13: compare Lanes 2 and 7), although not completely in either case (see Lanes 3, 8, which correspond to the supernatant (SN) after immunoprecipitation). As expected, GST-hE2F1 was phosphorylated only when purified GSK3β and γ^{32}P-labelled ATP were present (lanes 1, 2 and 3).

In order to determine whether GSK3β is responsible for the phosphorylation of E2F1, we performed radioactive labeling with ^{32}P-ortophosphate, this time in untransduced cells, together with pre-incubation with a GSK3β inhibitor (SB 216763), a PI 3-kinase inhibitor (LY 294002), and DRB (5,6-dichloro-1-β-D-ribofuranosylbenzimidazole), a cdk7 inhibitor. SB 216763 is a selective small molecule that acts as an ATP-competitive inhibitor of GSK3 activity with and IC_{50} of around 34 nM [Coghlan et al., 2000; Croos, D. A. E., et al., 2001]. LY 294002 is a highly specific inhibitor of the PI 3-kinase that directly inhibits the activity of the p110 catalytic subunit by competing with the ATP binding site [Vlahos et al., 1994]. DRB is an adenosine analogue that inhibits RNA polymerase II elongation by inhibiting the TFIIH-associated kinase, cdk7 [Yankulov et al., 1995].







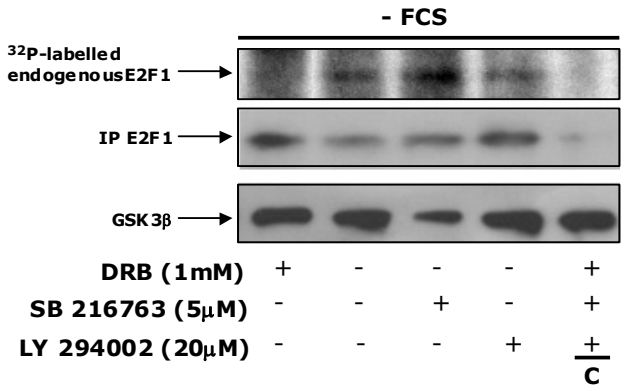


Figure 1.14. Effect of SB 216763, LY 294002 and DRB on the phosphorylation of endogenous E2F1 *in vivo*.

HeLa cells (250,000 cells/p35 well) were plated and incubated at 37ºC in a 5%CO_2 humidified atmosphere for 24 hours. Cells were rinsed in sterile PBS and DMEM with 10% FCS was added with the corresponding inhibitors at appropiate concentrations for one hour. Next, cells were rinsed twice in RPMI without phosphates and 0% FCS and further incubated for another two hours with this same medium together with the corresponding inhibitors. Later, radioactive orthophosphate (200 μCi/ml RPMI) was added and the cells were returned to the incubator for a further five hours. Following incubation, cells were extensively rinsed with cold TBS to eliminate unincorporated ^{32}P-ortophosphate. The *in vivo* phosphorylation was analysed by immunoprecipitation of endogenous E2F1 using either an antibody against E2F1 or against His tagged as a control (marked as **C**), and autoradiography. Western blotting of E2F1 and GSK3β proteins was done, as described in Materials and Methods. Quantities were normalized by volume since determination by Bradford was difficult to perform due to problems of radioactive contamination.

Neither inhibition of PI 3-kinase nor inhibition of endogenous GSK3β led to a change in endogenous E2F1 phosphorylation (see Figure 1.14, upper panel). The inhibition of the phosphorylation signal by DRB treatment is consistent with the proposed phosphorylation of E2F1 by cdk7 [Vandel and Kouzarides 1999].

In our model, other proteins besides GSK3β may contribute to the phosphorylation of endogenous E2F1 *in vivo*. The effects of cdk7 on E2F1 phosphorylation could then be predominant over the effect of GSK3β phosphorylation *in vivo*. Further studies would have to confirm this point.

Taken all the results together presented in this chapter we can conclude that, although GSK3β phosphorylates human E2F1 *in vitro* at residues 403 and 433, we did not detect such phosphorylation *in vivo*. Nevertheless, the possibility of a phosphorylation with physiological relevance *in vivo* cannot be ruled out completely.

According to Frame and Cohen (2001), seven criteria must be fulfilled in order to establish that a protein is a physiological substrate for GSK3 [Frame and Cohen 2001]:

1. *The substrate should be shown, by phosphopeptide mapping and sequence analysis, to be phosphorylated by GSK3* in vitro *at the same residue(s) that is (are) phosphorylated in vivo.*

2. Phosphorylation should be abolished by mutagenesis of this site to a non-phosphorylatable residue.

3. The phosphorylation of the endogenous protein in cells should decrease in response to one or more signals that are known to inhibit GSK3.

4. The endogenous protein should become dephosphorylated at the relevant site(s) in vivo *when cells are incubated with cell-permeant inhibitors of GSK3.*

5. Phosphorylation of the residues targeted by GSK3 should affect the function of the protein in a manner that is consistent with physiological effects of the agonist(s) that regulate(s) GSK3 activity.

6. Phosphorylation of the protein at the GSK3 site(s) should not occur in cells that carry targeted disruptions of the genes that encode GSK3.

7. Dephosphorylation of the protein at the GSK3 site(s) should not occur in response to the appropriate signal in cells that do not express one of the protein kinases that lies upstream of GSK3.

Genetic evidence (corresponding to the last two criteria) for the *in vivo* phosphorylation of GSK3β substrate has not yet been obtained. Furthermore, the majority of the proteins that have been proposed as candidate substrates of GSK3β (e.g. cyclin D1, CREB, HSF-1, etc), only meet the criteria that establish their role as substrates *in vitro* [Diehl et al., 1998; Fiol et al., 1994; and Chu et al., 1996, respectively]. Consequently, to show

unequivocally that a particular protein is a physiological substrate for a particular protein kinase is difficult. Many proteins currently considered as hypothetical kinase substrates might therefore be re-classified in the future.

For E2F1 as a GSK3β target, we fulfilled the first four criteria *in vitro*, but not completely *in vivo*. The goal of the two chapters that follow is to obtain more information to test the hypothesis of a relation between E2F1 and GSK3β.

Results 2. Binding assays of GSK3β and E2F1

BINDING ASSAYS OF GSK3β AND HUMAN E2F1.

INTRODUCTION

Although we did not detect phosphorylation *in vivo*, the fact that GSK3 phosphorylates E2F1 *in vitro* suggests that these two proteins could bind.

The aim of this chapter is to determine by co-immunoprecipitation whether E2F1/HA-E2F1 and GSK3β bind *in vivo*. The capacity of E2F1 and HA antibody to co-immunoprecipitate GSK3β, and the capacity of GSK3β antibody to co-immunoprecipitate E2F1 were assessed.

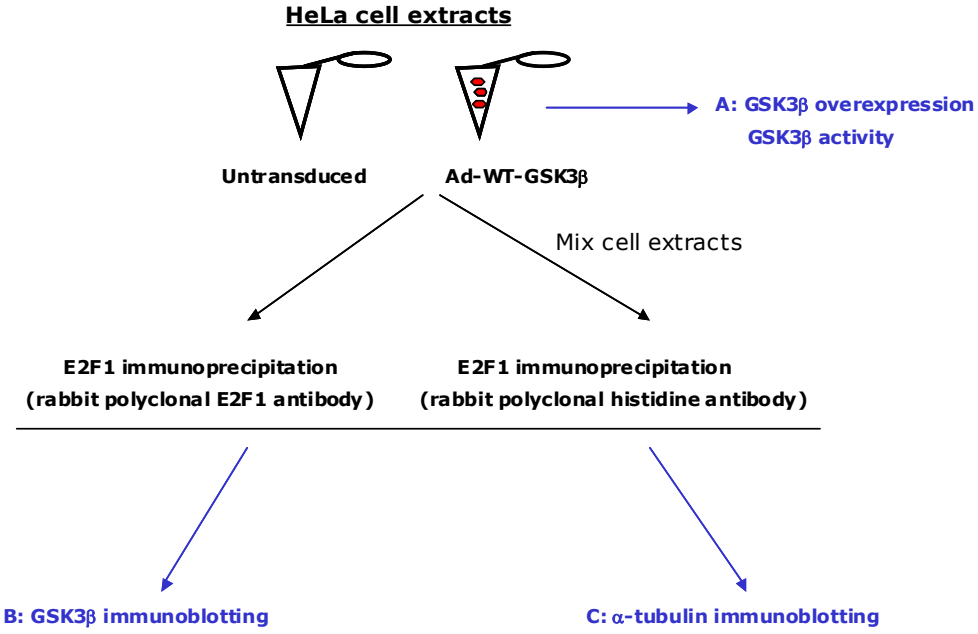
RESULTS

2.1 Detection of GSK3β in E2F1 immunoprecipitates.

The presence of GSK3β in E2F1 immunoprecipitates from whole HeLa cell extracts was analyzed. The HeLa cells used were previously transduced with an adenovirus GSK3β construct, Ad-WT-GSK3β, or left untransduced. The appropriate amount of adenovirus needed had already been established, as described in Chapter 1 (see Figure 1.10). After transduction with the specific adenoviral construct encoding WT-GSK3β, endogenous E2F1 was immunoprecipitated with E2F1 antibody. GSK3β co-immunoprecipitation was analyzed by Western blot using an antibody against GSK3β protein (Figure 2.1 panel B). Overexpression and kinase activity of Ad-WT-GSK3β was checked (see Figure 2.1 panel A). To rule out unspecific immunoprecipitation, cell extracts from all the conditions were mixed and E2F1 was immunoprecipitated using an antibody against an unrelated protein (histidine tag as polyclonal antibody or GST as a monoclonal antibody). A second control was included to ensure that the rinsing was sufficient to remove unbound GSK3β from the immunoprecipitation products. To this end we blotted the E2F1 immunoprecipitates against tubulin, a constitutively expressed

protein. These controls were included in all the co-immunoprecipitation assays shown in this chapter (see scheme of Figure 2.1).

Scheme of Figure 2.1



The results from the co-immunoprecipitation experiments in HeLa cells are shown in Figure 2.1. GSK3β was detected when using an antibody against E2F1 to immunoprecipitate E2F1 (Panel B). The amount of GSK3β co-immunoprecipitated was higher in the GSK3β transduced cells than in the untransduced cells (compare Lanes 1 and 2 from Panel B). Analysis of GSK3β protein after E2F1 immunoprecipitation using an antibody against the histidine tag confirmed that there was no unspecific GSK3β co-immunoprecipitation (lane 3, panel B). Western Blot analysis of cell extract confirmed that WT-GSK3β was correctly overexpressed in Ad-WT-GSK3β transduced HeLa cells (Panel A, top) and the correct GSK3β activity from the overexpressed protein was demonstrated (Panel A, bottom). Furthermore, absence of tubulin immunoreactivity indicates lack of supernatant protein contamination in the immunoprecipitation products (Panel C). These results are consistent with specific binding between GSK3β and E2F1 in HeLa cells.

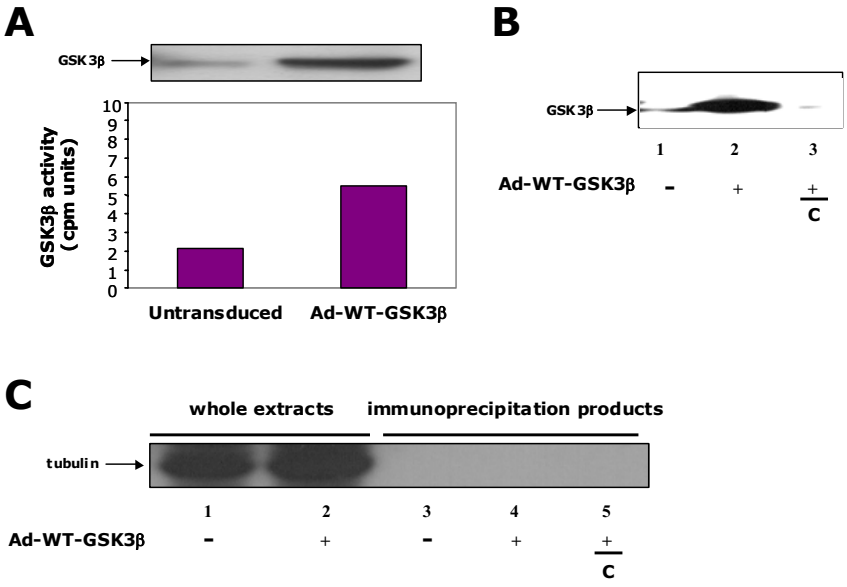
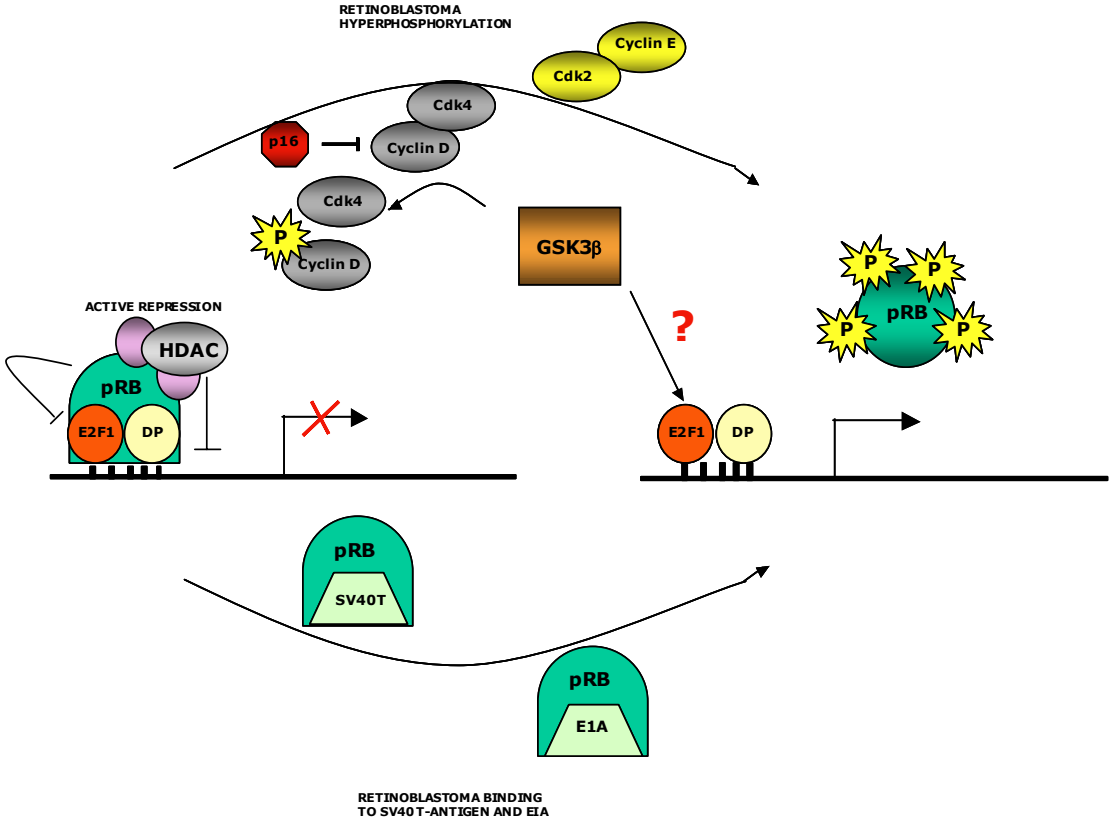


Figure 2.1 Co-immunoprecipitation analysis of overexpressed GSK3β in HeLa cells.
HeLa cells (80% confluent; 150-mm culture dish) were transduced with the adenoviral GSK3β construct Ad-WT-GSK3β. 48 hours post-infection, equal amounts of whole cell extract (3mg) were subjected to immunoprecipitation of E2F1 protein using an antibody against E2F1 or histidine tag as a control (underlined as C). (A) GSK3β overexpression in whole HeLa cell extracts was assessed by Western Blot using GSK3β antibody. Cell extract GSK3β kinase activity was measured by immunoprecipitating GSK3β and performing an *in vitro* kinase assay using Phospho-Glycogen Synthase Peptide-2 as substrate. (B) Co-immunoprecipitation was determined by immunoblotting detection of GSK3β protein in the immunoprecipitation products. (C) Tubulin presence in whole HeLa cell extracts and immunoprecipitation products was determined by Western Blot.

We followed the same experimental approach in HEK293T cell line to determine whether the effect observed was tissue-specific. These cells were chosen because they are transformed human embryonic kidney cells that synthesize E1A and SV40 T-antigen. Both proteins bind to the retinoblastoma gene product (pRB) and this interaction precludes the interaction of pRB and E2F1 and thus control of E2F1 activity [Nevins 1992; Martelli and Livingston 1999]. Consequently, E2F1 is not bound to the retinoblastoma protein (pRB) in these cells, since this is retained by E1A and the antigen T protein. (See Scheme 2.1).



Scheme 2.1. Schematic representation of mechanisms controlling E2F1 activity in HEK293T cells.

The retinoblastoma protein (pRB) binds to an E2F-DP complex in G0/G1 cells and this leads to repression of E2F1-responsive genes through two mechanisms. First, pRB inhibits E2F1 activated transcription by binding to its transactivation domain and preventing its interaction with the core transcriptional machinery. Second, the resulting complex binds to the promoters of E2F1-responsive genes and enforces their "active repression" through the recruitment of histone deacetylases (HDACs), which facilitate nucleosome packing. Cell-cycle entry is dependent on the sequential activation of the cell cycle-dependent kinases, cyclin D-cdk4/6 and cyclin E-cdk2, which phosphorylate pRB and cause it to release E2F1. This activation can be inhibited either by p16 or by GSK3β-induced cyclin D degradation. On HEK293T cells, the viral oncoprotein SV40 T-antigen and the adenoviral protein E1A bind to pRB, precluding pRB and E2F1 interaction. Scheme partially adapted from Trimarchi and Lees (2002).

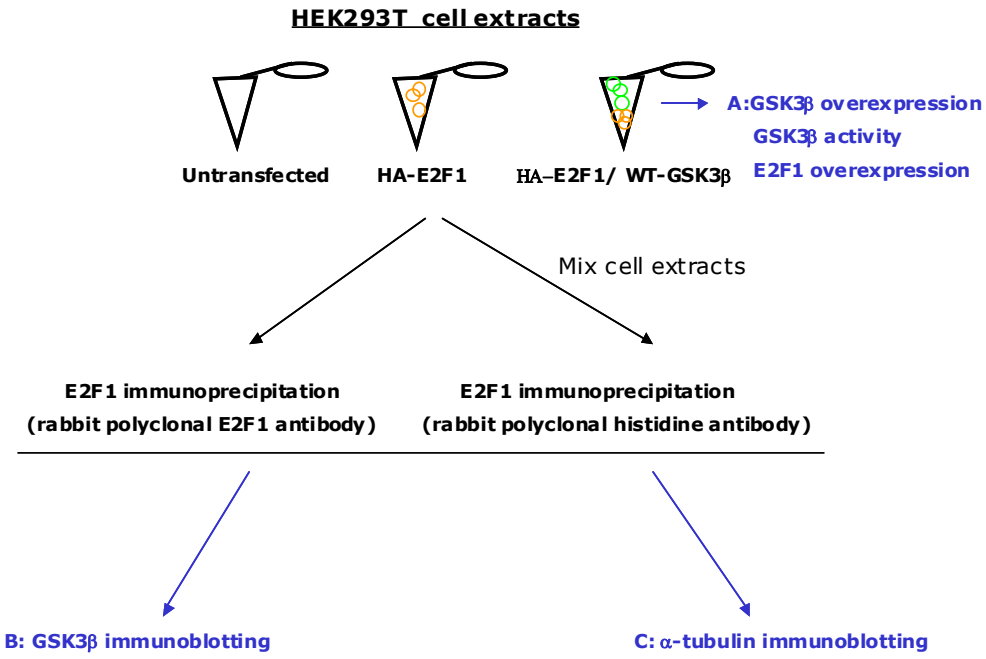
Adenovirus infection cannot be used in HEK293T cells since the lytic cycle of these viruses is not inhibited in this cell line. The HEK293T cell line was originally transformed with Ad5 and contains the E1 gene, which is essential for viral propagation and is deleted in most recombinant adenovirus [Graham et al., 1977],

Because the function of the E1 must be provided in *trans*, HEK293T cells are used to amplify E1-deleted recombinant virus (since the whole lytic virus cycle is completed) and they cannot be used in studies involving transient overexpression of proteins by means of adenovirus. Thus, in this new set of experiments, we constructed a new vector by inserting the adenoviral GSK3β forms into plasmids in order to express the three GSK3β forms in this cell type.

Viral DNA was extracted, and the DNA fragments containing the three GSK3β coding sequences were cut out of the adenovirus constructs with appropriate restriction endonucleases and cloned into a pcDNA3.1(-) vector (Invitrogen) between *Xho*I and *Not*I sites from the multiple cloning site, as described in Material and Methods. Correct orientation of the insert was confirmed by sequencing and subsequently, overexpression of GSK3β in HEK293T cells was analyzed (see Material and Methods for further details). Proteins expressed show a higher molecular weight than the endogenous proteins due to ten C-terminal extra amino acids.

HEK293T cells were transiently calcium phosphate transfected with HA-E2F1 plasmid (HA-tagged full human E2F1 sequence) and WT-GSK3β-pcDNA3.1.(-). Similarly treated, 48 hours post-transfection cells were pooled and equal amounts of whole cell extracts were immunoprecipitated using an E2F1 antibody. Controls to ensure the specificity of the co-immunoprecipitation were performed as previously described. GSK3β co-immunoprecipitation together with E2F1 and GSK3β protein level, as well as kinase activity was surveyed as before (see scheme of Figure 2.2.).

Scheme of Figure 2.2



The results obtained in HEK293T cells are shown in Figure 2.2. GSK3β was detected when immunoprecipitating endogenous and overexpressed E2F1 (Panel B) and the amount of GSK3β was proportional to its expression (compare Lane 1 with 2 and 3 from Panel B). E2F1 overexpression slightly increased the amount of GSK3β co-immunoprecipitated. Analysis of GSK3β protein after E2F1 immunoprecipitation using an antibody against histidine tag confirmed that no unspecific binding occurred (Lane 4, Panel B). In addition, WT-GSK3β was correctly overexpressed in WT-GSK3β-pcDNA3.1(-) transfected HEK293T cells (Panel A, top) and the overexpressed protein was active (Panel A, bottom). Furthermore, absence of tubulin immunoreactivity in the immunoprecipitation products indicates lack of supernatant protein contamination in the immunoprecipitation products (Panel C). All in all, these results are very similar to those obtained for HeLa cells, indicating that the co-immunoprecipitation of GSK3β with E2F1 protein is cell-line independent.

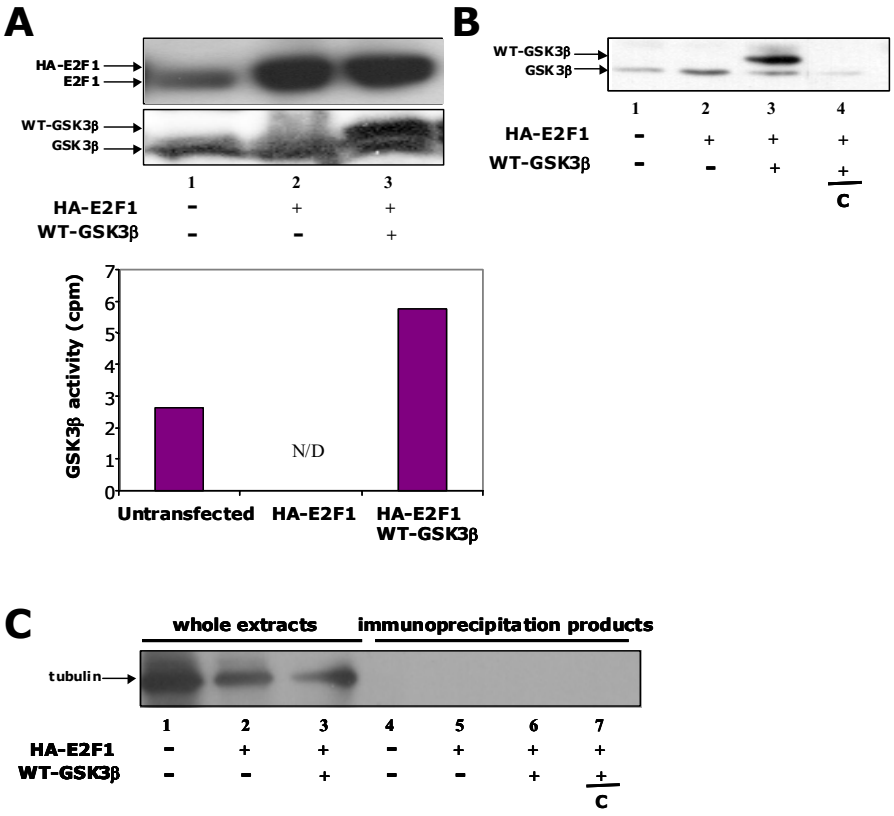


Figure 2.2 Co-immunoprecipitation analysis of overexpressed GSK3β and overexpressed HA-E2F1 in HEK293T cells.

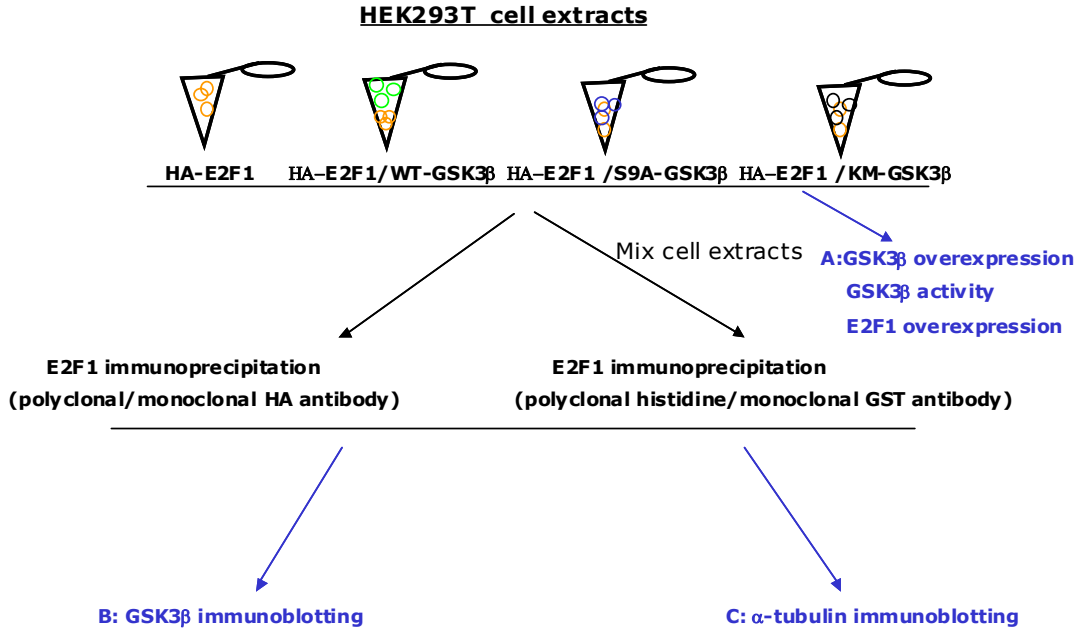
HEK293T cells (70% confluent; 100-mm culture dishes) were transiently transfected with 5μg of HA-E2F1 plasmid, and 15μg of WT-GSK3β-pcDNA3.1 (-) plasmid or CMV-Luc plasmid as carrier. 48 hours post-transfection, equal amounts of whole cell extract (3 mg) were subjected to immunoprecipitation of E2F1 protein using an antibody against E2F1 or against histidine tag as a control (underlined as C). (A) GSK3β and E2F1 overexpression in whole HEK293T cell extracts was assessed by Western Blot using GSK3β or E2F1 antibody. Cell extract GSK3β kinase activity was measured by immunoprecipitating GSK3β and performing an *in vitro* kinase assay using Phospho-Glycogen Synthase Peptide-2 as substrate. (B) Co-immunoprecipitation was determined by immunoblotting detection of GSK3β protein in the immunoprecipitation products. (C) Tubulin presence in whole HEK293T cell extracts and immunoprecipitation products was determined by Western Blot.

N/D, not determined.

2.2. Study to determine whether GSK3β activity is required for its binding to E2F1.

In order to assess whether the binding of GSK3β to E2F1 depends on its activity, we carried out co-immunoprecipitation experiments using an active, inactive and a wild-type form of the GSK3β enzyme. S9A-GSK3β is the active form, or uninhibitable, since the serine at position nine is mutated to alanine and therefore this form cannot be inhibited by phosphorylation at this position. On the other hand, KM-GSK3β is catalytically inactive since it contains two point mutations in its catalytic domain. To prevent endogenous levels of E2F1 from being limiting we overexpressed HA-E2F1. This time, HA-E2F1 was immunoprecipitated using a rabbit polyclonal antibody against the HA tag (Figure 2.3a). To ensure correct immunoprecipitation using an antibody against the HA tag, overexpressed HA-E2F1 was immunoprecipitated in parallel using a monoclonal HA tag antibody (Figure 2.3b). The use of monoclonal antibody has two main advantages: increased specificity and lack of IgG crossreaction with the rabbit polyclonal antibody used in the Western blot to detect E2F1. Controls were carried out as usual (see scheme of Figures 2.3.a and 2.3.b).

Scheme of Figures 2.3a and 2.3b



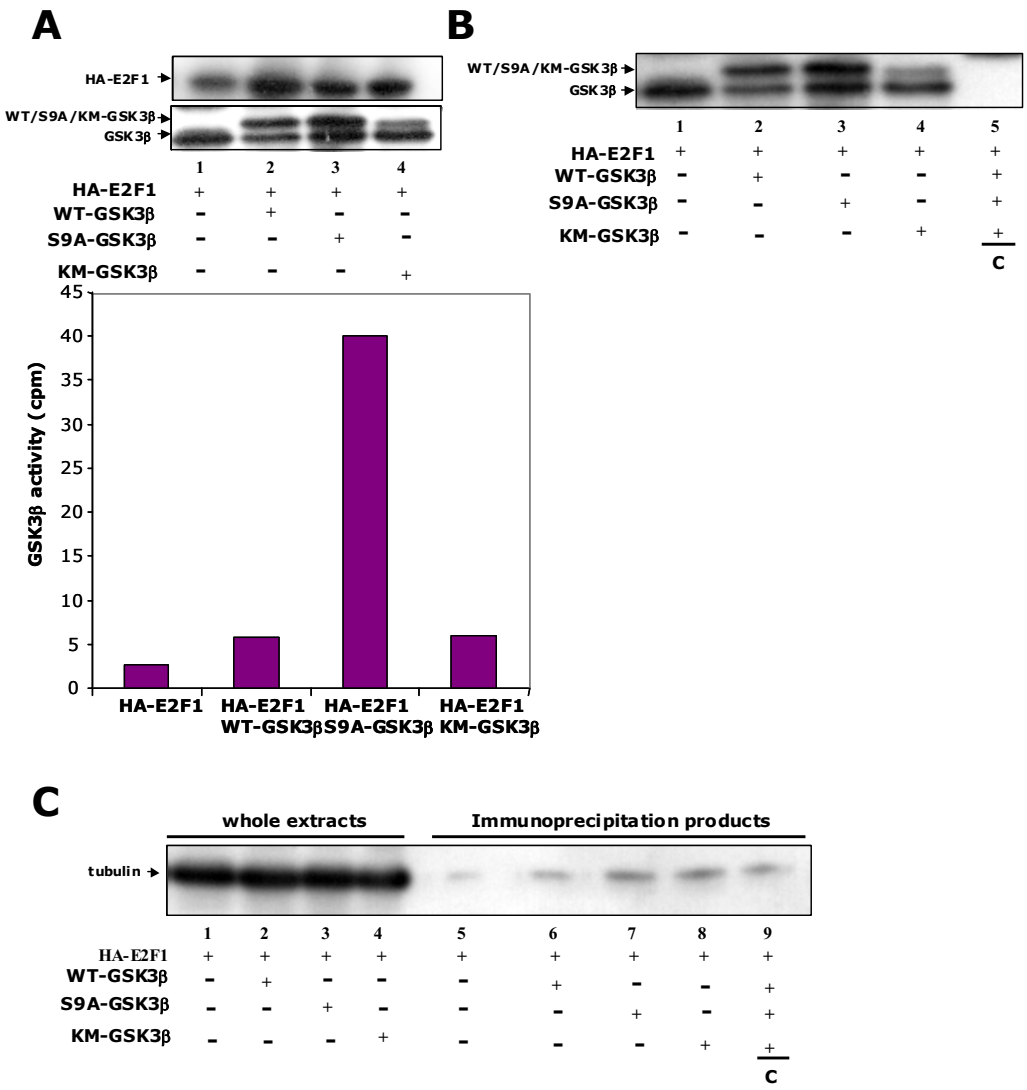


Figure 2.3a. Co-immunoprecipitation analysis of active and inactive GSK3β and overexpressed HA-E2F1 in HEK293T (HA tag polyclonal antibody).

HEK293T cells (70% confluent; 100-mm culture dishes) were transiently transfected with 5μg of HA-E2F1 plasmid, and 15μg of WT-GSK3β-pcDNA3.1(-) plasmid, S9A-GSK3β-pcDNA3.1(-), KM-GSK3β-pcDNA3.1(-) plasmid, or pcDNA3.1(-) plasmid as carrier, as described in Materials and Methods. 48 hours post-transfection equal amounts of whole cell extract (3 mg) were subjected to immunoprecipitation of E2F1 protein using a polyclonal antibody against HA tag or against histidine tag as a control (underlined as C). (A) GSK3β and E2F1 overexpression in whole HEK293T cell extracts was assessed by Western Blot using GSK3β or E2F1 antibody. Cell extract GSK3β kinase activity was measured by immunoprecipitating GSK3β and performing an *in vitro* kinase assay using Phospho-Glycogen Synthase Peptide-2 as substrate. (B) Co-immunoprecipitation was determined by immunoblotting detection of GSK3β protein in the immunoprecipitation products. (C) Tubulin presence in whole HEK293T cell extracts and immunoprecipitation products were determined by Western blot.

Co-immunoprecipitation results using a polyclonal HA-tag antibody are presented in Figure 2.3a. GSK3β was detected when immunoprecipitating overexpressed E2F1 (Panel B) and the amount of GSK3β was proportional to its expression (compare GSK3β co-immunoprecipitated, Panel B, with the amount overexpressed, Panel A, top). Moreover, GSK3β co-immunoprecipitation levels obtained when immunoprecipitating E2F1 through its HA tag were comparable to those achieved earlier, using an antibody against E2F1 protein. Interestingly, GSK3β activity did not seem to influence its binding to E2F1, since active (S9A) and inactive (KM) forms co-immunoprecipitate equally. Negative controls, shown in Panel B, lane 5 and Panel C, rule out unspecific binding to the antibody and the presence of unbound contaminating GSK3β, respectively.

The same experiment was performed on cell extracts where E2F1 was immunoprecipitated using a monoclonal HA-tag antibody (figure 2.3b), instead of the polyclonal antibody, and equal results were obtained. This finding adds further evidence that GSK3β co-immunoprecipitation with E2F1 is independent of GSK3β activity and independent of the antibody against HA tag used to immunoprecipitate E2F1.

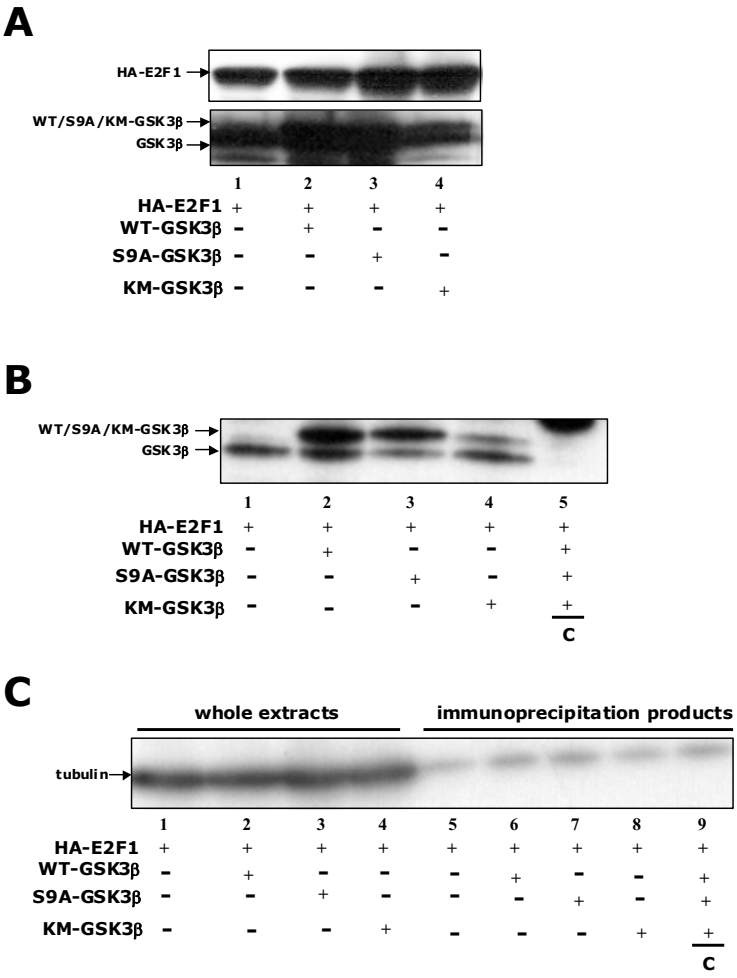


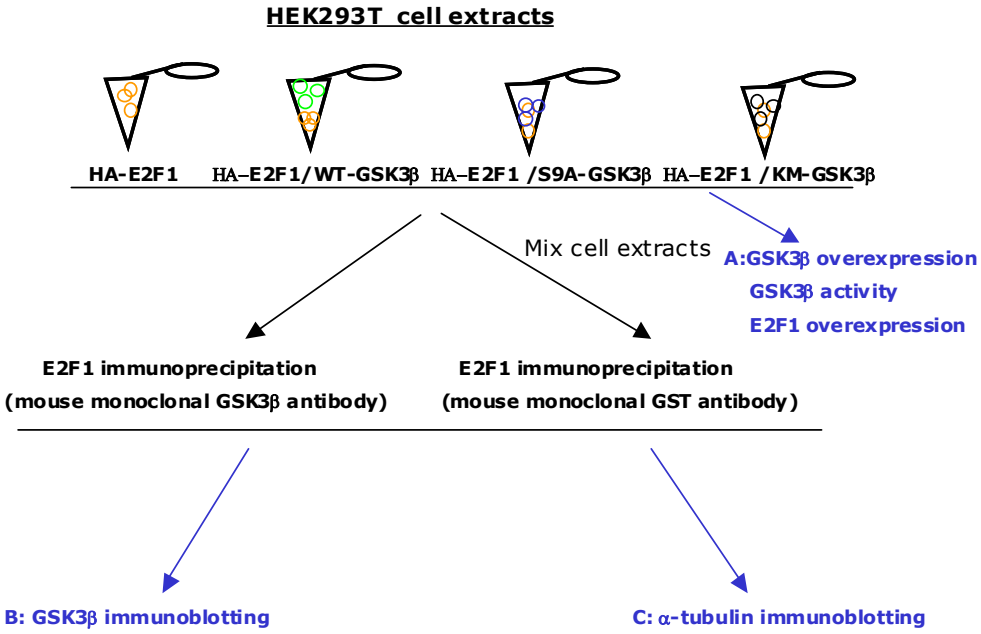
Figure 2.3b. Co-immunoprecipitation analysis of active and inactive GSK3β and overexpressed HA-E2F1 in HEK293T (HA tag monoclonal antibody).

HEK293T cells (70% confluent; 100-mm culture dishes) were transiently transfected with 5μg of HA-E2F1 plasmid, and 15μg of WT-GSK3β-pcDNA3.1(-) plasmid, S9A-GSK3β-pcDNA3.1(-), KM-GSK3β-pcDNA3.1(-) plasmid, or pcDNA3.1(-) plasmid as carrier, as described in Materials and Methods. 48 hours post-transfection equal amounts of whole cell extract (3 mg) were subjected to immunoprecipitation of E2F1 protein using a monoclonal antibody against HA tag or against GST as a control (underlined as C). (A) GSK3β and E2F1 overexpression in whole HEK293T cell extracts was assessed by Western Blot using GSK3β or E2F1 antibody. (B) Co-immunoprecipitation was determined by immunoblotting detection of GSK3β protein in the immunoprecipitation products. (C) Tubulin presence in whole HEK293T cell extracts and immunoprecipitation products were determined by Western blot.

2.3 Detection of E2F1 in GSK3β immunoprecipitates.

To confirm the binding results obtained we tested whether E2F1 from whole HEK293T cell extracts co-immunoprecipitates when using an antibody against GSK3β. HEK293T cells were transfected in the same conditions as described in section 2.2. Briefly, HEK293T cells were transfected with HA-E2F1 together with one of the GSK3β forms: wild-type, uninhibitable (S9A) or catalytically inactive (KM). GSK3β was immunoprecipitated using a monoclonal antibody against GSK3β and the presence of E2F1 in the immunoprecipitates was analyzed. Correct overexpression of the transfected forms was also examined. Negative controls were performed as described previously (see scheme of Figure 2.4).

Scheme of Figure 2.4



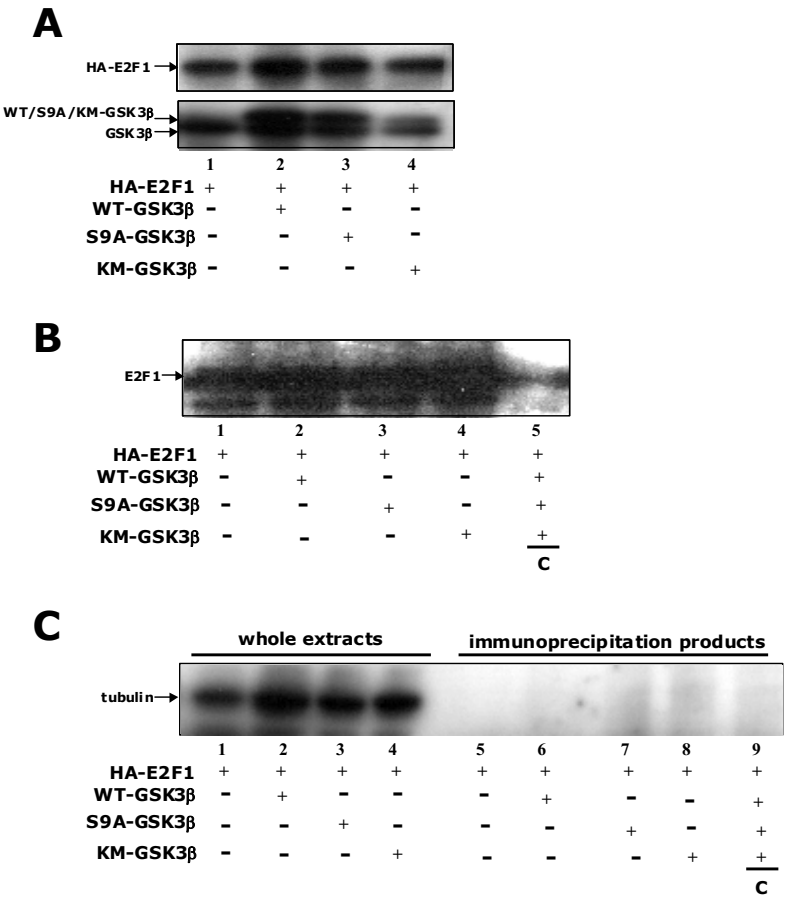


Figure 2.4 Co-immunoprecipitation analysis of overexpressed E2F1 and overexpressed GSK3β in HEK293T cells.

HEK293T cells (70% confluent; 100-mm culture dishes) were transiently transfected with 5μg of HA-E2F1 plasmid, and 15μg of WT-GSK3β-pcDNA3.1(-) plasmid, S9A-GSK3β-pcDNA3.1(-), KM-GSK3β-pcDNA3.1(-) plasmid, or pcDNA3.1(-) plasmid as carrier, as described in Materials and Methods. 48 hours post-transfection equal amounts of whole cell extract (3 mg) were subjected to immunoprecipitation of GSK3β protein using a monoclonal antibody against GSK3β or against GST as a control (underlined as C). (A) GSK3β and E2F1 overexpression in whole HEK293T cell extracts was assessed by Western Blot using GSK3β or E2F1 antibody. (B) Co-immunoprecipitation was determined by immunoblotting detection of E2F1 protein in the immunoprecipitation products. (C) Tubulin presence in whole HEK293T cell extracts and immunoprecipitation products was determined by Western blot.

As shown in Figure 2.4 E2F1 protein was detected to the same extent independent of whether immunoprecipitation was performed in HEK293T whole cell extracts transfected with GSK3β or extracts left untransfected (Panel B). As shown in prior results, the binding efficiency was independent of GSK3β activity. Analysis of E2F1 protein after GSK3β immunoprecipitation using an antibody against GST, confirms there is no unspecific binding (Lane5, Panel B). All GSK3β forms used (WT, S9A and KM) as well as HA-E2F1 were correctly overexpressed (Panel A). Moreover, absence of tubulin immunoreactivity in the immunoprecipitation products indicates lack of supernatant protein contamination in the immunoprecipitaion products (Panel C).

By comparing the results obtained in this section with those obtained in sections 2.1 and 2.2, we conclude that E2F1-GSK3β binding is detected when immunoprecipitating with an antibody against either protein. Therefore, the data presented in this chapter show strong evidence of binding of E2F1 and GSK3β *in vivo*, independently of GSK3β kinase activity.

Results 3. Effect of GSK3β on E2F1 activity

EFFECT OF GSK3β ON E2F1 ACTIVITY.

INTRODUCTION

GSK3β is a key regulator of a broad array of transcription factors, thereby extending its regulatory influence to the control of the expression of numerous genes [Grimes and Jope 2001 and references therein]. Indeed, transcription factors such as Activator protein-1 (AP-1), Cyclic AMP response element binding protein (CREB), c-Jun, c-Myc, Heat-shock factor-1 (HSF-1) among others are considered putative GSK3β substrates [Frame and Cohen 2001 and references therein; Grimes and Jope 2001]. A large number of these factors have been found to be regulated by GSK3β directly through phosphorylation, phosphorylation being the mechanism by which the activity of the transcription factor is regulated. Hence, considering results obtained in the first and second chapter we hypothesize that GSK3β could participate directly in the control of E2F1 activity as a transcription factor.

To study the direct influence of GSK3β on E2F1 activity, we chose the HEK293T cell line. As described in the previous chapter, this cell line expresses E1A protein, which sequesters pRB. This should avoid pRB-E2F1 interactions and its indirect regulation by GSK3β through CycD degradation, and therefore pRB hyperphosphorylation.

RESULTS

3.1. Effect of GSK3β inhibitors on E2F1 activity.

Results from Chapters 1 and 2 provide evidence that GSK3β binds to E2F1 and that this interaction, in our cell context, is independent of GSK3β kinase activity. Here we evaluate the effect of GSK3β on the transcriptional activity of E2F1-responsive promoters. To this end, we performed a series of experiments to examine the influence

of GSK3β kinase activity, protein concentration and binding to E2F1 on this possible modulation.

To assess the effect of endogenous GSK3β on E2F1 activity as a transcription factor (further referred to as E2F1 transcriptional activity), HEK293T cells were transfected with $[E2F]_3$-LUC plasmid together with HA-E2F1 plasmid. The former consists of a luciferase reporter gene under the control of an artificial promoter, with three copies of the E2F1 consensus binding sites (kind gift from Dr. Gstaiger) and the later consists in complete human E2F1 fused with HA tag (kind gift from Dr. Lam). HEK293T cells were treated with the PI 3-kinase and GSK3β inhibitors in the absence of foetal calf serum (-FCS): GSK3β is active under these conditions. LY 294002, as described previously in chapter one, was used as a highly specific inhibitor of p110 [Vlahos et al., 1994]. SB 216763 and SB 415286 were used as ATP-competitive small molecules that inhibit GSK3β [Cross et al., 2001]. The concentration of each inhibitor used was based on previous results of similar experiments reported in the literature [Fernandez de Mattos et al., 2000; Cross et al., 2001]. The carrier for the inhibitors (DMSO) was added as a control. The effect of the chemical inhibitors on E2F1 transcriptional activity was assessed by measuring relative luciferase units (RLU) in each of the treatments. The luciferase measurement corresponding to each condition was normalized to the protein amount in the sample, and expressed as a ratio to the luciferase value of untreated cells (control with DMSO), which was considered one arbitrary unit. To validate the correct effect of the inhibitors used, we measured the decrease in serine 473 Akt/PKB phosphorylation by the PI 3-kinase inhibitor LY 294002 and the accumulation of β-catenin protein in case of the GSK3β inhibitors SB 216763 and SB 415286 (Figure 3.1).

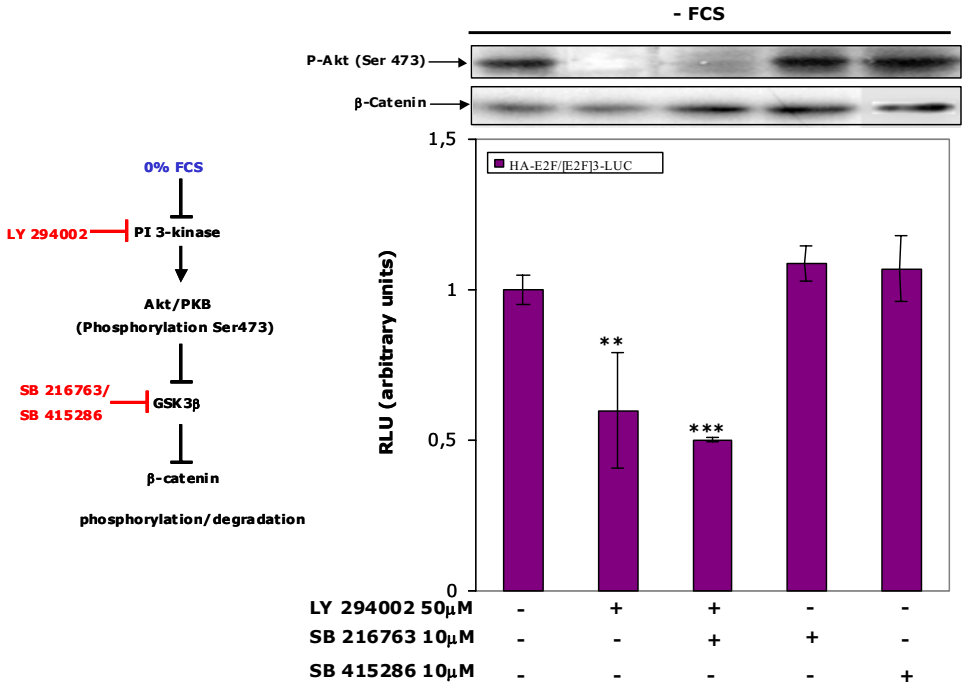


Figure 3.1. Effect of GSK3β activation and inhibition on E2F1 activity when overexpressing E2F1.

HEK293T cells (90% confluent; 100-mm culture dishes) were transiently transfected with 14 μg $[E2F]_3$-LUC plasmid together with 10 μg HA-E2F1 plasmid using Lipofectamine 2000. Cells were treated with the indicated inhibitors and incubated in the absence of foetal calf serum (-FCS). Luciferase activity was measured and expressed as relative luciferase units (RLU). Values were normalized to micrograms of proteins and expressed as a ratio to the value of untreated cells, which was considered one arbitrary unit. Data are means ± S.E.M. from 3 independent experiments. Statistically significant differences compared with untreated cells (control with DMSO) are indicated by ***p<0.001 and * *p<0.01. Western blots were performed to analyze accumulation of β-catenin, and phosphorylation of Akt/PKB (top panels).

We observed no variation when treating the cells with GSK3β inhibitors SB 216763 and SB 415286, but a considerable reduction of E2F1 transcriptional activity when using PI 3-kinase inhibitor LY 294002 (Figure 3.1). Moreover, combined treatment of LY 294002 and SB 216763 showed about the same reduction (40-50%) on E2F1 transcriptional activity as LY 294002 treatment alone. These results suggest that GSK3β kinase activity is not responsible for the modulation of E2F1 transcriptional activity under our experimental conditions. Results obtained with LY 294002 point out that there is one or several effectors downstream of PI 3-kinase that can inhibit E2F1

transcriptional activity. The fact that the addition of SB 216763 together with LY 294002 does not revert this inhibition implies that the effect of PI 3-kinase is not dependent on GSK3β kinase activity. Western blot of Akt/PKB phosphorylated on serine 473 shows inhibition of this phosphorylation when treating the cells with LY 294002, and therefore inhibiting PI 3-kinase. On the other hand, the Western blot of β-catenin shows an accumulation 6 hours after inhibiting GSK3β with SB 216763 or SB 415286 (Figure 3.1, top).

3.2. Effect of GSK3β depletion by RNA interference on E2F1 activity.

The previous results indicate that modulation of E2F1 appears to be independent of GSK3β kinase activity. However, since GSK3β and E2F1 interact, (see Chapter 2) we attempted to assess whether modulation of the transcriptional activity of E2F1 could be affected by GSK3β protein concentration. As a first approach we depleted GSK3β in HEK293T cells using RNA interference (RNAi).

In our particular case we used GSK3β siRNAs designed by Novartis (sequences under patent) to reduce GSK3β concentration in HEK293T. In order to determine the best sequence to be used, titration of three different siRNAS was performed (figure 3.2). Increasing concentrations of siRNAs were transfected into exponentially growing HEK293T cells using lipofectamine. 48 hours post-transfection cell extracts were obtained and subjected to SDS/PAGE followed by Western blot using an antibody against GSK3β (Figure 3.2).

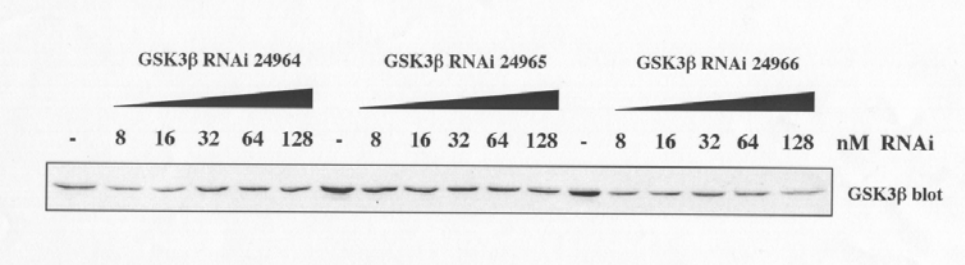


Figure 3.2. Tritration of siRNA.

Three siRNA: 24964, 24965 and 24966 were transfected using lipofectamine into exponentially growing HEK293T cells, at the indicated concentrations. Western blot using an antibody against GSK3β was perform to assess depletion of GSK3β protein.

As shown in Figure 3.2., RNAi 24964 and 24966 knockdown GSK3β protein when used at 8nM or higher concentrations. Although a reduction of GSK3β protein can be observed when using RNAi 24965 the knockdown percentage obtained is far from the other two siRNA employed. Considering initial endogenous GSK3β levels and the dose response behavior, GSK3β RNAi with sequence 24966 appears to be the most efficient, with the highest correlation between RNA knockdown and protein knockdown, and with a decrease of GSK3β protein of around 60%.

Following siRNA titration, HEK293T cells were transfected with $[E2F]_3$-LUC plasmid alone, together with HA-E2F1 or DP1, or a combination of both. GSK3β RNAi was performed using GSK3β siRNA 24966 and luciferase activity was determined. The results of GSK3β RNAi in HEK293T are summarized in Figure 3.3. and show that depletion of GSK3β protein levels to about 60% reduces E2F1 transcriptional activity in the same proportion. This strongly suggests that the presence of GSK3β protein is necessary to affect E2F1 transcriptional activity. Co-transfection of DP1 protein did not significantly alter the transcriptional activation achieved by overexpression of E2F1 alone. E2F1 and DP1 overexpression are confirmed by respective immunoblots (Figure 3.3. top).

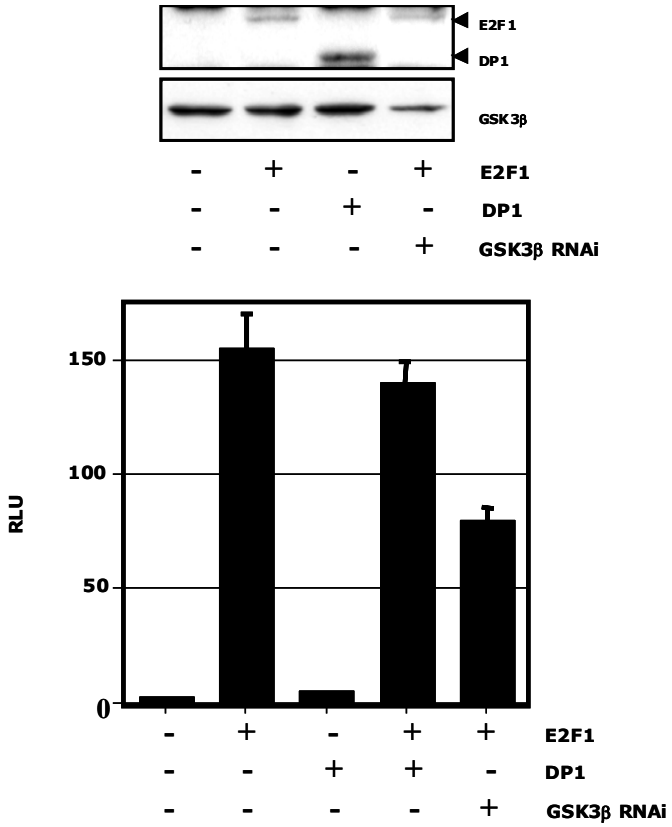


Figure 3.3. Effect of knockdown of GSK3β by RNAi in HEK293T.

HEK293T cells (100000 cells/35-mm culture dishes) were transfected with 16 nM of GSK3β siRNA alone or together with 0.25 μg $[E2F]_3$-LUC, 1 μg HA-E2F1 and/or 0.1 μg DP-1. Cells were harvested 2 days post-transfection. Luciferase activity was measured and expressed as relative luciferase units (RLU). Values were normalized to micrograms of proteins and expressed as a ratio to the value of untransfected cells.

3.3 Effect of GSK3β "overexpression" on E2F1 activity.

Previous results reported in Sections 3.1. and 3.2. describe the effects of GSK3β inhibition and depletion on E2F1 transcriptional activity. We showed that modulation of E2F1 activity is independent of GSK3β kinase activity but dependent on total GSK3β protein concentration. As another approach to examine the effect of GSK3β protein concentration we used expression vectors coding for different forms of GSK3β in order to increase total GSK3β protein concentration.

Two expression vectors for GSK3β were used, one coding for an uninhibitable form of the protein (S9A-GSK3β-pcDNA3.1(-)), and the other coding for a kinase-dead catalytic form of the protein (KM-GSK3β-pcDNA3.1(-)). As described in Chapter 2 S9A-GSK3β-pcDNA3.1(-) has serine 9 mutated to alanine which abrogates the inhibitory phosphorylation of GSK3β by PKB and gives a constitutively active GSK3β, whereas KM-GSK3β-pcDNA3.1(-) contains two point mutations on the catalytic domain.

We analyzed the effects of the expression of S9A-GSK3β-pcDNA3.1(-) and KM-GSK3β-pcDNA3.1(-) on E2F1 transcriptional activity in a co-transfection experiment. HEK293T cells were co-transfected with both the plasmid coding for S9A-GSK3β-pcDNA3.1(-) or KM-GSK3β-pcDNA3.1(-), in different concentrations, and the E2F1-responsive promoter $[E2F]_3$-LUC. Alternatively, another set of HEK293T cells was transfected under the same conditions together with HA-E2F1 plasmid. Luciferase activity was measured and the effect on $[E2F]_3$-LUC transcription was expressed as relative luciferase units (RLU) and normalized for β-Galactosidase for each of the transfection conditions. The Western blot technique was used to confirm that HA-E2F1 and the GSK3β constructs were correctly overexpressed. To confirm the GSK3β kinase activity of the constructs, we immunoprecipitated the transfected proteins and performed kinase assays (Figure 3.4., Panel C).

The two mutant GSK3β forms have an equal effect on E2F1 transcriptional activity (Figure 3.4, Panels A and B), indicating that GSK3β activity was not required for the modulation of E2F1 transcriptional activity under our experimental conditions. At high concentrations of GSK3β (5 μg), E2F1 transcriptional activity increased. This effect seems dose-dependent, increasing at elevated GSK3β and E2F1 concentrations. Upon E2F1 overexpression the luciferase activity detected at high GSK3β concentration was approximately five-fold higher than in endogenous E2F1 conditions. Immunoblotting analysis performed on the cell extracts confirmed the correct expression of both GSK3β constructs transfected (Figure 3.4. Panels A and B, top). In addition, the kinase assay *in vitro* confirmed the higher basal kinase of S9A-GSK3β-pcDNA3.1(-) and the null activity of KM-GSK3β-pcDNA3.1(-) (Figure 3.4. Panel C). The expression of E2F1

was also surveyed by immunoblotting against E2F1 (Figure 3.4. Panel A, top) or HA tag in the case of HA-E2F1 expression (Figure 3.4.,Panel B, top).

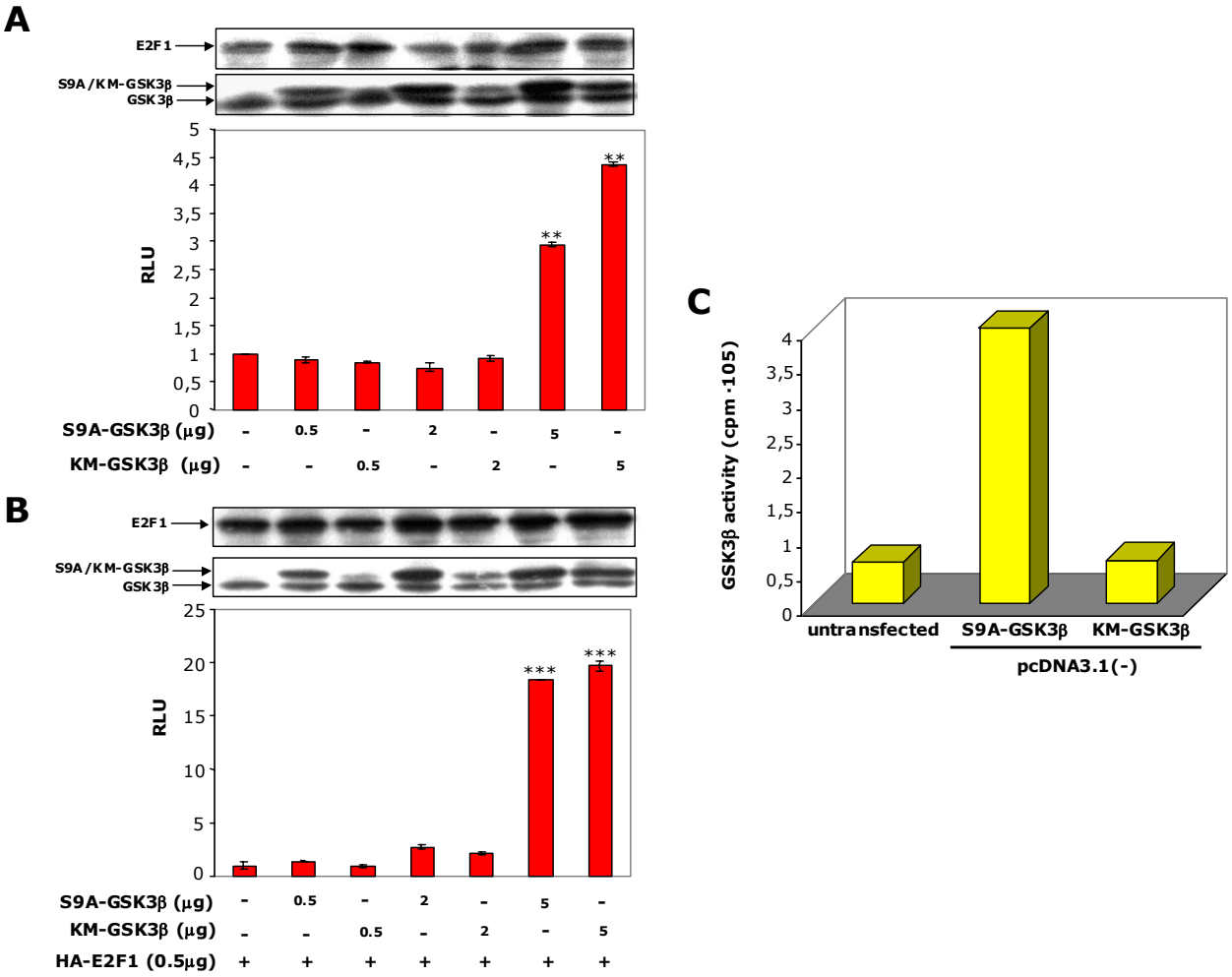


Figure 3.4. Effect of mutant GSK3β expression on E2F1 transcriptional activity.

(A)Luciferase activity in extracts from HEK293T cells (400,000 cells per 35-mm culture dishes) transiently transfected with 1,5 μg of $[E2F]_3$-LUC plasmid and 0,5 μg of pCMV-βGal plasmid and different concentrations of S9A-GSK3β-pcDNA 3.1 (-) or KM-GSK3β-pcDNA 3.1 (-) constructs as indicated (B). Luciferase activity in cells overexpressing HA-E2F1 (0.5 μg) and transfected with the same conditions as in (A). Luciferase activity was measured and expressed as relative luciferase units (RLU). Values, corrected for relative β-Galactosidase milliunits and expressed as a ratio to the value of luminescence of cells in which no mutant GSK3β was expressed (one arbitrary unit). Data are means ± S.E.M. from three independent experiments. Significant differences are indicated by ***p<0.001 and ** p<0.01 compared cells transfected with $[E2F]_3$-LUC plasmid only (case of (A)) or $[E2F]_3$-LUC plasmid and HA-E2F1 plasmid (case of (B)).Western blot of E2F1 and GSK3β protein (A and B, top panels). (C) Kinase activity of GSK3β confirmed by kinase assay as described in Materials and Methods.

It has been shown that high plasmid concentrations can generate artifacts on the transfection [Hofman et al., 2000]. Therefore, to rule out this possibility in our experimental setup, we repeated the assay reducing total plasmid concentrations to a fifth part. We decided to perform the following experiments in HEK293T cells overexpressing HA-E2F1, as shown in Figure 3.4, Panel B. Note that the plasmid concentrations are reduced but proportion between them is maintained.

The results are presented in Figure 3.5 and are equal to the ones shown in Figure 3.4. Upon expression of either S9A-GSK3β-pcDNA3.1(-) or KM-GSK3β-pcDNA3.1(-) the transcriptional activity of overexpressed E2F1 is increased to the same degree. This confirms the trend observed in Figure 3.4 and indicates that the relation between the amount of GSK3β protein and E2F1 proteins is key for the increase in E2F1 transcriptional activity detected. Immunoblotting analysis performed on the cell extracts confirms the correct expression of both GSK3β constructs transfected (Figure 3.5., top). The expression of E2F1 was also surveyed by immunoblotting against HA tag (Figure 3.5., top). On the basis of these results we used this same range of total plasmid concentration and relative proportion for further co-transfection experiments.

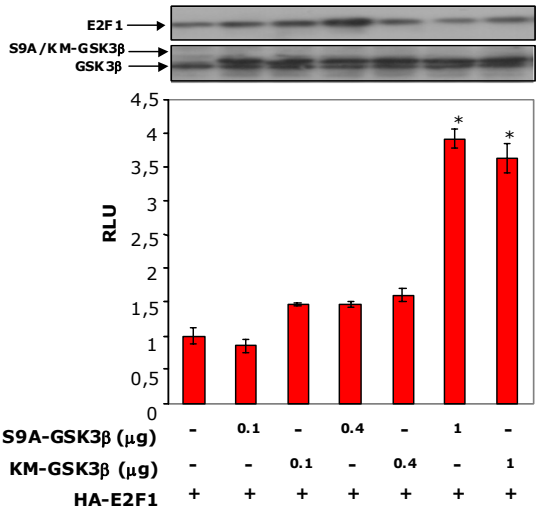


Figure 3.5. Effect of mutant GSK3β expression on E2F1 transcriptional activity (reducing total plasmid concentration)

Luciferase activity in extracts from HEK293T cells (400000 cells/35-mm culture dishes), transiently transfected with 0.3 μg of $[E2F]_3$-LUC plasmid, 0,1 μg of HA-E2F1 plasmid and 0.5 μg of pCMV-βGal plasmid and different concentrations of S9A-GSK3β-pcDNA 3.1 (-) or KM-GSK3β-pcDNA 3.1 (-) constructs as indicated. Luciferase activity was measured and expressed as relative luciferase units (RLU). Values were corrected for relative β-Galactosidase milliunits and expressed as a ratio to the value of luminescence of cells in which no mutant GSK3β was expressed, which was considered one arbitrary unit. Data are means ± S.E.M from three independent experiments. Statistically significant differences are indicated by * $p<0.05$ when compared to GSK3β untransfected cells. Western blots were performed to check expression of transfected constructs.

3.4. Effect of GSK3β localization on E2F1 activity.

E2F1 has been described to be primarily nuclear in cycling cells [Müller et al., 1997; Verona et al., 1997; Gill and Hamel 2000; Trimarchi and Lees 2002 and references therein]. In contrast, GSK3β is mainly cytoplasmatic [Bijur and Jope 2003a and references therein] although it is also present in other subcellular compartments, including the nucleus [Diehl et at., 1998; Bijur and Jope 2001] and mitochondria [Hoshi et al., 1996].

Cell cycle-dependent changes in the intracellular localization of GSK3β were detected by Alt and colleagues, who found an increase in nuclear GSK3β during S phase in

NIH3T3 cells [Alt et al., 2000]. Increased nuclear accumulation of GSK3β was induced by heat shock [Xavier et al., 2000] and induced by endothelin-1 in myocytes [Haq et al., 2000]. In cell lines like, for example, PC12 there is a GSK3β translocation from cytoplasm to nucleus at G1/S phase of the cell cycle and this translocation correlates with an increase of E2F1 transcriptional activity [Crowder and Freeman 2000; Katayama et al., 2001].

Taking this into account, we examined whether nuclear localization of GSK3β would affect its action on E2F1. To this end we cloned the uninhibitable and catalytically inactive GSK3β forms into plasmids containing a nuclear localization signal (NLS). The strategy chosen was very similar to that used to obtain the S9A-GSK3β-pcDNA3.1(-) and KM-GSK3β-pcDNA3.1(-) constructs (see Materials and Methods). Viral DNA was extracted and the DNA fragments containing the GSK3β coding sequences were cut out from the adenovirus constructs by appropriate restriction endonucleases. The DNA fragments were then cloned into a pCMV/myc/nuc vector (Invitrogen). Correct orientation of the insert was confirmed by sequencing, and subsequently overexpression of GSK3β in HEK293T cells was analyzed. The pCMV/myc/nuc vector contains a myc tag and a nuclear localization signal (NLS) which directs the cloned and translated protein to the nucleus.

The re-targeting of the constructs was analyzed by cell fractioning. Transfected HEK293T were fractioned into cytosolic, nuclear and mitochondrial fractions following the protocol described in 'Materials and Methods', and the different fractions were immunoblotted against GSK3β. Results in Figure 3.6. show that S9A and KM-GSK3β pcDNA3.1(-), are localized mainly in the cytoplasm while S9A and KM pCMV/myc/nuc, containing the NLS, are mainly localized in the nuclear fraction. Correct division of the cytosolic, nuclear, and mitochondrial fractions was verified by immunoblotting the fractions with antibodies for tubulin, PARP and cytochrome oxidase (the latter not shown). All in all, these results confirm correct cellular fractioning and the correct distribution of the GSK3β constructs tested.

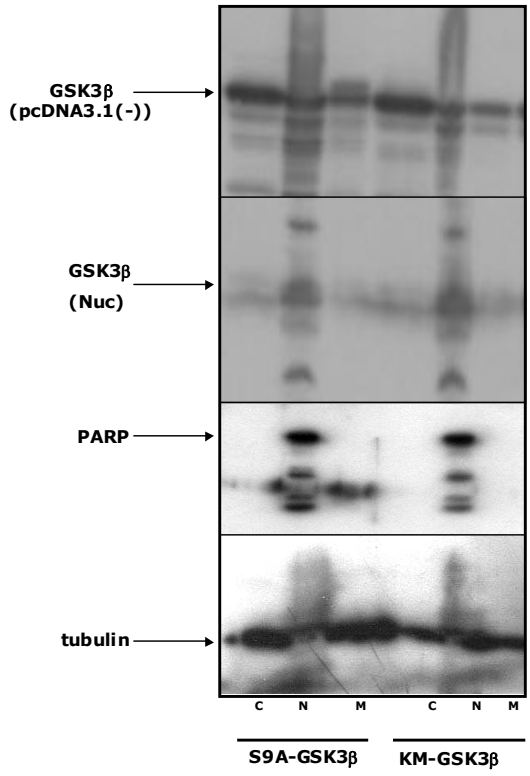


Figure 3.6. Analysis of the correct localization of the nuclear targeted and cytosol GSK3β proteins, by subcellular fractioning.

In order to confirm the nuclear localization of the nuclear-targeted S9A-GSK3β and KM-GSK3β forms cloned, and the cytoplasmatic localization of the S9A-GSK3β and KM-GSK3β proteins (pcDNA3.1(-) constructs), subcellular fractioning of transfected HEK293T was performed (details described in Materials and Methods). Western blots were carried out. Antibody against PARP was used as nuclear marker and antibody against tubulin as cytoplasmatic marker. C: cytoplasm; N: nucleus; M: mitochondria.

To ensure that the NLS did not affect GSK3β kinase activity, GSK3β protein was immunoprecipitated from HEK293T cells transfected with the nuclear constructs, and a kinase assay was performed *in vitro* (Figure 3.7). Results confirmed the higher basal kinase expected for S9A-GSK3β-nuc and the null activity of KM-GSK3β-nuc. These are coincident with the activities observed for their cytoplasmatic homologue constructs (see Figure 3.4. Panel C) and therefore demonstrate that the nuclear localization signal does not interfere with GSK3β kinase activity.

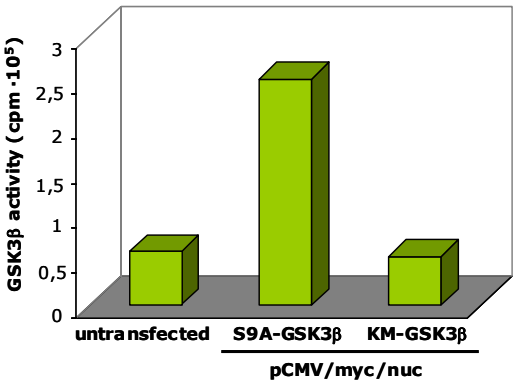


Figure 3.7. GSK3β activity in extracts from HEK293T cells transfected with the pCMV/myc/nuc GSK3β constructs.

Kinase activity was determined in extracts from HEK293T cells transfected with the constructs encoding the nuclear targeted GSK3β proteins: S9A-GSK3β-nuc and KM-GSK3β-nuc.

Once the correct functioning of the constructs was confirmed, HEK293T cells were transiently transfected with the GSK3β constructs with the NLS (pCMV/myc/nuc constructs) or their cytoplasmatic homologues (pcDNA 3.1(-) constructs), together with $[E2F]_3$-LUC and HA-E2F1 as indicated in Figure 3.8. The GSK3β concentration chosen was the highest used in Figure 3.5 (1μg). Luciferase activity was assayed (Figure 3.8.).

As shown in Figure 3.8., the two mutant GSK3β forms, uninhibitable (S9A) and catalytically inactive (KM) have an equal effect on E2F1 transcriptional activity. Cytoplasmatic and nuclear-targeted GSK3β constructs showed the same capacity to increase the transcriptional activity of overexpressed E2F1. Expression of HA-E2F1 and the several GSK3β forms transfected was confirmed by immunoblots using antibodies against HA tag or GSK3β proteins, respectively (Figure 3.8., top). Western blot of GSK3β protein gave 3 bands: the lowest corresponds to endogenous GSK3β protein, the middle to the GSK3β protein cloned into the pcDNA3.1(-) plasmid, and highest to GSK3β protein containing a myc tag and a nuclear localization signal.

Taken together, the data in Figure 3.8. indicate that neither GSK3β kinase activity nor its re-targeting to the nucleus influences the increase in E2F1 transcriptional activity when overexpressing GSK3β, at least under our experimental conditions.

Although we cannot rule out that translocation of GSK3β to the nucleus is necessary for E2F1 activation, under our experimental conditions there may already have been a sufficient amount of GSK3β localized or translocated (when overexpressing any of the GSK3β forms) into the nucleus to modulate E2F1 transcriptional activity.

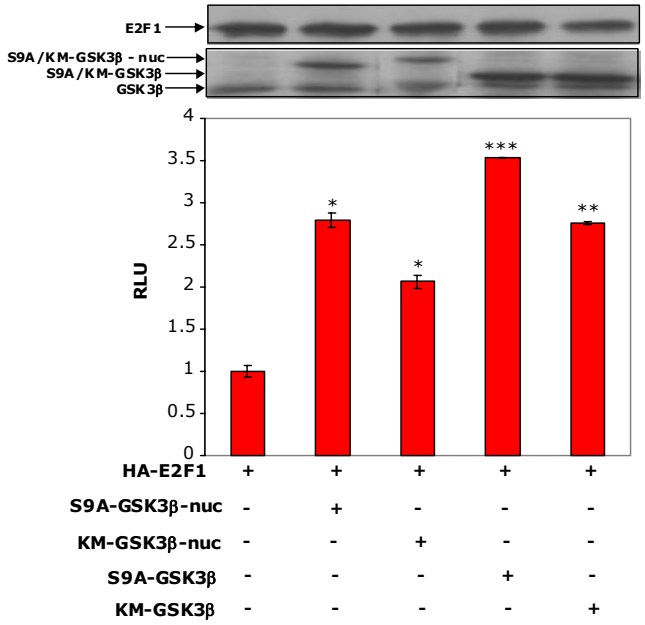


Figure 3.8. Role of the nuclear localization of GSK3β on E2F1 transcriptional activity. Luciferase activity in extracts from HEK293T cells (400,000 cells per 35-mm culture dishes) transiently transfected with 0.3 μg of $[E2F]_3$-LUC plasmid, 0.1 μg of HA-E2F1 plasmid, 0.5 μg of pCMV-βGal plasmid and either 1μg of S9A-GSK3β-pcDNA 3.1 (-) or KM-GSK3β-pcDNA 3.1 (-) constructs or 1μg of S9A-GSK3β-nuc or KM-GSK3β-nuc, as indicated. Luciferase activity was measured and expressed as relative luciferase units (RLU). Values were corrected for relative β-Galactosidase milliunits and are expressed as a ratio to the value of luminescence of cells in which no mutant GSK3β was expressed, which was considered one arbitrary unit. Statistically significant differences are indicated by * $p< 0.05$; ** $p< 0.01$; and ***$p< 0.001$ when compared with GSK3β untransfected cells. Western blots were performed to check expression of transfected constructs.

3.5. Effect of the interaction between GSK3β and E2F1 transactivation domain (AD).

Phosphorylation assays *in vitro* (see Chapter 1) show serine 403 and threonine 433, localized in the transactivation domain of E2F1, are the two residues phosphorylated by GSK3β. Although this phosphorylation does not appear to influence GSK3β modulation of E2F1 transcriptional activity, it suggests that GSK3β binds to E2F1 at its transactivation domain since the two amino acids phosphorylated by GSK3β *in vitro* are localized in this domain. For this reason we focused our study on the effect of GSK3β on this domain in the absence of the E2F1 DNA-binding domain.

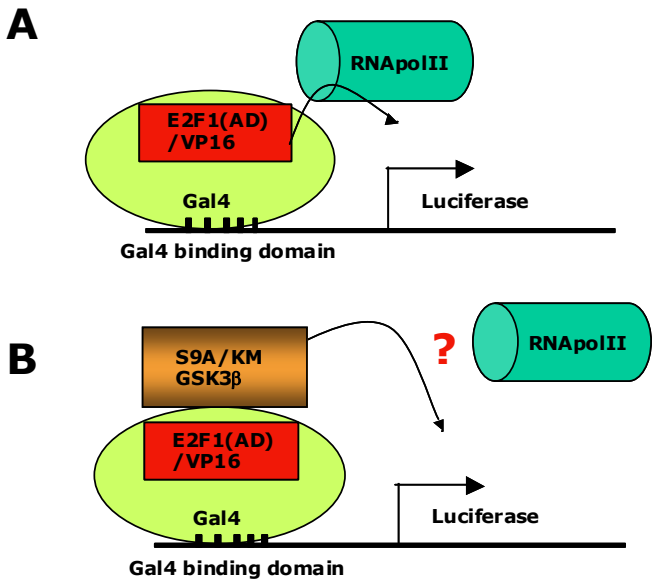
To this end we used expression vectors coding for the E2F1 transactivation domain: pHKGal4-E2F1(AD) and pHKGal4-E2F1(AD)S403A/T433A. Both encode for a fusion protein with DNA-binding domain of the Gal4 protein (present in the plasmid pHKG) and the E2F1 transactivation domain (cloned in frame). In the second construct, pHKGal4-E2F1(AD)S403A/T433A, serine 403 and threonine 433 were both mutated to alanine [Hagemeier et al., 1993; Vandel and Kouzarides 1999].

As a reporter we used Gal4-LUC, which contains the luciferase reporter gene under the control of a promoter inducible by Gal4 protein [De los Pinos et al., 2001]. In addition we also used Gal4-VP16, which encodes for a fusion protein that contains the DNA-binding domain of Gal4 protein fused to the active domain of the herpes virus transactivator protein, VP16 [Dickens et al., 1997; Lai and Herr 1997]. The GSK3β nuclear constructs S9A and KM described above were also used. Since cytoplasmatic and nuclear-directed GSK3β constructs displayed the same effect we decided to transfect only the nuclear constructs.

HEK293T cells were transfected with one or a combination of the constructs described above, respectively. We analyzed the effects of their expression on the induction of the Gal4-LUC expression. Luciferase activity was measured in the transfected cells.

Expression of HA-E2F1 and the GSK3β forms transfected was confirmed by immunoblots using antibodies against the HA-tag or GSK3β proteins, respectively.

Scheme 3.1 represents the model of induction of Gal4-LUC. The fusion protein Gal4-E2F(AD), its double mutant homologue, and Gal4-VP16 all bind Gal4-LUC. They all activate transcription by interacting with the transcriptional machinery through their transactivation domains. Our purpose was to evaluate whether binding of S9A/KM-GSK3β-nuc to E2F1 and VP16 transactivation domains could affect this interaction with the transcriptional machinery (see Scheme 3.1).



Scheme 3.1. Schematic representation of the system Gal4-Luciferase.
(A) In the Gal4 system used in this case, the fusion protein Gal4-E2F1(AD) (and the double mutant, not indicated to simplify the figure) or Gal4-VP16, binds to Gal4-LUC through its Gal4 binding domain and activates the transcription through its E2F1 or VP16 transactivation domain interacting with the transcriptional machinery. (B) With this system we aimed to evaluate the influence of GSK3β on E2F1 (and its mutants). The effect of GSK3β on VP16 transactivation domain will determine the specificity of the influence on E2F1 transactivation domain, serving as a negative control.

The results obtained are shown in Figure 3.9. and reveal that Gal4-E2F1(AD), Gal4-E2F1(AD)S403/T433 and Gal4-VP16 induce transcription of Gal-LUC to the same extent, under our experimental conditions, in transfected HEK293T cells. Both GSK3β

mutants (S9A and KM) inhibit E2F1 transcriptional activity with similar efficiency, suggesting that this modulation is independent of GSK3β kinase activity. The double point mutation 'S403A/T433A' on the transactivation domain of E2F1 does not change the effect by the GSK3β mutants. In contrast, there is no significant modulation of Gal4-LUC transcription by the two GSK3β mutants when transfecting with Gal4-VP16. This indicates GSK3β is acting through the E2F1 transactivation domain in a specific manner. HA-E2F1 and the two nuclear GSK3β constructs (S9A and KM) were correctly expressed, as confirmed by Western blot analysis (Figure 3.9., top).

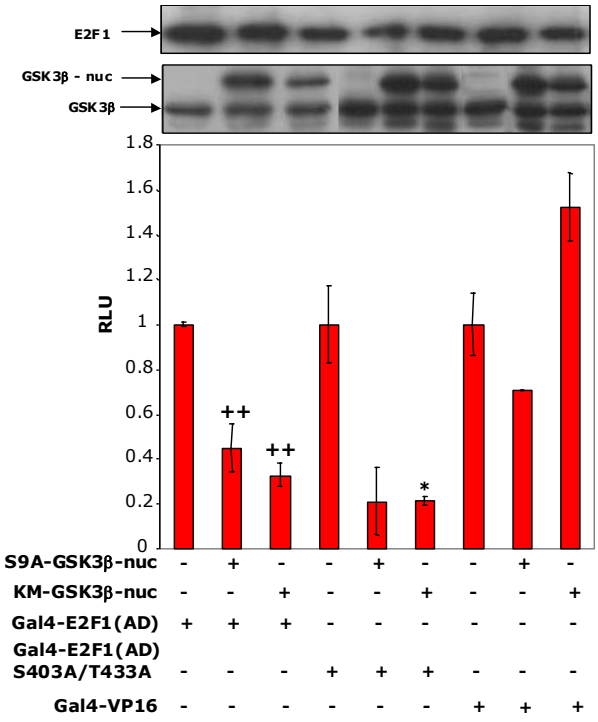


Figure 3.9. Role of GSK3β modulation of E2F1 transactivation domain (AD).

HEK293T cells (400,000 cells per 35-mm culture dishes) were transiently transfected with 0.3 μg Gal4-LUC and 0.5 μg pCMV-βGal, together with a series of different plasmid combinations: A first group was transfected with 0.1 μg of pHKGal4-E2F1(AD) and 1 μg S9A-GSK3β-Nuc or KM-GSK3β-Nuc. A second group was transfected with 0.1 μg of pHKGal4-E2F1(AD)S403A/T433A and 1 μg S9A-GSK3β-nuc or KM-GSK3β-nuc. And a third group was transfected with 0.1 μg of Gal4-VP16 and 1 μg S9A-GSK3β-nuc or KM-GSK3β-nuc. Luciferase activity was measured and expressed as relative luciferase units (RLU). Values were corrected for relative β-Galactosidase milliunits and expressed as arbitrary units. Data are means of three independent experiments. Statistically significant differences are indicated by *$p<0.05$ compared with Gal4-LUC/ pHKGal4-E2F1(AD) transfected cells, and ++$p<0.01$ compared with Gal4-LUC/ pHKGal4-E2F1(AD)S403A/T433A transfected cells.

The fact that the double point mutation 'S403A/T433A' on the transactivation domain of E2F1 does not change the effect by the GSK3β mutants and the equal activation of Gal4-LUC is consistent with what has been found earlier for pRB [Vandel and Kouzarides 1999]. Vandel and Kouzarides observed that phosphorylation or lack of phosphorylation of E2F1 (AD) at serine 403 or/and threonine 433 does not play a major role either in the transactivation capacity of E2F1 (AD) or in the binding and repression by pRB.

When analyzing the effect of GSK3β on E2F1(AD), E2F1 transcriptional activity is inhibited. In contrast, when using the whole E2F1 protein the transcriptional activity is enhanced (see Sections 3.3. and 3.4) However, these findings are not necessarily contradictory. It is known that the transcriptional properties of a particular factor are influenced either by its position relative to other factors bound to a given promoter or by the abundance of transcriptional cofactors in a given cell type. This cell context-dependent regulation can be lost when transferring a binding site to a heterologous promoter (as in our case), when the position of the binding site is changed within a natural promoter, or when the cellular expression levels of critical transcriptional cofactors are altered [Fry and Farnham 1999].

In the light of the results obtained in this chapter, we conclude that GSK3β modulates E2F1 transcriptional activity by binding to E2F1 transactivation domain (AD). This binding is independent of GSK3β activation state, and point mutations of serine 403 and threonine 433 do not preclude this binding nor its effect. Addition of a nuclear localization signal (NLS) to GSK3β does not significantly increase its effect over E2F1 transcriptional activity, under our experimental conditions.

IV DISCUSSION

DISCUSSION

Whether a cell enters the cell cycle, undergoes apoptosis or survives, is a consequence of the integration of several extracellular signals. Growth factors, cell adhesion and apoptosis inductors regulate a complex system of signal transduction pathways that modulate the activity of a large number of genes. The response of the cell requires the regulation and synchronization of a variety of processes. For example, not only mitogenic transduction pathways, but also other pathways associated with metabolic processes, cell survival and growth participate in cellular proliferation. The contribution of signal transduction pathways in these processes is a priority focus of research. In this context an essential role has been suggested for the PI 3-kinase/Akt pathway as an integrating and transferring pathway of mitogenic, metabolic, survival, apoptotic and growth processes [Dudek et al., 1997; Rodriguez-Viciana et al., 1994; Kozma and Thomas 2002; Fernández de Mattos et al., 2000]. The E2F family of transcription factors have a central role in commitment to cell proliferation or apoptosis [La Thangue et al., 2003; Bell et al., 2003]. Previous results obtained by others and our group implicate the PI 3-kinase/Akt pathway in E2F transcriptional activity [Brennan et al., 1997; Fernández de Mattos et al., 2002]. In this context we began to examine whether E2F was modulated by GSK3β, a downstream kinase from Akt/PKB in the PI 3-kinase/Akt pathway.

GSK3β phosphorylates E2F1

We first searched for consensus GSK3β phosphorylation sites on E2F1 protein. Scansite studies revealed ten putative GSK3β phosphorylation sites. Further analyses of the capacity of GSK3β to phosphorylate E2F1 *in vitro* were decisive Purified GSK3β showed specificity for E2F1 phosphorylation as well as other GSK3β substrates such as p53 and cyclin D1. Phosphorylation of human E2F1 by GSK3β was detected *in vitro*. This phosphorylation does not require priming phosphorylation. LC-MS studies were performed to discover the residue/s being phosphorylated. These studies gave three possible phosphopeptides, two of which we identified (serine 403 and threonine 433) were phosphorylated by GSK3β. These amino acids are localized in the transactivator

domain of E2F1. Point mutations of these sites reverted E2F1 phosphorylation *in vitro,* validating the mass spectrometry studies. Serine 403 appears to be more strongly phosphorylated than threonine 433 since the mutation of the former to alanine reverts the phosphorylation state to a greater extent than mutation of the latter. Neither of these two residues coincides with any of the ten *in silico* predictions. However, as mentioned above, electrospray mass spectrometry gave three peaks of radioactivity. Phosphopeptides of two of them were identified while the other gave no signal. The unidentified fraction might include one of the residues embedded in the consensus cluster -SXXXS(P)-, which Scansite uses to predict putative GSK3β phosphorylation sites in E2F1.

Most substrates of GSK3 must first be phosphorylated by another protein kinase at a serine or threonine residue located four residues carboxy-terminal to the site of GSK3 phosphorylation [Frame et al., 2001]. GSK3β target proteins known to require priming phosphorylation are glycogen synthase and β-catenin. Roach reported that glycogen synthase has multiple GSK3β phosphorylation sites which are located in clusters following the motif –SXXXS(P)- and that multiple phosphorylation occurs in a hierarchal fashion [Roach 1990]. Four GSK3β phosphorylation sites were defined, 3a, 3b, 3c, and 4. Priming phosphorylation of site 5 by casein kinase II is necessary for GSK3β to phosphorylate these four sites [Roach 1990; Fiol et al., 1990]. In the case of β-catenin, threonine 41, serine 37, and serine 33 are also located in a cluster with the motif –SXXXS(P)- and their phosphorylation is mediated by GSK3β in a sequential manner, beginning from the C-terminal threonine 41. The need for priming phosphorylation of serine 45 to allow GSK3β sequential phosphorylation has been shown, and casein kinase I alpha (CKIalpha) is the candidate priming kinase [Hagen and Vidal-Puig, 2002; Liu et al., 2002]. Other examples of GSK3β substrates which require priming phosphorylation are shown in Table 1.

Metabolic and signaling proteins	**Structural proteins**	**Transcription factors**
ATP-citrate lyase	NCAM	AP-1 (Jun family)
Cubitus interruptus	Neurofilaments	β-catenin/Armadillo
eIF2B	Telokin (KRP)	C/EBP
Glycogen synthase		CREB
hnRNP		HSF-1
Presenilin-1		c-Myb
Protein phosphatase I		c-Myc
		NFAT
		Notch
		TCF

Table 1. GSK3β primed substrates.

GSK3β primed subtrates include metabolic and signaling proteins, structural proteins and transcription factors.

C/EBP, CCAAT/enhancer-binding protein; CREB, cyclic AMP response element-binding protein; eIF2B, eukaryotic initiation factor 2B; hnRNP, heterogenous nuclear ribonucleoprotein D; NCAM, neural cell-adhesion molecule; NFAT, nuclear factor of activated T cells.

The substrates listed can be found in the following references: Frame and Cohen 2001; Grimes and Jope 2001; Doble and Woodgett 2003; Jope and Johnson 2004, and references therein.

As mentioned above, Scansite *in silico* predictions consider -SXXXS(P)- to be the recognition motif of GSK3. However, our *in vitro* results do not bear out/confirm this consensus site and clearly demonstrate that a primer phosphate is not necessary for phosphorylation of E2F1 by GSK3β *in vitro.* Nevertheless, it has been reported that a negatively-charged amino acid such as glutamate or aspartate at position n+4 can mimic the negative charge of the phosphate group introduced by the priming kinase [Casanova et al., 1990; Napper et al., 1996; Lee et al., 2004]. Sequence analysis of human E2F1 shows there is a glutamate at position n+4 carboxy-terminal from serine 403.

Although priming phosphorylation is considered the major mechanism by which GSK3 phosphorylates its targets, not all GSK3 substrates require a primer phosphate. This is the case of cyclin D1 and Tau protein. Diehl and collaborators observed that purified GSK3β or GSK3β recovered from mouse fibroblasts phosphorylates recombinant cyclin D1 only at threonine 286 *in vitro* [Diehl et al., 1998]. They report that phosphorylation of cyclin D1 on threonine 286 depends on the integrity of proline 287 but not on a previously primed phosphate. Although cyclin D1 also contains a threonine at residue 288, threonine 286 is the only residue phosphorylated in mammalian cells [Diehl et al., 1997]. GSK3β can act as a proline-directed kinase, and the ability of the purified enzyme to phosphorylate a bacterially expressed, affinity-purified GST fusion protein containing only the C-terminal 41 amino acids of cyclin D1 supports this principle. Several other proline-directed kinases described did not phosphorylate cyclin D1 on threonine 286. In addition the serines closest to threonine 286 lie some distance away, at residues 257 and 258. Therefore, although processive phosphorylation of GSK3β cannot be ruled out, data suggested that phosphorylation of cyclin D1 on threonine 286 depended on proline 287 integrity rather than on priming phosphorylation. Moreover, GSK3β also phosphorylates Tau protein at threonine 212 and the minimal consensus sequences described for GSK3β are serine-proline (Ser-Pro) and threonine-proline (Thr-Pro) [Zheng-Fischhöfer et al., 1998].

As mentioned above, in the case of non priming phosphorylation, the presence of a proline can induce the phosphorylation of a proline-directed kinase. Considering that neither serine 403 nor threonine 433 from E2F1 constitutes part of the recognition motif –SXXXS(P)-, but both contain a proline at position n+1, a proline-directed mechanism could account for GSK3β phosphorylation of E2F1 at these residues *in vitro,* rather than a primed substrate mechanism.

In our experimental conditions, E2F1 phosphorylation by GSK3β *in vivo* was not detected. Overexpression of GSK3β protein did not increase the E2F1 phosphorylation detected *in vivo*. No difference in E2F1 phosphorylation was observed when expressing the different GSK3β forms (WT, KM and S9A). Moreover, E2F1 phosphorylation *in vivo* , in which the endogenous GSK3β activity is modulated using chemical inhibitors,

showed no clear change when using Ly 294002 (PI 3-kinase inhibitor) or SB 216763 (GSK3β inhibitor). Thus we cannot confirm that E2F1 is phosphorylated *in vivo* by GSK3β. The *in vitro* phosphorylation observed at serine 403 and threonine 433 could be a consequence of the docking of GSK3β with E2F1 that could expose these amino acids to the catalytic domain and therefore facilitate phosphorylation *in vitro*. Still, our data do not formally exclude a possible endogenous phosphorylation *in vivo*.

Since GSK3 is a multi-task kinase involved in different signaling pathways, the list of proposed substrates of GSK3 is growing continually. In order to establish the integrity of these substrates, Frame and Cohen indicated the criteria that need to be fulfilled before a protein can truly be regarded as a physiological substrate for GSK3 [Frame and Cohen 2001]. These criteria are listed at the end of Chapter 1 of this thesis. As commented Chapter 1, genetic evidence (corresponding to the last two criteria) for the *in vivo* phosphorylation of GSK3β substrate has not yet been obtained. Furthermore, the majority of the proteins that have been proposed as substrates of GSK3β only meet the criteria that establish their role as substrates *in vitro.* Considering E2F1 as a putative GSK3β substrate to be confirmed, it fulfills the criteria to be considered a GSK3β substrate *in vitro*. Phosphopeptide mapping and sequence analysis indicated two amino acids phosphorylated by GSK3β. Mutations of these amino acids abolished *in vitro* phosphorylation. It should be noted that the proposed criteria to identify a physiological GSK3β substrate are based on the fact that GSK3β exerts its function on its targets by phosphorylating them. Our results indicate that GSK3β may affect E2F1 independently of phosphorylation. However, other experimental approaches, like the use of specific antibodies against E2F1 phosphorylated in serine 403 and/or threonine 433 GSK3β, may establish whether of E2F1 is phosphorylated by GSK3β *in vivo*.

GSK3β binds to E2F1 independent of its kinase activity.

Our co-immunoprecipitation assays using antibodies against E2F1, GSK3β and the tag present in the overexpressed E2F1 proteins show that GSK3β and E2F1 interact *in vitro* and *in vivo*. To our knowledge this is the first time that this binding has been demonstrated. As mentioned above, this binding was independent of GSK3β kinase activity since uninhibitable (S9A-GSK3β) and catalytically inactive (KM-GSK3β) forms of GSK3β bind to E2F1 to the same extent as wild-type GSK3β. The interaction between E2F1 and GSK3β also appears to be cell line independent, since results were reproduced in two cell lines. Furthermore, the binding experiments were conducted in the presence of FCS, and thus in conditions in which kinase activity of wild-type GSK3β is inhibited due to its phosphorylation at serine 9 by PKB. As a consequence, the N-terminal tail of GSK3β is transformed into an inhibitory pseudosubstrate. The phospho-serine occupies the same pocket as the priming phosphate of some of the substrates, blocking the access of these substrates to the active site. The fact that phosphorylation in serine 9 at the amino terminus tail does not abolish E2F1 binding to GSK3β supports that E2F1 binds GSK3β at a different site from the primed substrates. We thus conclude that E2F1 binds tightly to GSK3β, forming a stable complex independently of the phosphorylation at serine 9 or its catalytic activity (see figure 1).

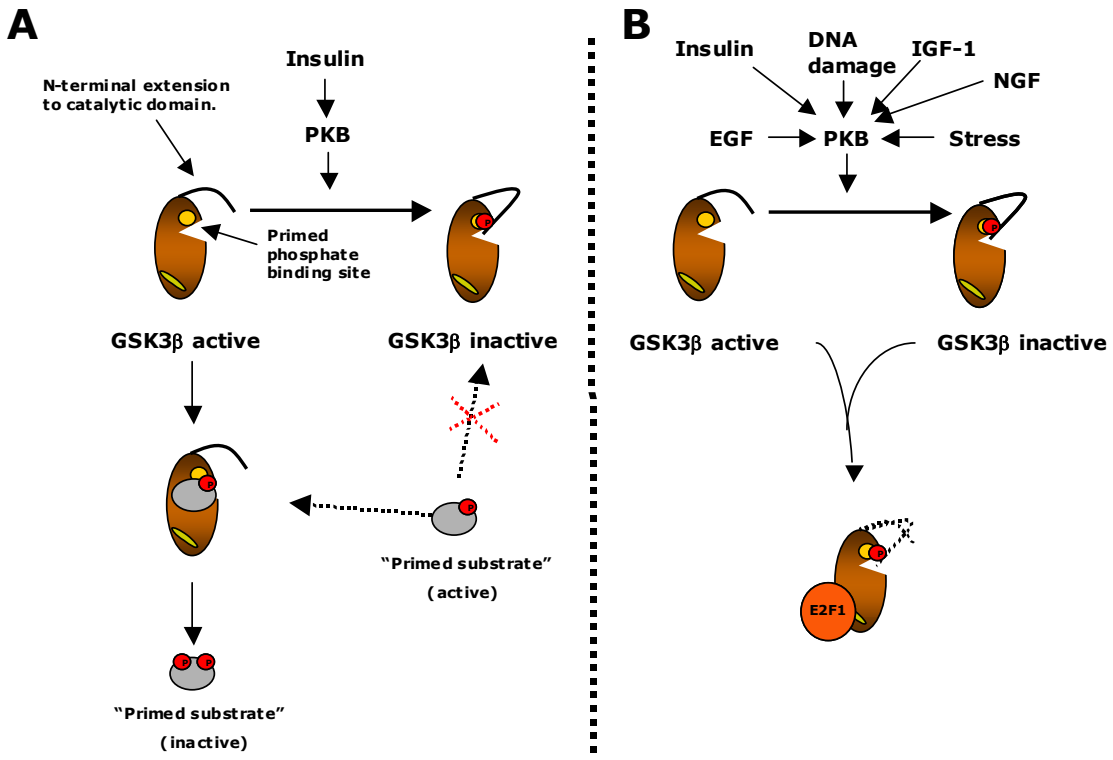


Figure 1. Docking mechanism of primed substrates with GSK3β and possible docking mechanism of E2F1.

(A) Non-phosphorylated GSK3 is fully active. Substrates that have a priming phosphate bind to a specific GSK3 pocket and become phosphorylated on a serine or threonine located four amino acids N-terminal to the priming phosphate. Upon cell stimulation by insulin (or growth factors), PKB can phosphorylate serine 9 located on the N-terminal tail of GSK3β, transforming the tail into a pseudosubstrate which blocks the access to the active site. (B) In response to cell stimulation by insulin, growth factors or DNA damage, E2F1 binds to GSK3β in a docking site different from the primed phosphate binding site. The interaction is independent of the phosphorylation state of serine 9 from GSK3β N-terminal tail or its catalytic activity.

Binding sites other than that bound by the primed substrates have been proposed for other proteins. This is the case for Axin and FRAT, whose binding site with GSK3β co-localizes in the C-terminal domain of GSK3β, hence their binding is mutually exclusive [Dajani et al., 2003]. This docking site seems exclusive to the GSK3β isoform and appears to be regulated neither by insulin nor by phosphorylation at the N-terminal residues (N-tail whose phosphorylation blocks the primed phosphate binding site) [Behrens et al., 1998; Li et al., 1999; Ding et al., 2000; Farr et al., 2000].

Although signal transduction pathways use protein kinases for the modification of protein function by phosphorylation, docking interactions with these also contribute to their specificity and regulation. Therefore in the case of GSK3β, not only its kinase activity is responsible for its effects on the cellular processes, but also its association with other proteins. This could be the case for GSK3β binding to E2F1.

GSK3β phosphorylates E2F1 on the same residues as cdk7: serine 403 and threonine 433.

Interestingly, both serine 403 and threonine 433 of E2F1 are also phosphorylated by RNA polymerase II transcription factor TFIIH [Vandel and Kouzarides 1999]. This complex is composed of at least 9 subunits, which include cdk7, cyclin H and MAT1. These three subunits form a ternary complex: cdk-activating kinase (CAK). The kinase activity of the whole TFIIH complex is directed primarily towards the phosphorylation of the carboxy-terminal domain of RNA polymerase II; but the complex cyclinH-cdk7 and CAK can also phosphorylate and activate cdk2. The fact that E2F1 is a target of TFIIH suggests an additional role of this kinase in the regulation of the cell cycle progression. TFIIH binds to E2F1 specifically in S phase *in vivo* and results in E2F1 phosphorylation and rapid degradation by an ubiquitin-proteasome-dependent pathway [Campanero and Flemington 1997]. The results reported in this thesis demonstrate that GSK3β and TFIIH interact with E2F1 on the same specific domain; nevertheless, we did not detect any change in the half-life of E2F1 after GSK3β overexpression. We cannot rule out the possibility that this difference is due to a difference in experimental conditions.

Then who phosphorylates E2F1 *in vivo*? Our results suggest that TFIIH phosphorylates E2F1 *in vivo*. We did not detect phosphorylation of E2F1 by GSK3β. GSK3 inhibitors do not modify E2F1 phosphorylation *in vivo*. In contrast, when cdk7 (the kinase associated with TFIIH) inhibitor DRB was used there was a significant reduction of E2F1 phosphorylation. Despite these results, data from other studies suggest that a kinase other than cdk7 contributes to the phosphorylation of E2F on the serine 403 and threonine 433. Vandel and Kouzarides demonstrated phosphorylation of E2F1 during

middle G1 and G2 phases in HeLa cells. However this pattern of phosphorylation did not correlate with the binding of TFIIH to E2F. Immunoprecipitation experiments demonstrate that p62, one of the subunits of TFIIH which binds to E2F1, is not present in the E2F1 immunoprecipitated during G1 and G2 phases, but it is present in the early S phase. In addition, all the depletion cdk7 systems (antibody, dominant-negative or DRB) used to demonstrate the involvement of this kinase in E2F phosphorylation gave 30-40 % of residual activity. Is GSK3 responsible for this remaining activity?

The fact that GSK3 and TFIIH share the same substrate, E2F, is not surprising. Indeed, TFIIH (CAK) and GSK3β phosphorylate another common substrate: p53. Both kinases phosphorylate p53 at serine 33 *in vitro* and *in vivo*, without the requirement of a priming kinase. However, the functional relevance of this phosphorylation remains to be determined. Ko and colleagues suggested that phosphorylation of p53 by CAK affects the association of p53 with other proteins interacting with the N-terminus, such as MDM2 or the TAFs [Ko et al., 1997]. On the other hand, Turenne and Price reported that GSK3β phosphorylates serine 33 of p53 and activates p53 transcriptional activity [Turenne and Price 2001]. In contrast, Watcharasit and co-workers demonstrated that the GSK3-binding region of p53 is localized at residues 364-373 and GSK3 activity is not required for association with p53 [Watcharasit et al., 2003]. Indeed, inhibition of GSK3 stabilizes its association with p53. In this model, inactive GSK3 would repress p53-induced genes (MDM2, p21, Bax). Activation of GSK3β would be necessary for its dissociation and to fully induce transcriptional activity of p53. In contradiction with the activing role GSK3β, Qu and colleagues have demonstrated that endoplasmic reticulum (ER) stress induces GSK3β activity, destabilizes p53 and prevents cells from entering p53-dependent apoptosis. During this process, GSK3β phosphorylates p53 at serine 376 and promotes its cytoplasmic localization [Qu et al., 2004].

All the reports described above point out that GSK3β and TFIIH interact physically with p53 and suggest that, directly or indirectly, these kinases modulate p53 transcriptional activity. At the same time, it has been reported that p53 can also regulate the activities of these kinases. Interaction of p53 with TFIIH resulted in a significant downregulation of cdk2 and CTD phosphorylation [Schneider et al., 1998]. These

findings imply a direct involvement of p53 in triggering growth arrest by its interaction with the cdk activating kinase complex without the need of cyclin-dependent kinase inhibitors. The binding of p53 to GSK3β increased the activity of GSK3β [Watcharasit et al., 2002]. It has been suggested that this interaction provides a mechanism for p53 to downregulate several survival-promoting transcription factors that are known to be phosphorylated by GSK3 in the nucleus, the inhibition of which would promote p53 activation of the apoptotic program.

E2F1 and p53 are key transcription factors that mediate the cellular response. The results of this thesis suggest that, as reported for p53, GSK3β interaction could play a important role in E2F1 transcriptional activity. At present, we do not rule out any of the possibilities of regulation described for p53. The physical interaction of GSK3β to E2F1 could affect the association of E2F1 to other proteins (pRB family, transcription factors) and regulate their activity. The fact that serine 403 and threonine 433 are phosphorylated *in vitro* by GSK3β, and are localized on the E2F1 transactivation domain supports this model of modulation of transcriptional activity. GSK3β may also be able to phosphorylated E2F1 *in vivo*, but we did not detect it, perhaps due to the limited sensitivity of the assay or cell-type specificity. The experiments reported in this study were aimed at establishing whether GSK3β (protein or activity) regulates E2F1 transcriptional activity. However, the possibility that E2F1 could also regulate GSK3β substrate specificity remains to be explored.

Regulation of E2F1 transcriptional activity: role of GSK3β.

In the last part of this thesis, we evaluate the effect of GSK3β on E2F1 transcriptional activity. For this purpose we used GSK3β chemical inhibitors to control GSK3β kinase activity and RNAi to knock down the total GSK3β protein level in the cell. No change on E2F1 transcriptional activity was detected when using specific GSK3β inhibitors, SB 216763 and SB 415286. Nevertheless, depletion or knockdown of GSK3β protein by RNAi clearly showed a reduction of E2F1 transcriptional activity proportional to the reduction in GSK3β protein. These results strongly suggest that modulation of E2F1 transcriptional activity is independent of GSK3β kinase activity but dependent on

GSK3β protein concentration. This opens the possibility that GSK3β could bind E2F1 in a tetrameric complex GSK3β-E2F1-DP1 which interacts with the transcriptional machinery.

Interestingly, when we used PI 3-kinase inhibitor LY 294002 there was a reduction in E2F1 transcriptional activity. Combination of the LY 294002 and SB 216367 treatments led to the same reduction in E2F1 transcriptional activity. All this indicates PI 3-kinase pathway can regulate E2F1 transcriptional activity in a manner that is independent of GSK3β kinase activity but dependent on GSK3β protein concentration. This result is surprising if we consider that the HEK293T cell line was chosen because E2F1 escapes from the pRB control (see Scheme 2.1 in Chapter 2) and we expected a direct control of E2F1 by GSK3β. Nevertheless, the results obtained could be explained by a surplus control of E2F1 activity through hyperphosphorylation of pRB protein of pRB-E2F1 complexes (Figure 2). There could be still a small fraction of E2F1 protein in HEK293T controlled by the phosphorylation of pRB by the cyclin D-cdk4/6 complex. Cyclin D turnover is regulated in a complex manner. Its transcription is regulated by Ras-PI 3-kinase and mitogen-activated kinases and by β-catenin, which is negatively regulated by GSK3β phosphorylation. PKB (downstream effector of PI 3-kinase) is involved in the translation control of cyclin D through p70S6K and eIF4E activation. In addition p70S6K also controls the activation of eIF4E through 4E-BP1 phosphorylation [Albanese et al., 1995; Lavoie et al., 1996]. On the other hand, expression of cyclin D1 is mediated by PI 3-kinase through mTOR-p70S6K-independent signaling in growth-factor-stimulated NIH-3T3 fibroblasts [Takuwa et al., 1999]. As mentioned above, β-catenin can also increase cyclin D1 expression [Tetsu and McCormick 1999]. This is in contradiction with our results since inhibition of GSK3β corresponds with an accumulation of β-catenin but not with and increase in E2F1 transcriptional activity. The degradation of cyclin D is less understood but an increase in its degradation following GSK3β phosphorylation has been described (see Figure 2) [Diehl et al., 1998].

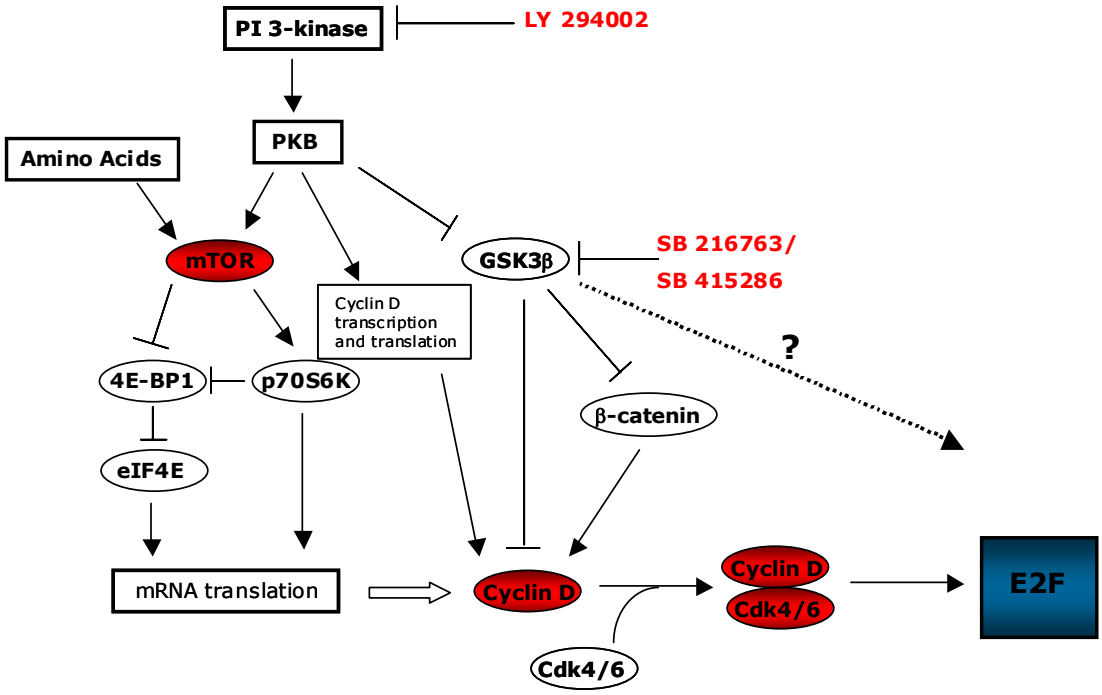


Figure 2. Various mechanisms of control of E2F1 transcriptional activity.

Therefore, the remaining repression of pRB on E2F1, through its binding to E2F1, could explain the pattern of regulation of E2F1 transcriptional activity in our experimental conditions. The PI 3-kinase-mediated effect on E2F1 transcriptional activity may be regulated by the action of this kinase on levels of cyclin D. Note, that although cells were incubated in the absence of foetal calf serum, their transformed nature allows them to escape the normal regulation of their signaling pathways. Thus a percentage of transformed cells could have their PI 3-kinase pathway activated independently of growth factors levels. GSK3β could then control E2F1 activation by competing with pRB for E2F1 binding and therefore liberating E2F1 from the pRB repression. In our model this mechanism seems to prevail over the control of cyclin D protein degradation through GSK3β phosphorylation [Diehl et al., 1998].

Conversely, when we overexpressed GSK3β protein we detected an increase of endogenous and overexpressed E2F1 transcriptional activity on the E2F-responsive construct $[E2F]_3$-LUC. Ten folds higher concentration of GSK3β protein compared to E2F1 protein was required. Higher amounts of total GSK3β protein lead to a higher luciferase activity. Furthermore, a comparable increase was obtained when overexpressing uninhibitable (S9A) and catalytically inactive (KM) forms of

GSK3β. These results indicate GSK3β kinase activity is not necessary to detect the effect over E2F1 transcriptional activity while the level of protein appear limiting. This is in agreement with the results obtained using PI 3-kinase and GSK3β chemical inhibitors and reducing GSK3β total protein with RNAi.

Overexpression of nuclear-targeted GSK3β forms does not increase the effects of GSK3β on E2F1 transcriptional activity.

GSK3β is mainly cytoplasmatic, but it is also present in other subcellular compartments such as the nucleus and mitochondria with a higher activity [Bijur and Jope 2003a]. The formation of specific multiprotein complexes can also regulate the translocation between compartments and influence the access of different signals to GSK3β.

Translocation of GSK3β from the cytoplasm to the nucleus has also been reported during cell cycle and apoptosis. During S phase an accumulation of GSK3β has been detected in the nucleus, indicating a role of this kinase during this phase. In addition, Bijur and Jope observed a rapid nuclear level increase of GSK3β in apoptotic conditions such as serum-free media, heat shock, or staurosporine [Bijur and Jope 2001]. The intracellular localization of GSK3β is dependent on the PI 3-kinase pathway, but independent of the phosphorylation state of serine 9 and/or tyrosine 216. Because GSK3β does neither have a nuclear localization signal (NLS) nor a nuclear export signal (NES), the PI 3-kinase could be regulating the binding to GSK3β of some importing or exporting proteins. An example is the case of Frat, which binds GSK3β and regulates its nuclear export. Frat contains a nuclear export sequence that promotes its own nuclear export and that of associated GSK3β.

On the overexpression of an uninhibitable (S9A) and catalytically inactive (KM) GSK3β form with a nuclear localization signal we aimed to assess the effect of nuclear re-targeting of the protein on E2F1 transcriptional activity. Nuclear GSK3β forms showed the same trend in activation of E2F1 transcriptional activity as their overexpressed homologous cytoplasmatic forms. These results suggest the increase of

the protein concentration itself may be enough to increase GSK3β translocation to the nucleus without the need of a nuclear localization signal. GSK3β could translocate to the nucleus by binding to a protein containing a nuclear localization signal. Therefore we cannot rule out the need of nuclear translocation of GSK3β to regulate E2F1 activity.

E2F1 transactivation domain (AD) is sufficient for GSK3β to modulate E2F1 transcriptional activity.

Since GSK3β phosphorylates *in vitro* residues serine 403 and threonine 433, located in E2F1 transactivation domain (AD), it is likely that the effect of GSK3β on E2F1 transcriptional activity takes place through the interaction of GSK3β with this domain. To address this question we measured the effect of GSK3β overexpressed forms (S9A and KM) on the transcriptional activity of an E2F1 fusion protein containing E2F1 transactivation domain fused to a Gal4 DNA-binding domain.

Both GSK3β forms employed were able to regulate in the same way E2F1 transcriptional activity in the absence of the E2F1 DNA-binding domain. This regulation is specific, since GSK3β showed no modulation of acidic transactivation domain of the helper virus protein, VP16. The results suggest an "action model" in which E2F1 transactivation domain is sufficient for GSK3β to exert its activity. This, together with the strong binding observed in the co-immunoprecipitation assays discussed previously would be in agreement with a binding of GSK3β to E2F1 on its transactivation domain. Furthermore, GSK3β effect appeared independent of the mutation of both serine 403 and threonine 433 in the transactivation domain and of GSK3β kinase activity. As a consequence the expected binding of GSK3β to E2F1 transactivation domain would not be affected by point mutation of serine 403 or threonine 433. GSK3β binding domain could overlap with the pRB binding domain. Conversely, it has been reported that neither binding nor action of pRB is affected by these two point mutations [Vandel and Kouzarides 1999].

In this case GSK3β causes an inhibitory effect over the luciferase expression of the Gal4-LUC construct used, contrary to the activation effect observed when using the whole E2F1 protein and the $[E2F]_3$-LUC construct. This can be easily explained by context-dependent transcriptional regulation. The transcriptional properties of a particular factor are influenced either by its position relative to other factors bound to a given promoter or by the abundance of transcriptional cofactors in a given cell type. This context-dependent regulation can be lost when transferring a binding site to a heterologous promoter, when the position is changed within a natural promoter, or when the cellular expression levels of critical transcriptional cofactors are altered [Fry and Farnham 1999]. The cell context is also important. Genes like cyclin E and Mcl-1 have the same sequence of binding to E2F1, but in the first case E2F1 activates the transcription while in the second case it inhibits it [Croxton et al., 2002]. Considering all this it is not surprising that we obtain different effects on the modulation of $[E2F]_3$-LUC construct versus Gal4-LUC construct, when introducing full human E2F1 protein or a Gal4 fusion protein containing E2F1 transactivation domain (AD).

Interaction between GSK3β and E2F1: a link between cell signaling and regulation of cell cycle progression and apoptosis?

Cell cycle progression and apoptosis is controlled by a complex network of signal transduction pathways. To date, examination of these pathways has revealed a cross-talk between them, increasing the interconnections between signals. A deeper analysis of some members of these pathways and of the cell cycle machinery has discovered key factors that act like switches between cell signaling and cell cycle progression control. This could be the case for GSK3β. Our results suggest a physiological role for GSK3β in cell cycle progression and apoptosis through the regulation of E2F1 transcription factor.

During cell cycle, growth factors activate diverse intracellular signaling pathways being PI 3-kinase pathway one of the main pathways involved. The PI 3-kinase pathway has a key role in the G1/S progression and its induction leads to the activation of PKB. PKB phosphorylates GSK3β and this phosphorylation promotes GSK3β inhibition. PKB

activation is periodic in early G1 but falls when the cell progresses into the S phase [Auger et al., 1989]. The decrease in PKB activity leads to GSK3β activation [reviewed in Plyte et al., 1992]. GSK3β translocates to the nucleus during the S phase in NIH3T3 cells, increasing nuclear GSK3β levels [Alt et al., 2000]. In the nucleus GSK3β is able to interact with a broad array of transcription factors such as AP-1, CREB and other nuclear proteins such as cyclin D1 and eIF2B. The data obtained in this study add considerably to this picture, and allows us to hypothesize a direct regulation of E2F1 transcription factory by GSK3β. GSK3β can regulate E2F activity indirectly through the increase of cyclin D1 [Diehl et al., 1998] and β-catenin degradation. Nevertheless our results point out the possible coexistence of another regulatory mechanism of E2F1 activity. We show GSK3β interaction with E2F1 transactivation domain is required for a full E2F1 transcriptional activity. According to the model we propose, E2F1 trancriptional activity would not only be controlled by the binding of pRB but also by GSK3β binding to its transactivation domain (Figure 3 A).

Under proapoptotic stimuli such as serum withdrawal and DNA damage, GSK3β also translocates to the nucleus [Bijur and Jope 2001]. The increased nuclear level of GSK3β facilitates the interactions with its nuclear targets involved in apoptosis like the tumor suppressor p53 [Watcharasit et al., 2002; Watcharasit et al., 2003]. E2F1 also exhibits the ability to trigger apoptosis through different mechanisms that are mainly divided into p53-dependent or independent mechanisms. This ability has been shown to be regulated by a PI 3-kinase dependent signaling pathway [Hallstrom and Nevins 2003]. Taking into consideration the implication of both proteins, GSK3β and E2F1, in apoptosis and their regulation through PI 3-kinase pathway, we consider GSK3β interaction with E2F1 may ultimately affect E2F1 apoptotic activity (Figure 3 B).

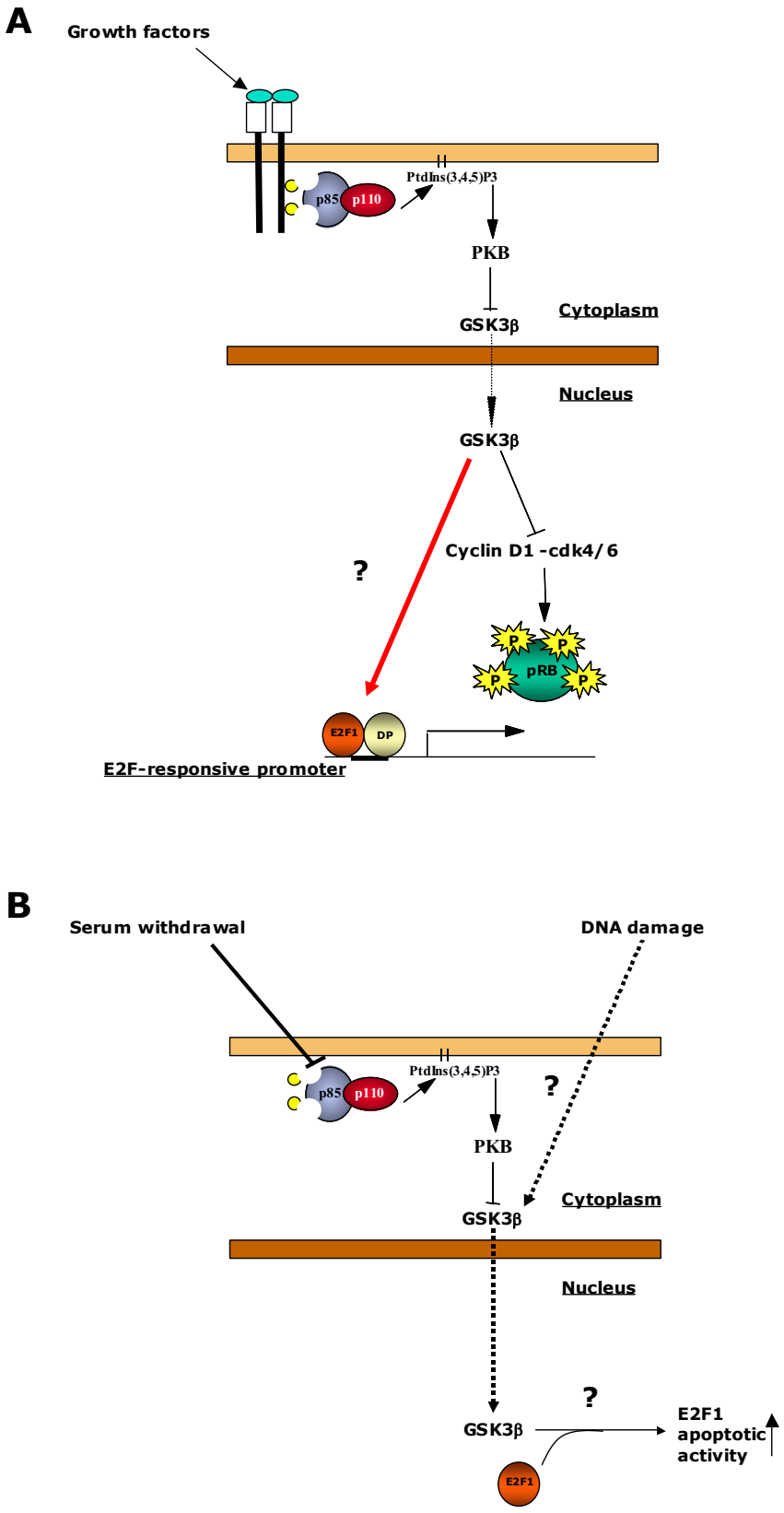


Figure 4. GSK3β effects on E2F1 transcriptional activity.

V CONCLUSIONS

CONCLUSIONS

1. GSK3β phosphorylates human E2F1 *in vitro*. This phosphorylation is specific and takes place without the requirement of priming phosphorylation. Previous phosphorylation by p38 did not entail synergistic activity.

2. LC-MS studies indicate that GSK3β phosphorylates serine 403 and threonine 433 on human E2F1 *in vitro*. This phosphorylation is lost when both of these two amino acids are mutated, confirming the mass spectrometry results.

3. Kinase assays demonstrate that E2F1 is phosphorylated *in vivo*. However, inhibition of GSK3β activity does not affect endogenous E2F1 phosphorylation *in vivo.*

4. Co-immunoprecipitation reveals the interaction between GSK3β and E2F1 in HeLa and HEK293T cell lines *in vivo*. This interaction is detected when immunoprecipitating either protein. The binding is not affected by GSK3β activity, as evidenced by co-immunoprecipitations using an un-inhibitable (S9A), a catalytically inactive (KM) and a wild-type form leading to the same result.

5. The use of chemical inhibitors to modulate endogenous GSK3β activity in HEK293T cells overexpressing E2F1 does not affect transcriptional activity of an E2F-responsive promoter ($[E2F]_3$-LUC). In contrast, its activity is reduced in a dose-dependent manner when knocking down GSK3β by RNAi.

6. Uninhibitable (S9A) and catalytically inactive (KM) forms of GSK3β modulate endogenous and overexpressed E2F1 transcriptional activity of the E2F1-responsive promoter equally. This suggests that kinase activity is not required for the regulation of E2F1 ability to activate transcription.

7. Functional studies using E2F1 constructs expressing the transactivation domain (AD) show that this domain is sufficient for transcriptional modulation mediated by GSK3β. Mutation of residues 403 and 433 in this domain do not abrogate this activity indicating that the phosphorylation state of these amino acids is irrelevant in this case.

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VII MATERIALS AND METHODS

CELL CULTURE

Principle: this term is used to denote the growing of cells *in vitro.* In cell cultures, the cells are no longer organized in tissues. These cells will grow, survive and maintain function in suspension or when attached to an inert surface such as a plastic plate.

Cell lines

The cell lines used in this study have been the following:

-**HeLa**: human epitheloid carcinoma cell line

-**HEK-293T**: human embryo kidney cell line

The cells were routinely grown in an incubator at 37^{o}C in a 5% CO_2 humidified atmosphere.

Media

For all the cell lines the media used has been:

DMEM (Dulbecco's Modified Eagle Medium; GibCo) with 4.5 g/l glucose supplemented with:

- 0.11 g/l sodium pyruvate
- 0.58 g/l L-Glutamine
- 100 U/ml penicillin
- 100 μg/ml streptomycin
- 10% (v/v) serum: *foetal calf serum* (FCS) (Biological Industries)

RPMI (without phosphates; Biological industries): in case of HeLa cells used for phosphorylation experiments *in vivo*. The medium was complemented with:

- 2 mM L-Glutamine
- 100 U/ml penicillin
- 100 μg/ml streptomycin
- 10% (v/v) serum *foetal bovine serum* (FBS) (Biological Industries)

*** Advice**

When starting with a new cell line it is recommended to first test the serum/s prior to any experiment. Once the result is satisfactory one should use the same batch in all experiments that are to be compared between them.

All serums were heat inactivated at 55ºC in a water bath for 30 minutes and filtered with a 0,2 μm cellulose acetate membrane filter.

For normal maintenance of the cell lines, 100 or 150-mm culture dishes were used. HeLa cells were normally subcultured (trypsinized) when the cells were near confluence (70-80%). The cells were then passed to another plate at a 1:5 - 1:10 dilutions. HEK-293T were rinsed with phosphate buffered saline (PBS) and passed to another plate at 1:5-1:10. Cells will just disloged when they are aspirated with a pipette, together with the medium onto the plate surface.

Trypsinization procedure

- Remove media by aspiration and rinse the plates twice with phosphate buffered saline (PBS). Serum inhibits trypsin activity, therefore complete removal of serum-containing medium is necessary.

Add trypsin as follows: 100-mm culture dish: 1 ml.

150-mm culture dish: 2 ml.

- Cover the monolayer thoroughly with trypsin. Leave it for approximately 1-2 min.

- When the cells have been dislodged, add 5-10 volumes of 10% serum-containing medium to inactivate the trypsin. Aspirate the medium plus cells with a pipette onto the surface to remove all remaining cells. It is important that this aspiration be done as completely as possible so as to obtain individual, dispersed cells.

- Transfer to a new plate the volume of medium plus cells necessary to obtain the desired dilution.

Although the line will be eventually lost at a high number of passages, it will not be lost for use, as frozen stocks can be obtained by the Cryopreservation procedure at almost every passage. Thus, the cell line can be restored again when reaching a determinate amount of subcultures.

Cryopreservation procedure

- A subconfluent plate of cells is selected.

- Cells should be removed by trypsinisation if necessary.

- Centrifuge the necessary amount of cells at 1000 rpm for 10 min. Consider that the frozen stocks should be done at a final concentration of 1-1.5x10^6 cells/ml.

- Resuspend the cell pellet in serum-containing medium plus 10% DMSO (prevents membrane cracking).

- Dispense the cell suspension into plastic screw-cap vials suitable for freezing in liquid nitrogen.

- Place the vials in a -70^{o}C freezer. Ideally, low the temperature at a rate of 1^{o}C/min.

- Transfer the cells within the next three days to a liquid nitrogen container.

Reconstitution of frozen cells

- A screw cap tube that is to be reconstituted is removed from the tank and thawed by slowly resuspension of the frozen cells in the growing media.

- The thawed cells are then transferred to a tube containing more medium in order to dilute the percentage of DMSO as soon as possible.

- The cells are then centrifuged at room temperature at 1000 rpm for 10 min.

- The cell pellet is then resuspended in the appropriate amount of serum-containing medium and plated in a culture dish. It is desirable to change the medium within 24 hours to remove any residual DMSO, and to refeed the culture periodically until cell growth is confluent.

The growth factors and inhibitors used in cell culture have been:

- LY 294002 (Sigma).
- SB 216763 (Tocris).
- SB 415286 (Tocris).

PCR FOR THE IDENTIFICATION OF MYCOPLASMA IN CELL CULTURES.

Principle: Besides possible bacteria and yeast contamination another problem one encounters when working with cell cultures is mycoplasma contamination. Mycoplasma are parasitic microorganisms possessing some characteristics of both bacteria and viruses. With no cell wall they can change shape easily and can be smaller than 0,2 μm in diameter. Their effect on cell cultures can be unpredictable as mycoplasma can alter nucleic acid metabolism and cause chromosomal aberrations.

Correct treatment of all cell culture devises as well as correct mycoplasma control and cell quarantine are necessary. PCR is one of the techniques that may be used to detect this type of contamination.

Procedure

- Keep culture at least 3 days with the same medium without antibiotics. Prepare another plate with media only.

- Take 1 ml of supernatant of each plate in an eppendorf.

- Samples for PCR:

2 µl of media alone

2 µl of culture medium

2 µl of diluted culture medium (dilution 1/100)

2 µl of a positive control

2 µl of water (negative control)

- Quantities for PCR:

Taq buffer 10X	5 µl
$MgCl_2$ (50mM)	1,5 µl
dNTPs (2mM)	5 µl
oligo Myc.for	2,5 µl
oligo Myc.rev	2,5 µl
Taq (1U)	0,25 µl
H_2O	31,25 µl (up to a total reaction volume of 50 µl)

- PCR program:

95ºC 5 min

94ºC 5 min

60ºC 1 min

72ºC 2 min

30 cycles from step 2

72ºC 10 min

4ºC 30 min

- Oligo Myc.for 5' XGC CTG XGT AGT AXX XXC GC 3'

- Oligo Myc.rev 5' GCG GTG TGT ACA AXX CCC GA 3'

A band of approximately 500 bp will indicate the presence of mycoplasm.

TREATMENT OF CELLS CONTAMINATED WITH MYCOPLASM

If possible it is always better to thaw a new clean cell line stock to proceed with the experiments. If not, treatment with BAYCIP (ciprofloxacino, Bayer ref.982421) can be applied:

Keep the cell culture three weeks with BAYCIP diluted in the cell medium at a final concentration of 0,01 mg/ml.

Cells are tripsinized or split as usual keeping the medium with the indicated BAYCIP concentration.

When the three weeks are over, leave the culture a minimum of three days with **medium without antibiotic** and do the PCR.

Repeat treatment until PCR results negative.

E.Coli CULTURES AND TRANSFORMATION PROCEDURES

For cloning and amplification of plasmids the bacterial strains DH5α and JM109 have been used.

Reagents

-LB growth media: 10 g/l tryptone pH: 7.5 (adjusted with NaOH)
5 g/l yeast extract
5 g/l NaCl

-Antibiotics: Ampicillin 50 μg/ml (25 mg/ml stocks in H_2O).
Chloramphenicol 200 μg/ml (100 mg/ml stocks in ethanol 100%)

For normal growth, LB media plus the required antibiotics has been used.

COMPETENT CELL PREPARATION

The cells were rendered competent following a protocol based in the Calcium chloride method.

Reagents

-Calcium chloride ($CaCl_2$) solution: 60 mM $CaCl_2$
(autoclave or filtrate) 15% glycerol
10 mM Tris-HCl pH 7.0

Procedure

1. Grow miniculture of the competent cells (just scrappe with a sterile tip the frozen stock): 1 ml LB without antibiotics overday at a shaker at 37ºC.

2. Spread on an LB plate (without antibiotic) and leave overnight in a 37ºC incubator.

3. The next day pick one colony from the LB plate and grow miniculture overnight at 37ºC in LB medium without antibiotics.

4. The following day dilute the culture 1:100 in LB and leave it until it reaches the exponential phase ($O.D._{600}$= 0.3-0.5).

5. Stop cell growth maintaining the cells on ice for 10 min.

6. Harvest cells by centrifugation at 3000 rpm for 7 min at 4^{o}C in GS-6R Beckman Centrifuge. Discharge supernatant.

7. On ice, carefully resuspend the cell pellet for each 50-ml of LB in 10 ml $CaCl_2$ solution.

8. Centrifuge at 3000 rpm for 5 min at 4^{o}C.

9. On ice, resuspend the cell pellet again as in step 5.

10. Leave the cell suspension on ice for 30 min.

11. Centrifuge at 2500 rpm for 5 min at 4^{o}C.

12. The pellet corresponding to 50-ml of LB is resuspended in 2 ml of $CaCl_2$ solution. This suspension is quickly aliquoted into microcentrifuge tubes (200-µl aliquots) and placed in an ethanol/dry ice bath. The tubes are then stored at -80^{o}C.

TRANSFORMATION PROCEDURE

1. Thaw a competent cell aliquot slowly on ice and add 10 ng of plasmid DNA (usually 1-2 µl). Incubate on ice for 30-90 min.

2. Heat the tube at 42^{o}C for 3 min. Place on ice to cool for 5 min.

3. Recover cells by adding 500 µl of LB medium without antibiotics and place at 37^{o}C for 30-60 min with gentle shaking.

4. Plate 100-200 µl of the transformation mix onto selection plates. Incubate overnight at 37°C.

*** Advice**

While incubating in step 1, mollify (37ºC) the LB plate/s that are going to be used at the end of the transformation.

On step 4, once the transformation mix is spread on the selection plate, allow it to dry before taking the plate to the incubator.

PLASMID DNA PURIFICATION BY CESIUM CHLORIDE ULTRACENTRIFUGATION

Principle: plasmid DNA, after removal of host cell DNA, is isolated in supercoiled form by banding in CsCl gradients in the presence of Ethidium Bromide which separates it from nicked or linear DNA and RNA.

Reagents

- TES solution:	25% sacarose (w/v) 50 mM Tris-HCl pH 8.0 100 mM EDTA	Autoclave
- Triton solution:	3% Triton X-100 200 mM EDTA 150 mM Tris-HCl pH 8.0	Prepared extemporaneously
- TE solution:	10 mM Tris-HCl pH 8.0 1 mM EDTA	Autoclave

- Lysozim: 10 mg/ ml TE. Prepare extemporaneously.

- RNAse A: 10 mg/ml. Prepared and heated at 65°C for 20 min to inactivate the DNAses.

- Cesium Chloride (CsCl) solution: 1.08 g/ml TE. Density should be 1.55-1.59 g/ml. Autoclave.

- Ethidium Bromide solution (EtBr): 10 mg/ml Tris-HCl pH 7.5.

Procedure (the volumes of the solutions indicated are referred to 1 L of LB culture).

1. Obtain the bacterial culture:

· From a single colony of plasmid-bearing cells, grow a 3-ml LB culture over day at 37°C.

· Transfer this culture to 500 ml of fresh media and leave it shaking overnight at 37°C.

· The next morning, add chloramphenicol (200 μg/ml) and incubate at 37°C for 5-6 hours.

2. Harvest cells by centrifugation at 5000 rpm for 15 min at 4°C.

3. Resuspend cells in 8 ml of TES solution. From this point, cells are kept on ice.

4. Add 2.25 ml lysozim 10 mg/ml. Incubate on ice for 5 min.

5. Add 3.25 ml EDTA 0.5 M pH 8.0.

6. Add 25 μl RNAse A 10 mg/ml. Incubate 5 min at room temperature.

7. Add 4 ml of cold triton solution by gently dropping while slowly shaking the tube. Let stand on ice for 10 min.

8. Centrifuge in a SM24 rotor at 18000 rpm for 70 min at 4°C.

9. Transfer supernatant to corex tubes; add 1g CsCl per ml of solution and mix. Placing the tubes in a 30°C water bath can help to dissolve the CsCl, as it precipitates when the temperature is lower than 15°C.

10. Add 0.1 volumes of Ethidium Bromide solution. Protect tubes from light.

11. Centrifuge at 7000 rpm for 20 min at room temperature (eliminates EtBr-protein complexes).

12. Harvest supernatant with a Pasteur pipet and transfer it to Beckmann Quick-Seal polyallomer tubes (# 342413) used for ultracentrifugation.

13. Top tubes off with CsCl solution and heat seal the tubes.

14. Centrifuge in a NVT65 rotor at 60000 rpm for 16-18 hours at 20^{o}C.

15. Retrieval of plasmid DNA: clamp tube in front of a long-wave UV light source. There should be two bands; the upper being nicked DNA and the lower the desired intact DNA. Puncture the top of the tube with a syringe needle, and then insert a syringe needle (bevel up) just below the plasmid DNA band. Slowly draw out the band into syringe.

16. Transfer to a new ultracentrifugation tube and repeat steps 13-15.

17. After the second CsCl gradient centrifugation, transfer the DNA to a sterile tube and extract repeatedly with water-saturated isobuthanol, removing pink colored EtBr each time until lower extract is colorless.

18. Precipitate the plasmidic DNA as follows:

- Measure volume of DNA (v).
- Add 3v (volumes) of water: we have a final volume V.
- Add 2V of absolute Ethanol (room temperature).
- Precipitate at 4^{o}C for 1-2 hours.

19. Centrifuge in an AS4.14 rotor at 10000 rpm for 10 min at room temperature.

20. Wash the pellet with 70% Ethanol. Dry the pellet.

21. Resuspend the DNA pellet in 500 μl of TE solution.

22. Calculate DNA concentration: dilute an aliquot of DNA in TE and read absorbance at 260/280 nm.

1 O.D. $_{260\ nm}$ = 50 μg/ml.

Later on we started to use different commercial kits (**Quiagen; Nucleobond AX500**) in order to obtain maxi-preps since the purification levels were comparable to the one

obtained by cesium chloride ultracentrifugation. The basic steps in all of these DNA purification methods are:

Harvest of bacterial culture

Cell disruption

Precipitation of chromosomal DNA

Clarification of the bacterial lysate (by centrifugation or filtration)

Equilibration of the resin column

Adsorption of the plasmid DNA to the column

Wash of the column

Elution of the plasmid DNA

Precipitation of the plasmid DNA

DNA EXTRACTION FROM ADENOVIRUS CONSTRUCTS

(adapted from Souza and Armehtano, 1999)

At some point one may need to extract the DNA from an adenovirus construct in order to sequence or clone on another vector to further transfect instead of infecting, etc

Procedure

1. We can begin from cells infected with adenovirus or with purified virus (capsides). Mix together 181,5 µl of virus, 8 µl of EDTA 0,5M, 10 µl of SDS 10% and 0,5 µl proteinase K 50µg/ml

2. Digest one hour at 56ºC

3. Add 200µl of phenol suitable for DNA to the mixture from step 1

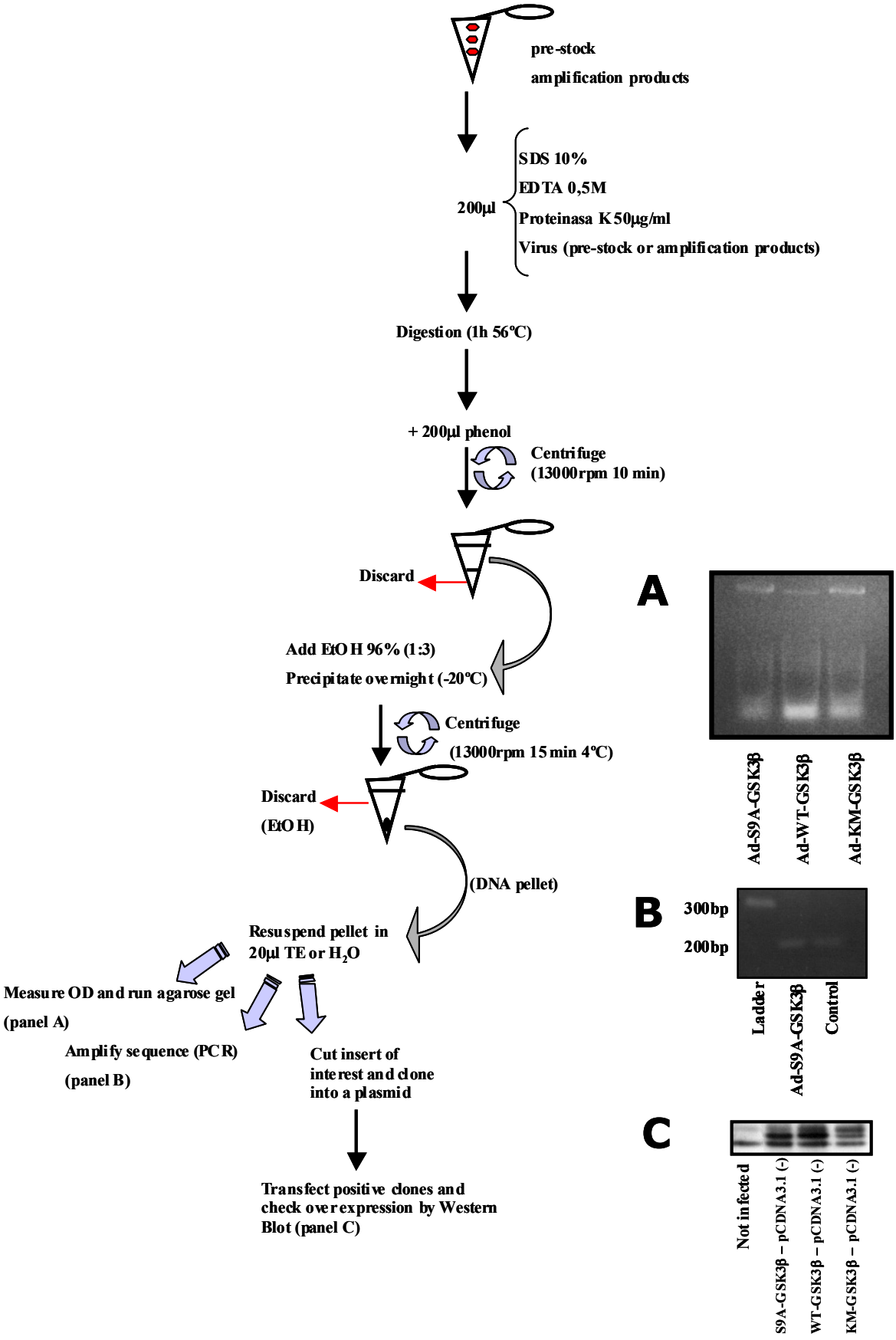
4. Centrifuge at 13000 rpm in a microcentrifuge for 10 minutes

5. Collect the upper fraction and discard the lower one (phenol)

6. Add ethanol 96º in a charge ratio of 1:3 with respect of the upper fraction recovered and leave at –20ºC in order to precipitate (from hours to days).

7. Centrifuge at 13000 rpm in a microcentrifuge at 4ºC for 15 min in order to pellet the precipitated DNA. Discharge supernatant (ethanol).

8. Let DNA pellet dry (not too much) and resuspend with 20 ml TE or milliQ water.


pre-stock
amplification products
200µl
SDS 10%
EDTA 0,5M
Proteinasa K 50µg/ml
Virus (pre-stock or amplification products)
Digestion (1h 56ºC)
+ 200µl phenol
Centrifuge
(13000rpm 10 min)
Discard
Add EtOH 96% (1:3)
Precipitate overnight (-20ºC)
Centrifuge
(13000rpm 15 min 4ºC)
Discard
(EtOH)
(DNA pellet)
Resuspend pellet in
20µl TE or H2O
Measure OD and run agarose gel
(panel A)
Amplify sequence (PCR)
(panel B)
Cut insert of
interest and clone
into a plasmid
Transfect positive clones and
check over expression by Western
Blot (panel C)
A
Ad-S9A-GSK3β
Ad-WT-GSK3β
Ad-KM-GSK3β
B
300bp
200bp
Ladder
Ad-S9A-GSK3β
Control
C
Not infected
S9A-GSK3β – pCDNA3.1 (-)
WT-GSK3β – pCDNA3.1 (-)
KM-GSK3β – pCDNA3.1 (-)

DNA extraction from the adenovirus constructs Ad-S9A-GSK3β, Ad-WT-GSK3β and Ad-KM-GSK3β, cloning into pCDNA 3.1(-) vector and transient tranfection of HEK293T cells.

General scheme of DNA extraction from an adenovirus. Cloning into a plasmid vector and transfection into cultured cell lines. (A) Status of extracted viral DNA. Isolated infectious plasmids were run on a 1% agarose gel and stained with Ethidium Bromide. (B) PCR of infectious plasmid from Ad-S9A-GSK3β using GSK3β internal plasmids. A full length GSK3β cloned in a pcDNA3 vector was also amplified as a control. (C) Positive clones checked by sequencing were used in a calcium phosphate transfection of HEK293T cells and whole cell extracts were immunoblotted against GSK3β protein as described in Materials and Methods.

TRANSFECTION OF DNA INTO EUKARYOTIC CELLS

Principle: introduction of nucleic acids into eukaryotic cells by physical and chemical methods.

Depending on the experiment, different methods for transfection have been used in this study:

Calcium Phosphate Transfection for HEK293T cells for binding experiments on Chapter 2 and for E2F1 transcriptional activity studies with overexpression of GSK3β of Chapter 3.

Lipofectamine-mediated transfection for HEK293T cells for E2F1 transcriptional activity studies with endogenous GSK3β (Chapter 3, figure 3.1).

CALCIUM PHOSPHATE TRANSFECTION OF HEK-293T CELLS

A precipitate containing calcium phosphate and DNA is formed by slowly mixing an HEPES-buffered saline solution with a solution containing calcium chloride and DNA. This precipitate adheres to the surface of cells and should be visible in the phase contrast microscope some hours after transfection. A determinate percentage of the cells on a dish will take up the DNA precipitate through an as yet undetermined mechanism.

Reagents

- $CaCl_2$ 2.5 M: prepare and filter-sterilise through 0.45-µm cellulose acetate membrane filter. Store at -20°C in aliquots. This solution can be frozen and thawed repeatedly.

- HEPES-buffered saline (HeBS) solution, 2X: 0.28 M NaCl
0.05 M HEPES
1.5 mM Na_2HPO_4

Add 800 ml of H_20. Titrate to pH 7.05 with NaOH. Add H_20 to 1 litter. Filter-sterilise through 0.45-µm filter. Test for transfection efficiency. Store at -20°C in 50-ml aliquots.

An exact pH is extremely important for efficient transfection. The optimal pH range is 7.05 to 7.12.

- Purified plasmid DNA.

- Sterile double distilled water, pH 7.0.

- Serum-containing medium.

Procedure

1. The day before transfection, split exponentially growing HEK-293T cells into 100-mm culture dishes (1:10 dilution).

2. The next day, feed cells with 9.0 ml of complete medium 2 to 4 hours prior to transfection.

3. Prepare the following mixture: 50 µl $CaCl_2$ 2.5 M
x µl plasmid DNA (20-25 µg)
add water to 500 µl.

4. Place 500 µl of 2X HeBS in a sterile 15-ml conical tube. Use a mechanical pipettor attached to a plugged 2-ml pipet to bubble the 2X HeBS and add the DNA/ $CaCl_2$ solution dropwise with a pipet. Immediately after, vortex the solution for 5 seconds.

5. Allow precipitate to sit for 20 min at room temperature.

6. Use a pipet to distribute the precipitate evenly over a 100-mm plate of cells and gently agitate to mix precipitate and medium.

7. Incubate the cells overnight under standard growth conditions.

8. The following day, remove the medium; wash the cells twice with 5 ml of PBS and feed cells with 10 ml complete medium.

Cells were splited 4 hours later, pooled and replated into 35-mm or 100-mm culture dishes. Harvest cells at the desired time points after appropriate treatment.

***Advices**

One should prepare enough HeBs 2X solution in order to use the same stock in experiments that need to be compared. Transfection efficiencies may vary a lot between stocks.

Cell density is an important factor for transfection efficiency. Confluence should be between 60-80% to allow enough cell surfaces for the transfection precipitate to enter. Incubation time can also vary.

LIPOSOME-MEDIATED TRANSFECTION OF HEK293T CELLS: LIPOFECTAMINE™ 2000 TRANSFECTION REAGENT.

Incubation of cationic lipid-containing liposomes and nucleic acids results in quick association and a compactation of the nucleic acid, presumably from electrostatic interactions. The liposome complex neutralizes the negative charge of the nucleic acids, allowing closer association of the complex with the negatively charged cell membrane. Entry of the liposome complex into the cell may occur by the process of endocytosis.

Reagents

-LipofectamineTM 2000 Transfection Reagent

-Purified plasmid DNA

-Opti-MEM^{R} I Reduced Serum Medium

Procedure

Volumes and amounts are given for transfections performed in 100-mm plates

1. Plate the cells one day before transfection in growth medium **without antibiotics** so that they will be 90-95% confluent at the time of transfection.

2. The day of transfection prepare DNA-lipofectamine^{TM} 2000 complexes as follows:

dilute 24 μg of plasmid/s DNA in 1.5 ml of Opti-MEM^{R} I Reduced Serum Medium. Mix gently.

Mix Lipofectamine^{TM} 2000 gently before use, then dilute 60 ml in 1.5 ml of of Opti-MEM^{R} I Reduced Serum Medium. Mix gently and incubate for 5 min at room temperature.

After the 5 min incubation, combine the diluted DNA with the diluted Lipofectamine^{TM} 2000 (total volume is 3 ml). Mix gently and incubate for 20 min at room temperature to allow the DNA-Lipofectamine^{TM} 2000 complexes to form.

3. Add the 3 ml of the DNA-LipofectamineTM 2000 complexes to each plate containing cells and medium. Mix gently by rocking the plate back and forth.

4. Incubate the cells at 37ºC in a CO_2 incubator until assay for transgene expression. It is not necessary to remove the complexes or change the medium. However growth medium may be replaced after 4-6 hours without loss of transfection activity.

Cells were kept overnight in the incubator. Cell plates with the same transfection condition were pulled together and plated again. Once cells were attached again the growth medium was changed to growth medium without foetal calf serum together with

different chemical inhibitors or their carrier. Cells were harvested at the desired time points.

INFECTION OF EUKARYOTIC CELLS WITH ADENOVIRUS.

Principle: the need of introducing gens in particular mammal cells, the inefficiency of other available techniques (calcium phosphate transfection, liposomes, electroporation, etc) and the particular characteristics of DNA viruses, turn viruses into a new vector to accomplish this aim.

Procedure:

A. Virus construction (not discussed in this Thesis)

B. Virus amplification

C. Checking the construct (southern or western blot of infected cells)

D. Virus titration

E. Virus purification (this step is only required in case of toxicity due to infection with whole extracts and *in vivo* application**)**

B. Virus amplification

In order to increase virus PFU/ml (plaque formation units/ml) we need to grow this adenovirus (which are E1-deleted recombinant virus) in the human HEK-293T cell line. This cell line was originally transformed with Ad5 and contains the left 14% of the adenovirus genome integrated into cellular DNA, including the E1 region (required for virus transformation).

Before infecting HEK-293T cells to amplify, measure $O.D_{260nm}$ to estimate the number of virus particles we have knowing:

1 O.D $_{260nm}$ virus not diluted = 10^{12} particles

Infect one 150-mm plate of HEK293T cells (roughly 20 X 10^6 cells) with 1-10 PFUs. It is consider that only 1-5% of total particles are active. To infect just mix the amount of virus extract necessary with the cell medium.

Incubate cells at 37ºC and 5% CO_2 atmosphere for 48 hours or until cells start to deattach (don´t allow them to lyse massively).

Collect cells aseptically with a pipette and centrifuge at 1200 rpm for 8 minutes.

Pass supernatant to another tube and resuspend the pellet with a little bit of this supernatant (aprox 1,3 ml/2 150-mm plates) and freeze at –20ºC.

Thaw virus extract (a water bath can be used).

Mild sonication in order to break the cells (if a sealed type sonicator is not available then thaw-freeze cycles should be done).

Remove cell debris by centrifugation at 3500 rpm 5 min at 4ºC (the pellet should be small).

Take supernatant (carefully) and aliquote in small volumes. Keep at –20ºC.

C. Checking the constructs

Protein expression levels from adenovirus constructs was checked by Western blot (for a detail protocol see *Electrophoretic transfer of proteins and western blot* section).

Infect target cells with different virus quantities and incubate at 37ºC and 5% CO_2 and collect for western blot at different times (normally start with 24, 48 and 72 hours).

Check by Western blot the expression of the protein of interest of each time point. The goal is to achieve a good expression level with little virus extract volume (to reduce cytotoxic effects due to the adenovirus itself) in the shortest time. If the amount of virus needed is too high or the incubation periods too long, a re-amplification of the virus must be done.

D. Virus titration

When working with different virus constructs it is sometimes necessary to infect with the same PFU/ml for further comparison of the data. We can either titrate whole infected cell extracts (like in our case) or purified virus.

There are two main ways to titrate the virus:

1. **Plaque formation method.**
2. **TCID $_{50}$ method (as described in Takara Biomedicals Kit)**

1. Plaque formation method.

Principle: calculation of the active particles we have of a virus based on the number of isolated lysed cells (plaque) it generates. It is also a way of purifying the virus since it is considered that one plaque is due to only one virus type.

Reagents:

- Cells extracts from infected HEK-293T cells or purified virus

- HEK-293T cells

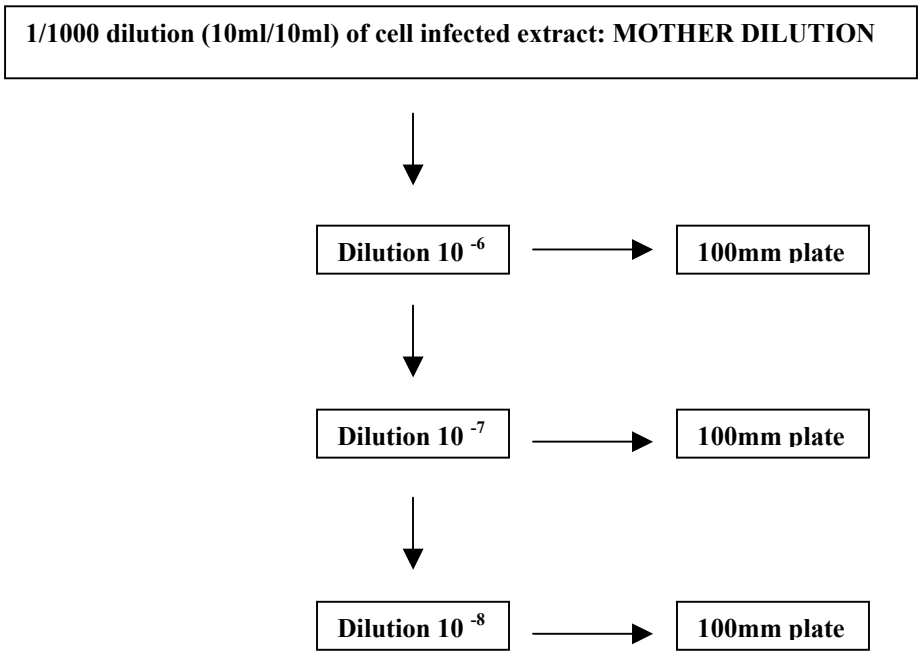
- Medium DMEM 2% FBS

- Agarose: SEAKEM ME FMC BioProducts). Must be sterile.

Procedure:

Seed HEK-293T at 80% confluence (don't allow higher confluence in order to avoid cells growing on top of each other during the assay).

Prepare 3 dilutions per virus in 15ml sterile tubes as follows: (all dilutions done with DMEM 2% FBS):



Remove medium from HEK-293T to be infected and add 4ml of one virus dilution (three 100-mm plate/virus).

Incubate for an hour at 37ºC and 5% CO_2 atmosphere.

Rinse twice with DMEM 2% FBS.

Add 8 ml per 100-mm plate of 0,76 % agarose dissolved in DMEM 2% FBS.

Leave dishes under the hood until the agarose solidifies (about and hour).

Return cells to the incubator.

Check for the formation of plaques (it takes about 10 days).

Every two to three days add more agarose onto the dishes (4 ml/100-mm plate).

In order to calculate the PFU (plaque formation units) count the plaques of at least one of the dilutions and consider the dilution factor:

PFU/ml = (n plaques x dilution factor)/4 ml of the dilution

***Advices:**

To dissolve the agarose: dissolve 0,38 g of agarose in 10 ml water and melt in the microwave. Place agarose in a 65ºC water bath to avoid solidification. In the meantime warm at 37ºC 40 ml of DMEM 2% FBS for every 10 ml of agarose. Quickly mix the medium with the agarose and keep it at 42ºC in a water bath while working. Add 8 ml of this agarose-medium mixture per 100-mm dish on the side and shake the plates (Don´t do a lot of plates at the same time not to let the agarose cool down too much).

It is quite difficult to identify the plaques. They look like small calcifications when observed against the light. Under the microscope you must see the cells with lised morphology.

Another way of expressing infectivity is MOI (multiplicity of infection) being:

MOI = PFU/cell

2. TCID $_{50}$ method (as described in Takara Biomedicals Kit)

Principle: calculation of the active particles we have of a virus based on the dose needed to generate a cytopathic effect on HEK-293T greater than 50% of the population.

Reagents:

- Cells extracts from infected HEK293T cells or purified virus

- HEK293T cells

- Medium DMEM 5% FBS

- Medium DMEM 10% FBS

Procedure:

Culture HEK293T cells in 100mm dish.

Dilute the viral solution 10-fold repeatedly using DMEM 5% FBS and prepare a 10^4 dilution of the viral solution (i.e. 0,1 ml of a viral solution with 0,9 ml of DMEM 5% FBS).

Place 50 µl each of the DMEM 5% FBS in all wells of collagen-coated 96-well plate.

Add 25 µl of a 10^4-fold dilution of recombinant virus to each well of the first row.

Transfer 25 µl to the wells of the second row using an 8-channel multi-pipette. Repeat this procedure to the 11^{th} row and discard the last 25 ml. This results in serial dilutions of 3^n . Since we prepared a 104 dilution of our virus stock, the wells from the first row will have a dilution of 3 x 10^4, from the second row 3^2 x 10^4 and so on up to 3^{11} x 10^4. Place uninfected cells in the 12^{th} row as a control.

Suspend the cultured HEK293T cells in 6 ml of DMEM 5% FBS.

Add 50 ml of this cell suspension to each well.

Add 50 ml of DMEM 10% FBS gently to each well after 4-5 days and 7-8 days.

Judge the end point of complete cytopathic effect by microscopy after 11-13 days. The cells of the wells in which the virus is present show cytopathic effect: rounded cells in clusters and some dislodged cells.

To calculate the virus concentration in the used medium, the following formula is applied (see figure 1).

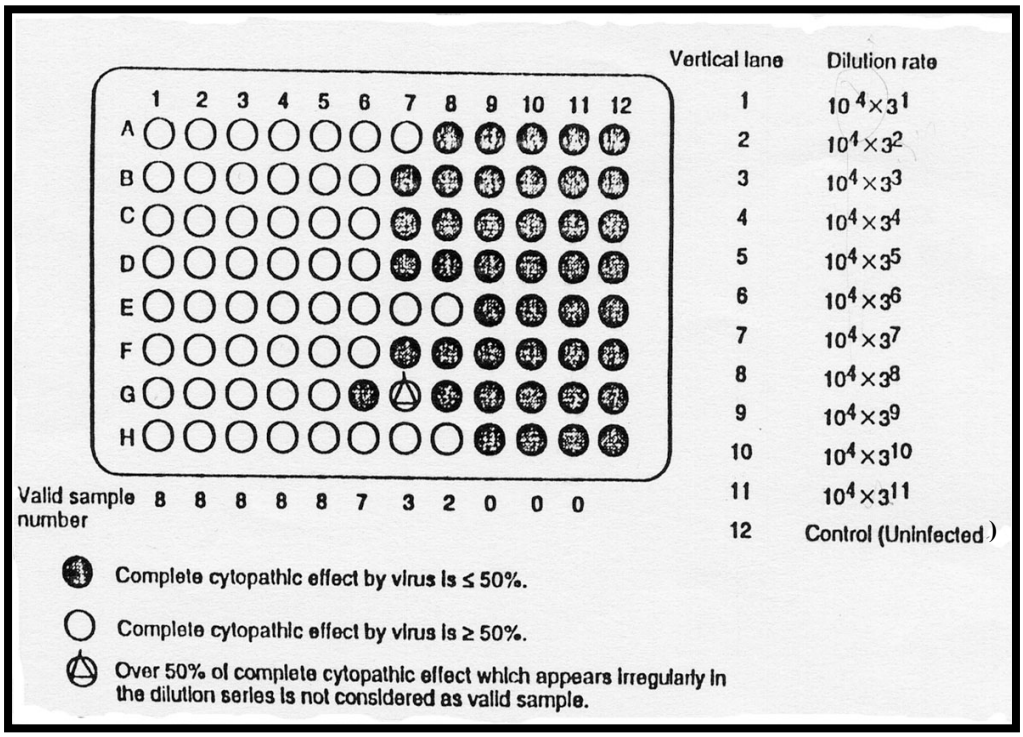


Fig.1 TCID $_{50}$ method (as described in Takara Biomedicals Kit)

$TCID_{50}$ = (dilution rate in the first row) x (dilution rate) $\Sigma^{-0,5}$

Σ= total sum of (number of wells showing cytopathic effect)/ (number of samples) at each degree of dilution.

In the above example,

$\Sigma = 8/8 + 8/8 + 8/8 + 8/8 + 8/8 + 7/8 + 3/8 + 2/8 = 6,5$

Therefore,

$TCID_{50} = 3 \times 10^4 \times 3^{6,5-0,5} = 2,2 \times 10^7$

Assuming that **TCID50 = PFU** and since 50ml of virus solution was used, the titer of the undiluted virus solution is calculated as below:

2,2 x 107 x 1ml/0,05ml = 4,4 x 108 (PFU/ml)

E. Virus purification

Principle: for infection of certain cells lines as well as for infection of animals, cell lysate can't be used (citotoxic) and the virus has to be purified. This differs from the lysate in the absence of proteins and cell membranes. Purification consists in a CsCl gradient and a further concentration of the virus through a Sephadex column.

For our particular experiments there was no need of purification.

EXPRESSION AND PURIFICATION OF RECOMBINANT PROTEINS IN *E.Coli*

Principle: use of the pET System to express recombinant proteins in *E.coli*. Target genes are cloned in pET plasmids under control of strong bacteriophage T7 transcription. Expression is induced by providing a source of T7 RNA polymerase in the host cell. The desired product can comprise more than a 50% of the total cell protein after a few hours of induction. Target genes are transcriptionally silent in the uninduced state avoiding possible toxicity of the host cell. Expression hosts contain a chromosomal copy of the T7 RNA polymerase gene and expression is induced by the addition of IPTG.

Reagents

Plasmids: pGEX

Expression host: BL21(DE3)pLysS

Media LB:	Bacto tryptone	10 g/L
	Bacto yeast extract	5 g/L
	NaCl	10 g/L
NET solution:	20mM Tris-HCl pH 8.0	
	100mM NaCl	
	1mM EDTA	
NETN solution:	20mM Tris-HCl pH 8.0	
	100mM NaCl	
	1mM EDTA	
	0,5% NP-40 (or Igepal)	
NETN(I) solution:	NETN + 10 μg/ml aprotinin, 5 μg/ml leupeptine, 1 mM PMSF	
Elution buffer:	50 mM Tris pH 8,5	
	100 mM NaCl	
	10 mM gluthatione	
	2 mM DTT	
Dialisi buffer:	50 mM Tris pH 8	
	100 mM NaCl	

Procedure

A. Obtain the bacterial culture

1. Inoculate 3 ml of LB-Ampicillin-Chloramphenicol with a single colony of BL21(DE3)pLysS transformed with a pGEX construct. Leave it at 37ºC and agitaton for 3 hours.

2. Dilute 1 ml of cells to a final volume of 100 ml with the same media and incubate overnight in a shaker at 37ºC.

3. The next morning, check culture O.D. (λ600 nm). When it reaches 0,8-1,0 dilute 10-40 ml of this culture into a total of 250 ml of the same media and leave shaking for an hour at room temperature.

4. Add IPTG to the culture to a final concentration of 0,4 mM and leave the cultures at room temperature for 4 hours with vigorous agitation.

5. Harvest cells in 250 ml tubes and centrifuge at 6000 rpm with Sorvall GSA rotor at 4ºC for 10 min. Discard the supernatants and continue the process with the pellet or keep at –80ºC.

B. Obtain cell lysates

1. Thaw the pellets on ice and Resuspend in 10 ml of NENT(I) and transfer to a 50 ml polypropylene tube.

2. Wash the tube with 10 ml of NETN(I). Cells are then spontaneously lysed due to the T7 lysozyme.

3. Pool and sonicate the suspensions at top speed for 2 x 15s (seconds) three times (or until lysate looses its viscosity).

4. Centrifuge the suspension for 15 min at 15000 rpm in a Sorvall ss-34 rotor at 4ºC. Transfer supernatant into a 50 ml polypropylene tube. Take small fraction to checking on SDS gel.

C. Purify GST construct.

1. Add 0,5 ml of gluthation-sepharose beads (previously washed twice in 10 ml PBS and once in 10 ml NETN) to the pooled supernatants and leave on a rotation wheel for 1 hour at 4ºC.

2. Collect the nonbound supernatant for later check and wash the beads twice with 30 ml of NETN and once with NET. Last wash in eppendorf with only 1 ml of NET.

3. We elute twice with 300 µl of elution buffer at 4ºC and rotating for 30 min each time.

4. Pool separately both eluted fractions and store at –80ºC. Take a small fraction of each elution for checking on SDS-gel and protein quantification.

Check the process on a SDS-gel and either stain with coomasie or check by western blot.

D. Dialyze GST construct.

One has to consider what are the GST constructs going to be use for. As substrates of an *in vitro* kinase assay they can be dissolved in the elution buffer containing glutathione but in case of future application in a pulldown experiment, glutathione must be eliminated. Therefore we have to dialyze the elutions.

The possible dialysing solutions vary. One has to account for a possible precipitation of the eluted proteins that must be avoided. In our case we decided to use elution buffer without glutathione, nor DTT.

1. Put elution in a Slide-a-Lyzer minidialisi unit 7000 mW co (Pierce). Make sure the membrane is in good condition.

2. Leave dialisi unit floating in five liter dialisi buffer at 4ºC overnight with agitation.

3. The following day change the buffer for another five liters of fresh buffer and continue dializating for 5 more hours at 4ºC.

4. Aliquote the dialized elution and freeze at –80C.

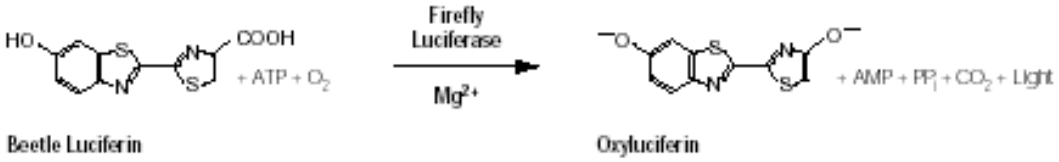
*** Advices:**

In case your GST construct expressed poorly or not at all with this “standard” protocol, one could try to improve the protein yield by:

- Rise the IPTG concentration
- Increase the incubation time with IPTG
- Change the growing or/and the induction temperature of the bacterial culture
- Rise the glutathione concentration in the Elution Buffer (dialization obligatory then)

LUCIFERASE ACTIVITY DETERMINATION

Principle: reporter gene system used to measure the level of expression of a transfected gene. The firefly (*Photinus pyralis)* luciferase enzyme catalyses a reaction using D-luciferin and ATP in the presence of oxygen and Mg^{2+} resulting in light emission. The luciferase reaction is quantitated using a luminometer, which measures light output. The total amount of light measured during a given time interval is proportional to the amount of luciferase reporter activity in the sample.



Reagents

- Luciferase Assay System with Reporter Lysis Buffer (Promega kit) containing:

* Luciferase Assay Reagent: prepared mixing Luciferase Assay Substrate and Luciferase Assay Buffer as described by the manufacturer, and stored in aliquots at -70°C.
* Reporter Lysis Buffer 5X.

- PBS buffer.

Procedure

1. Remove the growth medium from the transfected cells to be assayed. Wash the cells twice with ice-cold PBS, being careful not to dislodge any of the cells. Add 1 ml PBS.

2. Scrape the cells off the plate with a cell scraper. Transfer them to a 1.5-ml microcentrifuge tube on ice.

3. Microcentrifuge cells at maximum speed for 3 min at 4^{o}C.

4. Resuspend the cell pellet in 200 µl of 1X Reporter Lysis Buffer per 60-mm culture dish (600 µl per 100-mm dish).

5. Perform a single freeze/thaw cycle in liquid nitrogen and a room temperature bath (Reporter Lysis Buffer is a mild lysis agent and requires a freeze/thaw cycle to achieve complete cell lysis).

6. Vortex the tube 10-15 seconds. Centrifuge at maximum speed for 2 min at 4^{o}C to pellet the cell debris. Transfer the supernatant to a new tube (can be stored at -70^{o}C).

The sample to be assayed and the Luciferase Assay Reagent should be at room temperature (not above 25^{o}C).

7. Mix 20 µl of the room temperature extract with 50 µl of room temperature Luciferase Assay Reagent. Place the reaction in a luminometer.

8. Measure the light produced for a period of 15 seconds with a delay time of 5 seconds and 100 % sensitivity or lower depending on the sample in order to be in the luminometer range.

β-GALACTOSIDASE ENZYME ASSAY

Principle: method for assaying β-galactosidase (commonly used reporter molecule) activity in lysates prepared from cells transfected with b-galactosidase reporter vectors. The β-galactosidase enzyme hydrolyzes the colorless substrate ONPG (o-nitrophenyl-β-D-galactopyranoside) to o-nitrophenol, which is yellow. The reaction is terminated by addition of sodium carbonate, and the absorbance (indicative of enzyme activity) is read at 420 nm with spectrophotometer.

In our case, we cotransfect with β-galactosidase reporter vector in order to normalize transfections.

Reagents:

- E3971 Reporter Lysis Buffer 5X (Promega): same buffer as in luciferase activity determination (therefore both activities can be measure from the same cell extract)

- **Assay 2X Buffer:** 200 mM sodium phosphate buffer (pH 7.3)
  2 mM $MgCl_2$
  100 mM β-mercaptoethanol
  1.33 mg/ml ONPG
  (Store at –20ºC)

- Sodium Carbonate 1 M

- PBS buffer

– β-Galactosidase (if a standard curve is desired) Store at 4ºC or –20ºC.

Procedure:

1. Add 4 volumes of water to 1 volume of 5X RLB (Reporter Lysis Buffer) to produce a 1X stock solution.

2. Remove the growth medium from the transfected cells to be assayed. Wash the cells twice with ice-cold PBS, being careful not to dislodge any of the cells. Remove as much of the final wash as possible using a pipet tip.

3. Add a sufficient volume of 1X RLB to cover the cells (400 µl for a 60-mm culture dish, 900 µl for a 100-mm dish). Ensure complete coverage of the cells.

4. Incubate at room temperature for 15 minutes, slowly rocking the dish.

5. Scrape the cells off the plate with a cell scraper. Transfer the cell lysates to a microcentrifuge tube on ice.

6. Vortex the tube for 10-15 seconds, then centrifuge at top speed in a microcentrifuge for 2 minutes at 4ºC. Transfer the supernatant to a fresh tube. If the lysates are not to be assayed directly they can be stored at –70ºC at least two months.

7. Thaw system components and mix well. Place 2X Assay Buffer on ice. If a standard curve is desired, use standards between 0 and 6,0 x 10^{-3} units of β-Galactosidase.

8. It may be necessary to dilute the cell lysates in 1X Reporter Lysis Buffer. In our case a 1:4 dilution of lysates to 1X Reporter Lysis Buffer (30 ml of lysates plus 120 RLB) was optimal, but 2:1 is a good starting dilution. Up to 150 µl of cell lysates can be used per reaction. As a negative control, prepare the same dilution of a cell lysates made from cells that have not been transfected with β-galactosidase gene.

9. Pipet 150 µl of the diluted (or undiluted) cell lysates into labeled tubes.

10. Add 150 µl of Assay 2X Buffer to each of the tubes.

11. Mix well by vortexing briefly.

12. Incubate the reactions at 37ºC for 30 min or until a faint yellow color has developed. Color development continues for approximately 3 hours. If enzyme activity is low, samples may be incubated overnight (close the tubes tightly).

13. Stop the reactions by adding 500 µl of 1M Sodium Carbonate. Vortex briefly.

14. Read absorbance at 420 nm immediately after addition of 1M Sodium Carbonate.

SDS POLYACRYLAMIDE GEL ELECTROPHORESIS OF PROTEINS

Principle: proteins are denatured by heating them in buffer containing SDS and a thiol reducing agent. The proteins are then separated by electrophoresis on polyacrylamide gels in the presence of SDS.

Reagents

- Lysis Buffer: see *Protein extracts* section for recipes.

- Bradford Reagent (BioRad protein assay dye reagent concentrate, BioRad).

- 30% Acrylamide/Bisacrylamide solution, 37.5:1 mixture (BioRad).

- Lower (Resolving) Buffer : 0.75 M Tris-HCl, pH 8.8.

0.29 % SDS

- Upper (Stacking) Buffer : 0.25 M Tris-HCl, pH 6.8.

0.2 % SDS

- 10% (w/v) ammonium persulfate (APS).

- Sample Buffer 4X: 40% glycerol Store frozen aliquots at -20^{o}C

50 mM Tris-HCl, pH 6.8

200 mM dithiothreitol (DTT)

5 mM EDTA

10% SDS

0.5% (w/v) bromphenol blue

- Electrode (Running) Buffer: 25 mM Tris-HCl (unadjusted pH should be 8.3)

192 mM glycine

0.1% SDS

Procedure

Protein extracts

In order to obtain protein extracts from culture cells, several Lysis Buffers can be used depending on the cellular localization of the proteins, their stability, or their phosphorylated state. It should also be considered what for the protein extract is going to be used: western blot, immunoprecipitation, pulldown, kinase assay or a combination of more than one technique.

In this study, several **Lysis Buffers** have been used:

A. Western Blot:

20 mM Tris-HCl, pH 8

2.5 mM MgCl2

10 mM EDTA

1% NP-40

1 mM DTT

200 mM PMSF

10 μg/ml of Aprotinin, Leupeptin, Benzamidine

10 mM Pepstatine A

B. Immunoprecipitation, Kinase Assay and Western Blot:

50 mM Hepes, pH 7.4

150 mM NaCl

1.5 mM $MgCl_2$

1 mM EGTA

1% Triton X-100

10% Glycerol

100 mM NaF

25mM β-glycerophosphate

1 mM DTT

1 mM Na_3VO_4

1 mM Benzamidine

10 μg/ml Aprotinin, Leupeptin

C. IP of endogenous E2F1, In vivo kinase assay and Western Blot (RIPA):

50 mM Tris-HCl pH 8

150 mM NaCl

1 mM EDTA

1% NP-40

1 mM PMSF

1 mM Na_3VO_4

1 mM NaF

1 μg/ml each : aprotinin, leupeptin, pepstatin

***Advices:**

Do not add Na-deoxycholate or SDS when preparing lysates for kinase assays. Ionic detergents can denature enzymes, causing them to lose activity.

Please note that adding DTT rapidly inactivates sodium orthovanadate (inhibitor of protein tyrosine phosphatases).

D. Immunoprecipitation of endogenous GSK3β and Western Blot (no detergent, need to homogenize):

50 mM Hepes pH 7.5

10 mM $MgCl_2$

1 mM EGTA pH 8

1 mM DTT

90 mM β-glicerophosphate

1 mM PMSF

0.4 mM NaF

0.4 mM Na_3VO_4

1. Wash cells twice with ice-cold PBS. Add 1 ml of PBS.

2. Scrape plate with a cell scraper.

3. Spin down cells by microcentrifugation at maximum speed for 5 min at 4°C.

4. Add 100-200 µl (per 100-mm dish) of ice-cold Lysis Buffer to the pellet. Vortex.

5. Shake the tubes for 30 min at 4°C (buffer A) or use a homogenizer (buffer D) to resuspend well the pellet. Vortex again.

6. Centrifuge at maximum speed for 5 min at 4°C. The supernatant fluid is the protein cell lysate.

7. Determine the extract protein concentration by the Bradford method using bovine serum albumin as a standard.

8. Add equal amounts of protein (30-50 µg) from each sample to a new tube. Adjust the volume of each sample to the same final volume with Lysis Buffer. Add one-fourth final volume of Sample Buffer 4X.

9. Boil samples for 5 min and place immediately on ice. After a short spin, the extracts are ready to be loaded on to SDS-polyacrylamide gels.

Protein electrophoresis

The resultant denatured polypeptides are now in a uniform charge-to-mass ratio proportional to their molecular weights and are separated in polyacrylamide gels according to their molecular mass.

The proteins are run on two consecutive gels: a stacking gel with acid pH (6.8) and low acrylamide concentration (4%), and a resolving gel with basic pH (8.8) and high acrylamide concentration (7-15%).

10. Set up glass plate moulds as described by manufacturer.

11. Prepare acrylamide RESOLVING gel mixture. The percentage of acrylamide depends on the molecular weight of the protein to be studied. In our case, percentages of 10% to 15% acrylamide were used. Gels were prepared as follows (see Table):

	10%	12%	15%
Lower Buffer pH8.8 (ml)	2.5	2.5	2.5
30% Acrylamide/Bisacrylamide (ml)	1.7	2	2.5
H_2O (ml)	0.8	0.5	-
TEMED (µl)	7	7	7
PSA 10% (µl)	25	25	25
Total Volume (ml)	5	5	5

12. Quickly pour solutions into mould, overlay with water or water-saturated butanol and leave undisturbed until polymerised (30 min).

13. Prepare acrylamide STACKING gel solution as follows:

· Prepare the monomer solution
- 0.18 ml 30% Acrylamide/Bis
- 0.75 ml Upper Buffer
- 0.6 ml H2O
- 3.75 µl TEMED
- 15 µl PSA 10%

14. Rinse off butanol and unpolymerized acrylamide with a stream of water. Pour the stacking solution between the glass plates. Insert the comb between the spacers. Allow to polymerise for 30 min.

15. Gently remove the comb and rinse the wells with distilled water.

16. Place the gel cassette inside the running tank and fill the tank with Running Buffer.

17. Load boiled protein samples on to the gel.

18. Run proteins at 100 V in the stacking gel and at 150 V in the resolving gel.

Once the proteins are separated, proceed to the Electrophoretic Transfer Protocol (next section).

ELECTROPHORETIC TRANSFER OF PROTEINS AND WESTERN BLOT

Principle: after electrophoresis in SDS-polyacrilamide gels, proteins are transferred by transverse electrophoresis to a membrane. The remaining sites of the membrane are blocked with dried milk and it is incubated with the primary antibody. The membrane is washed extensively and then incubated with the secondary antibody (directed against immunoglobulins of the primary antiserum, and conjugated with Horseradish peroxidase). The membranes are washed and the location of the protein (antigen)-primary antibody-secondary antibody-peroxidase visualised by chemiluminescence with ECL reagents.

Reagents

-Transfer Buffer: 25 mM Tris-HCl (unadjusted pH should be 8.3)
192 mM Glycine
20% Methanol

- PVDF membranes (*Immobilon P*, Millipore).

- Tris-buffered saline (TBS) buffer: 20 mM Tris-HCl, pH 7.5
137 mM NaCl

- Tris-buffered saline Tween (TBS-T) buffer: 20 mM Tris-HCl, pH 7.5
137 mM NaCl
0.1% Tween-20

- Phosphate-buffered saline (PBS) buffer

- Phosphate-buffered saline Tween (PBS-T) buffer: PBS
0.1% Tween-20

- Blocking solution: 5% skimmed dry milk in TBS-T or PBS-T

- Antibody solution: 5% skimmed dry milk in TBS-T or PBS-T buffer without milk (add 0.02% sodium azide to preserve the buffer from contaminations). In case of Phospho-Akt (Ser473) antibody (Cell Signaling) a solution of 5% BSA and TBS-T was used.

- Primary antibody: the primary antibodies used in this study have been:

GSK3β (Mouse IgG1, BD Transduction Laboratories)

E2F1 (C-20) (rabbit polyclonal, sc-193, Santa Cruz Biotechnology)

Hemagglutinin epitope (HA, Y-11 rabbit polyclonal sc-805, Santa Cruz Biotechnology)

Hemagglutinin epitope (HA 12CA5 mouse monoclonal, Roche)

Histidine (rabbit polyclonal, Santa Cruz Biotechnology)

GFP (rabbit polyclonal ab6556, Abcam)

α-tubulin (mouse, Oncogen^{TM})

Phospho-Akt (Ser473) (rabbit polyclonal, Cell Signaling)

β-Catenin (Mouse IgG1, BD Transduction Laboratories)

GST (mouse monoclonal, Santa Cruz Biotechnology)

PARP (rabbit polyclonal, Boehringer Mannheim)

- Horseradish peroxidase-conjugated secondary antibodies.

- ECL chemiluminescence reagents (Amersham).

* TBS/TBS-T based buffers were used for GFP, α-tubulin antibodies, Phospho-Akt (Ser473), β-catenin, GST and PARP antibodies. For all other antibodies PBS/PBS-T based buffers were used with excellent result, although TBS/TBS-T solutions can be use as well.

Procedure

***Transfer of proteins*.**

1. Wet PVDF membranes in methanol, transfer to water and place in Transfer Buffer for some minutes.

2. Place the gel previously wet with transfer buffer carefully over a PVDF membrane placed on a wet filter paper. Do not trap any air bubbles between gel and membrane. Place a second wetted filter paper over the gel to give a paper-gel-membrane-paper sandwich and place in the Transblotter between two soaking pads. Place the sandwich inside the holder so that the gel is closer to the black side (positive) of the holder. Fill with cold transfer buffer.

3. Electrophorese overnight at 35 V at 4^{o}C.

4. Remove holder from cell and gel from membranes.

Western blot

5. Once proteins are transferred to PVDF filters, these are hydrated for 5 min in TBS-T/PBS-T.

6. Membranes are then blocked in blocking solution for 1-1.5 hours at room temperature.

7. After a quick rinse in TBS-T/PBS-T, the filter is incubated with the primary antibody diluted in antibody solution. Primary antibodies are diluted according to their specificity and purity, and incubations times can also be varied. The dilutions and times used in this study have been:

* 1:5000 dilution / 1 hour at room temperature for GSK3β, E2F1 and HA antibodies (when checking coimmunoprecipitation the incubation time was normally increase to an overnight incubation)

* 1:1000 dilution / overnight at 4^{o}C for GFP and α-tubuline antibodies.

8. After primary antibody incubation, the membrane is washed 4 times for 10 min each with TBS-T/PBS-T at room temperature.

9. Secondary antibodies conjugated to peroxidase are used according to the nature of the primary antibody. Usually, a 1: 2500-5000 dilution in Blocking solution or PBS-T is used. The incubation is performed for 45-60 min at room temperature with constant agitation.

10. Wash membrane three times for 10 min each with TBS-T/PBS-T and once for 10 min with TBS/PBS.

11. Incubate membrane in ECL chemiluminescence reagents following the manufacturer's instructions.

IMMUNOPRECIPITATION OF ENDOGENOUS GSK3β AND TRANSFECTED/INFECTED HA tagged GSK3β AND KINASE ASSAY.

Principle: a cellular protein (in this case GSK3β) is purified from a cellular lysate by immunoprecipitation with a specific antibody, coupled to protein A or G bound to sepharose. The GSK3β -antibody-protein A/G-sepharose complex is centrifuged and the kinase activity of the immunoprecipitated protein is assessed by incubation of the pellet with a specific substrate and radioactivelly-labelled ATP. The same procedure can be used to study the kinase activity of transfected proteins (in this case, HA-tagged GSK3β). These transfected/infected proteins can be immunoprecipitated by using an antibody directed against an epitope linked to the fusion protein (HA) or the protein itself.

Reagents

-Lysis buffer: (See section *SDS POLYACRILAMIDE GEL ELECTROPHORESIS OF PROTEINS)*

- GSK3β antibody (BD Transduction Laboratories)

- Hemagglutinin epitope antibody Y-11 (Santa Cruz).

-Protein A-sepharose and Protein G-sepharose (Sigma).

- Kinase buffer (2X) : 16 mM MOPS, pH 7.4

100 mM β-glycerolphosphate

0.4 mM EDTA

20 mM Magnesium Acetate

2 mM NaF

1 mM Benzamidine

2 μg/ml Aprotinin

- [γ-^{32}P] ATP : 3000 mCi/mmol [γ-^{32}P] ATP

- ATP (cold 2 mM)

- Phospho-Glycogen Synthase Peptide-2 (Upstate Biotechnology): rehydrated with distilled water to 1 mM, stored at -20°C.

- Whatman P81 phosphocellulose paper (Whatman int. LTD. Maidstone, UK).

- 0.75% phosphoric acid.

- Acetone.

Procedure

Immunoprecipitation of proteins

1. Wash cells twice with ice-cold PBS. Add 1 ml of PBS.

2. Scrape plate with a cell scraper.

3. Spin down cells by microcentrifugation at maximum speed for 5 min at 4°C.

4. Add 200-300 μl (per 100-mm dish) of ice-cold Lysis Buffer to the pellet. Vortex.

5. Shake the tubes for 15 min at 4°C.Vortex again.

6. Centrifuge at maximum speed for 15 min at 4°C. Transfer supernatant to a new tube.

7. Determine the extract protein concentration by the Bradford method using bovine serum albumin as a standard.

8. Add equal amounts of protein (200 μg) from each sample to a new tube. Adjust the volume of each sample to the same final volume (at least 500 μl) with Lysis Buffer.

9. Add 2 μg (if possible keep the relation 1 μg antibody/100μg total extract) of the corresponding antibody to each tube. Incubate with continuous shaking for 2 hours at 4°C.

10. Add 40 μl of 50% protein G-sepharose to the tubes corresponding to the endogenous GSK3β, or 40 μl of 50% protein A-sepharose to the tubes corresponding to the transfected/infected HA-tagged GSK3β. Incubate with continuous shaking for 1 hour at 4°C.

11. Centrifuge at 13000 rpm for 5 minutes at 4°C.

12. Wash the pellets three times with ice-cold Lysis Buffer.

Kinase assays

13. Prepare the following Total mix for each sample plus two:

- 1.25 μl Phospho-Glycogen Synthase Peptide-2 (final concentration of 15.62 μM/sample)
- 0.4 μl ATP (40 μCi final)
- 0.4 μl ATP 2 mM (final concentration 10 μM/sample)
- 37.95 ml Kinase Buffer 2X

14. To each immunoprecipitated pellet add: ·40 μl of the above Total mix

The synthetic peptide is similar to skeletal muscle glycogen synthase; it contains sites 3b, 3c and phosphorylated site 4 from glycogen synthase.

15. Incubate the kinase reactions for 30 min at 30°C.

16.Cut 2X2 cm-squares of P81 paper. Place each square on a non-absorbent material (aluminium foil).

17. Spot 25 µl of the contents of each assay tube quickly onto the surface of the prepared P81 papers.

18.Wash assay squares extensively three times with cold 0.75% phosphoric acid (30 minutes/wash).

19. Wash assay squares once with acetone. Allow the papers to dry.

20. Place each square in a 5-ml scintillation vial and read in a scintillation counter.

21. Compare CPM of enzyme samples to CPM of control samples that contain no enzyme (background control).

The ^{32}P-labelled peptide is separated from [γ-^{32}P] ATP after the protein kinase reaction by adsorbing the peptide to P81 phosphocellulose paper. P81 is an ion-exchange matrix with net negative charge at most pHs. At low pH (phosphoric acid), the excess [γ-^{32}P] ATP left will not bind to the paper, whereas the phosphorylated peptide will do so.

***Advice:**

The use of a positive control if possible is highly recommended (in this case one can use commercial purified rabbit recombinant histidine tagged GSK3β; Sigma). In this case, dilute 1.2 µl of this recombinant GSK3β (2 U/µl) into 38.8 µl Kinase buffer 2X and treat as an immunoprecipitated sample (Step 14).

Protein A-Agarose or protein G-Agarose is used according to the antibody emploid to immunoprecipitate: for mouse IgG_{2a} & IgG_{2b} and rabbit polyclonal antibodies, protein A-agarose is recommended while for mouse IgG_1 & IgG_3, rat IgG_1, IgG_{2a}, IgG_{2b} and IgG_{2c} and rabbit and goat polyclonal antibodies, protein G-agarose is recommended.

A negative control of the immunoprecipitation is as important. Idealy use another antibody from the same species as the one used for the target protein.

CO-IMMUNOPRECIPITATION OF ENDOGENOUS GSK3β, ENDOGENOUS E2F1 AND TRANSFECTED/INFECTED HA-tagged GSK3β AND HA-tagged E2F1.

Principle: a variation of the immunoprecipitation technique described previously. In this case the goal is not to measure the immunoprecipitate's kinase activity but to purified a cellular protein in such conditions that we also obtain all the other proteins physiologically bound to it. A kinase assay can be further performed if one of the bound proteins we expect is a kinase.

Reagents

-Lysis buffer: (See section *SDS POLYACRILAMIDE GEL ELECTROPHORESIS OF PROTEINS)*

- GSK3β antibody (BD Transduction Laboratories).

- E2F1 (C-20) antibody (Santa Cruz).

- Hemagglutinin epitope antibody Y-11 (Santa Cruz).

- Hemagglutinin epitope antibody 12CA5 (Roche).

- Protein A-sepharose and Protein G-sepharose (Sigma).

- Sepharose (Sigma)

Procedure

Co-immunoprecipitation of proteins

1. Wash cells twice with ice-cold PBS. Add 1 ml of PBS.
2. Scrape plate with a cell scraper.
3. Spin down cells by microcentrifugation at maximum speed for 5 min at 4^{o}C.
4. Add 1ml (per 150-mm dish) of ice-cold Lysis Buffer to the pellet. Vortex.

5. Shake the tubes for 15 min at 4°C.Vortex again.

6. Centrifuge at maximum speed for 15 min at 4°C. Transfer supernatant to a new tube.

7. Determine the extract protein concentration by the Bradford method using bovine serum albumin as a standard.

8. Add equal amounts of protein (500 μg up to 3 mg in case of endogenous proteins) from each sample to a new tube. Adjust the volume of each sample to the same final volume (at least 500 μl) with Lysis Buffer.

9. Add 20 ml of 50% protein A-sepharose or G-sepharose (the same we are going to use later on for immunoprecipitating) to the tubes and incubate with continuous shaking for 30 minutes at 4ºC (preclearing)

10. Centrifuge at maximum speed for 15 min at 4ºC. Transfer supernatant to a new tube.

11. Add 2 μg (if possible keep the relation 1 μg antibody/100 μg total extract) of the corresponding antibody to each tube. Incubate with continuous shaking for 2 hours at 4°C.

12. Add 40 μl of 50% protein A-sepharose or G-sepharose to the tubes. Incubate with continuous shaking for 2 hours at 4°C (overnight incubation may be required in case of endogenous proteins).

13. Centrifuge at 13000 rpm for 5 minutes at 4°C.

14. Wash the pellets three times with ice-cold Lysis Buffer (wash well to make sure that what we are detecting are really bounded proteins and not proteins left from the rinses).

15. Perform a western blot against the bounded protein.

RADIOACTIVE LABELING WITH ^{32}P TO DETERMINE *IN VIVO* PHOSPHORYLATION

Principle: use of an ATP precursor (^{32}P-ortophosphate) permeable to the cell to determine *in vivo* phosphorylation processes keeping all the cell compartments intact.

Reagents:

- ^{32}P-ortophosphate 10mCi/ml

- RMPI 1640 without phosphates (Biological Industries)

- TBS

Procedure:

1. Rinse treated cells three times with cold RPMI without phosphates

2. Add to the cells 1 ml RMPI without phosphates containing 20 ml of ortophosphate 10mCi/ml per well (p35)

3. Incubate for 5 hours at 37ºC and 5% CO_2

4. Rinse 4 times with cold TBS in order to eliminate all the ortophosphate not incorporated.

5. Proceed with the necessary protocol in order to analyze the phosphorylation (in our case immunoprecipitation of endogenous E2F1).

CLONING OF GSK3β

Principle: introduction of the sequence encoding for human GSK3β protein into adequate vectors (plasmids). Two commercially available vectors were used: pcDNA3.1(-) and pCMV/myc/nuc and the different GSK3β sequences (S9A and KM) were obtained from extraction of DNA from adenovirus containing them. [The cloning of the different constructs into both vectors was performed by Nuria Majòs].

Procedure: In all the clonings the strategy followed was the same.

1. Introduction of the restriction sites for enzymes XhoI and NotI in all the GSK3β sequences through PCR reaction using the following oligos which have the restriction sites wanted included.

-*Xho*I forward 5′-CGGGCCTCGAGCATGTCAGGGCGGCCCAGAACCACC-3′

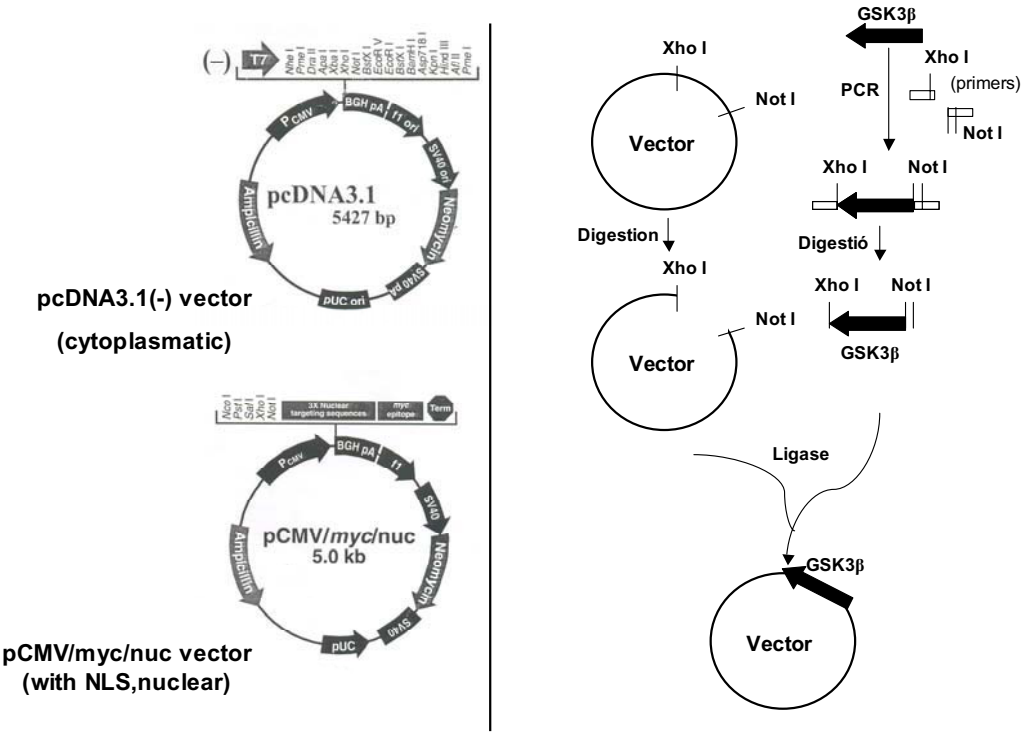
-*Not*I reverse 5′-CGCCGGCGGCCGCGGTGGAGTTGGAAGCTGATGCAGAAGC-3′

2. Isolation of the PCR products by running the products on an agarose gel and extraction of the band using a commercial extraction kit.

3. Purification of the different inserts by enzymatic digestion using XhoI and NotI.

4. Digestion of the vectors pcDNA3.1(-) and pCMV/myc/nuc with XhoI and NotI (see scheme below)

5. Ligation of the GSK3β onto each of the vectors using T4 ligasa.



Scheme of GSK3β cloning into pcDNA3.1(-) and pCMV/myc/nuc.

All the resulting constructs were confirmed by sequence analysis, the correct protein expression by Western Blot, and their expected subcellular localization by subcellular fractioning followed by Western Blot of the fractions.

SUBCELLULAR FRACTIONING

Principle: selective extraction of different intracellular compartments. Using different reagents and a centrifuging gradient, we are able to separate the nuclear, mitochondrial and cytoplasmatic fraction of a cellular culture.

Reagents

- PRE-isotonic buffer (final volum of 48,95 ml): 25 mM Tris (pH 6.8)
250 mM Sucrose
1 mM EDTA

- Proteases inhibitor cocktail (for 1 ml) 100X: 1 μg/ml leupeptin
1 μg/ml pepstatin (in ethanol)
1μg/ml aprotinin (in Hepes 0.01M)
0.1 mM Benzamidin

- 0.1 M Na_2CO_3 pH 11.5

-Hypotonic nuclear buffer (final volume of 50 ml): 10 mM Hepes·KOH pH 7.9
1.5 mM $MgCl_2$
10 mM KCl
0.2 mM PMSF

- 20% Triton X-100

- PBS 1X

LYSIS BUFFER

PRE-isotonic buffer	980 µl
Digitonin (in DMSO) 100mg/ml	5 µl (final 0.05%)
DTT 1 M	1 µl (final 1mM)
PMSF 100mM	1 µl (final 0.1 mM)
Proteases inhibitor cocktail	10 µl

COMPLETE NUCLEAR BUFFER

Hipotonic nuclear buffer	992 µl
DTT 1 M	2 µl
PMSF	2 µl
Proteases inhibitor cocktail	4 µl

Procedure

1. Harvest the cells (around $30x10^6$ cells) on their own medium and centrifuge for 5 min at 1500rpm at 4ºC and discard supernatant.

2. Resuspend the pellet with 3 ml of ice-cold PBS 1X carefully, not to break the cells. Centrifuge for 5 min at 1500 rpm at 4ºC (repeat three times)

3. Resuspend the pellet with 1 ml of ice-cold PBS 1X and transfer into an eppendorf. Centrifuge for 8 min at 2300 rpm at 4ºC.

4. Discard supernatant and resuspend pellet with 400 µl of Lysis buffer (200 µl for every $15x10^6$ cells). Leave for 30 seconds and centrifuge for 3 min at 13000 rpm at 4ºC.

5. Collect the supernatant (370 µl; cytoplasmatic fraction) making sure we do not get any part of the pellet (mitochondrial and nuclear fractions). Transfer the supernatant into an eppendorf tube and quick-freeze with liquid nitrogen.

6. Resuspend the pellet with 1 ml of PRE-lysis buffer without digitonin carefully not to break the nucleus. Spin for 15 seconds at 13000 rpm to pellet down the nucleus.

7. Collect all the supernatant (mitochondrial fraction) into a new eppendorf tube and centrifuge for 3 min at 13000. Discard supernatant and quick freeze pellet with liquid nitrogen. This is the mitochondrial fraction.

8. We resuspend the pellet obtained in step 6, with 1 ml of *Complete Nuclear Buffer* and we centrifuge for 7 min at 3300 rpm.

9. Discard supernatant and resuspend again the pellet with 300 µl of a mix of 300 ml of *Nuclear Buffer* + 2 ml of Triton X-100. Vortex for 10 seconds, incubate 10 min in ice and vortex for 10 more seconds. Following, we centrifuge for 10 min at 3300 rpm.

10. Discard supernatant and resuspend pellet with 400 µl of Loading buffer 1X. We quick-freeze all with liquid nitrogen. This is the nuclear fraction.

11. Usually to check the correct subcellular fractioning blotting against the following marker proteins is performed: tubulin (citoplasmatic fraction), citocrom-oxidase (mitochondrial fraction) and PARP (nuclear fraction).

RNA interference (RNAi)

Principle: gene silencing ("knock down" the expression of a gene) by small interfering RNAs that guide sequence-specific mRNA degradation.

Reagents

- Optimem

- Lipofectamine (Invitrogen)

- Titrate siRNA of the corresponding gene to be knockout

Procedure

1. Previous to the use of the siRNA, this has to be titrated to the adequate concentration in which it knocks down the gene of interest in our cell line. In our case this is verified by Western blot. It is recommended to prepare more than one siRNA from the same gene.

2. Seed cells two days before transfection (105 cells/ well; 6 well multiplates).

3. **Transfect cells (change medium to 1 ml antibiotic free DMEM/FCS fresh medium before adding the transfection mix**):

Prepare two tubes:

Tube 1

50 µl Optimem/transfection/well

-/+ siRNA (titrate between 8 and 64 nM, RNAi stocks are usually 20 mM double strand and annealed)

-/+ reporter plasmids

Let it sit for 5 min

Tube 2

50 µl Optimem/transfection/well

2 µl Lipofectamine (Invitrogen)/transfection/well

(Prepare a master mix for as many transfections as you may have.)

Let it sit for 5 min

Add 52 µl from the Optimem/Lipofectamine mixture on top of every tube 1 and let it sit for 20 min.

Add gently and dropewise to the wells and mix.

4. The following day to the transfection, change the medium again to DMEM/FCS fresh medium (this time with antibiotics); 2 ml/well.

5. Harvest the cells 2 days post-transfection. Normalize by protein content and run Westerns.

LC-MS (LIQUID CHROMATOGRAPHY INTERFACED WITH ELECTROSPRAY MASS SPECTROMETRY)

Principle: fractioning and analysis of a phosphorylated peptide to determine the residues phosphorylated.

Procedure: (performed at the LC-MS service of FMI Institute, Basel, Switzerland)

Peptides were extracted, fractionated and analyzed by liquid chromatography interfaced with electrospray mass spectrometry (LC-MS). A Rheos 4000 chromatograph was used, equipped with a 1x250mm Vydac (Hesperia, CA) C8 column and interfaced with a Sciex API 300 mass spectrometer (PE Sciex, Toronto, Ontario, Canada) operated in the single quadrupole mode. The column was equilibrated in 95% solvent A (2% CH3CN, 0.05% TFA in H_2O), 5% solvent B (80% CH3CN, 0.045% TFA in H2O), and a linear gradient was developed from 0 to 50% solvent B in 60 min at a flow rate of 0.05 ml/min as described elsewhere [Krieg et al., 1998].

NanoESI mass spectrometry (MS) was performed as described [Wilm and Mann 1996]. The masses of the phosphopeptides were identified by 79 Da precursor-ion scanning in the negative ion mode [Carr et al 1996]. The mass spectra were acquired on a API 300 triple quadrupole mass spectrometer (PE Sciex, Toronto, Ontario, Canada) equipped with a NanoESI source (Protana, Odense, Denmark) or on a Iontrap Deca XP (Thermo Finnigan, San Jose, CA).

VIII SPANISH SUMMARY (RESUMEN CASTELLANO)

PRESENTACION

La decisión de una célula de iniciar el ciclo celular, ir hacia apoptosis o sobrevivir es consecuencia de la integración de diferentes señales extracelulares. Los factores de crecimiento, los contactos entre células, así como diversos inductores de apoptosis regulan un complejo sistema de vías de transducción de señales que inducen la activación de un gran número de genes implicados en la respuesta celular de los procesos mencionados anteriormente. Una de las proteínas claves en la regulación del ciclo celular y/o de la entrada en apoptosis es el factor de transcripción E2F1. Se ha sugerido que los niveles de actividad E2F1 pudieran actuar como sensores de la entrada o parada del ciclo celular y apoptosis. En estas decisiones no sólo la actividad transcripcional es importante sino también la sincronización de E2F1 con vías de transducción específicas. Es en este contexto en el cual se ha sugerido que la activación de la vía de la fosfatidilinositol 3-quinasa (PI 3-quinasa) inhibe el efecto apoptótico causado por la sobreexpresión de E2F1. Debido a que la glicógeno sintasa quinasa-3-beta (GSK3β) es uno de los substratos fisiológicos más importantes de esta vía, se realizaron experimentos con el fin de analizar la existencia de una interacción entre estas dos proteínas.

Los resultados obtenidos en esta Tesis demuestran que GSK3β fosforila al factor de transcripción E2F1 humano *in vitro* en las posiciones serina 403 y treonina 433. Estos residuos ya han sido descritos anteriormente como substratos de la fosforilación por parte del complejo quinasa TFIIH, concretamente por uno de sus miembros: cdk7. A pesar de que no podemos detectar fosforilación *in vivo*, experimentos de immunoprecipitación confirman la existencia de una unión *in vivo* entre las proteínas GSK3β y E2F1. Mediante transfecciones transitorias, 'RNA interference (RNAi)', y la utilización de inhibidores específicos de PI 3-quinasa y GSK3β, demostramos que GSK3β regula la actividad de E2F1 a través de la interacción con su dominio transactivador y que la actividad quinasa de GSK3β no es requerida para esta activación.

Por tanto, nuestros resultados se integrarían en un modelo en el que la translocación de GSK3β al núcleo modularía la actividad E2F1 y, en consecuencia, la decisión de una célula de entrar en el ciclo celular o dirigirse hacia la apoptosis.

OBJETIVOS

Históricamente, E2F1 se ha relacionado exclusivamente con su función como factor de transcripción esencial para la transición G1/S. Resultados de los últimos años han cambiado esta visión, involucrando a E2F1 en otros procesos celulares como la parada del ciclo celular y la apoptosis. Se ha sugerido que los niveles de esta proteína pudieran ser el sensor biológico que determinaría el proceso a realizarse. También se ha comprobado la necesidad de otros efectores de transducción de señales para definir los procesos celulares.

La activación de la vía de la PI 3-quinasa ha demostrado ser determinante en las decisiones celulares en las cuales E2F1 participa. La activación de esta vía inhibe el efecto apoptótico de la sobreexpresión de E2F1. GSK3β es uno de los substratos fisiológicos de esta vía y se ha visto que participa en diferentes procesos apoptóticos. Considerando lo expuesto anteriormente, nuestra hipótesis de trabajo es que GSK3β es la señal, o una de las señales, que regula la actividad de E2F1 y, en consecuencia, la división celular.

Por tanto, los objetivos de esta Tesis son los siguientes:

-1. Análisis de la fosforilación *in vitro* e *in vivo* de E2F1 por parte de GSK3β.

-2. Estudio de la interacción *in vitro* e *in vivo* entre GSK3β y E2F1.

-3. Estudio de la regulación del factor de transcripción E2F1 por parte de GSK3β.

INTRODUCCIÓN

CICLO CELULAR Y SEÑALIZACIÓN CELULAR.

El ciclo celular es el proceso a través del cual todos los organismos se propagan. En organismos unicelulares, cada división celular da lugar a un nuevo organismo mientras en el caso de los organismos pluricelulares múltiples divisiones celulares son necesarias. En el caso de estos últimos, el ciclo celular también es necesario para generar nuevas células para sustituir a otras que se han perdido por daño o apoptosis.

Por tanto, el ciclo celular, debe se un proceso perfectamente controlado en el cual las células proliferan incrementando su masa y duplicando su cantidad de DNA. Esto obliga a la maquinaria de control del desarrollo del ciclo celular a estar en estrecho contacto con el medio externo para así interpretar correctamente los estímulos externos y activar el ciclo celular sólo cuando las condiciones sean favorables.

En la mayoría de los casos, la proliferación celular está regulada a través de agentes presentes en el medio externo (suero en el caso de organismos pluricelulares). Casi todos estos agentes son proteínas presentes en bajas concentraciones y que reciben el nombre general de factores de crecimiento.

Las células interpretan las distintas condiciones externas a través del estímulo de diferentes receptores, mucho de los cuales son receptores del tipo proteína-quinasa. La función de éstos y otros tipos de receptores es actuar como sensores celulares ante la presencia de factores de crecimiento y enviar una señal al interior de la célula, señal que debe alcanzar el núcleo dónde se encuentra la maquinaria del ciclo celular.

Considerando todo lo expuesto anteriormente, se concluye la enorme importancia del correcto funcionamiento de toda la maquinaria de señalización y su correcta integración con la maquinaria de control del ciclo celular. Una perfecta coordinación de las

diferentes vías de señalización con el ciclo celular es esencial para el progreso del ciclo celular y evitar la desregulación del crecimiento celular.

CICLO CELULAR

Los acontecimientos que conciernen a la replicación y partición de los cromosomas son comunes para todo los ciclos celulares. Con pocas excepciones, una célula recién dividida necesita recibir un genoma completo para sobrevivir. Por tanto, se requieren mecanismos especiales para asegurar la precisa replicación y partición de los cromosomas.

El ciclo celular eucariota se divide en dos fases importantes: la fase **S** y la fase **M**. La fase S es una parte de la interfase (periodo que tiene lugar entre una fase M y la siguiente) en la cual el DNA se replica. La fase M es la fase en la cual tiene lugar la mitosis, proceso de división celular. Entre estas dos fases, hay otras dos fases en las cuales la célula crece y duplica su masa proteica así como sus orgánulos. Estas fases intermedias son, la fase G1 (antes de la fase S) y la fase G2 (después de la fase S) y juegan un papel importante para iniciar los eventos de la fase S y de la mitosis en un orden correcto y en acuerdo con el crecimiento celular y las condiciones externas. Hay células que pueden parar su progreso antes de la fase S y mantenerse en lo que se denomina la fase G0 incluso durante años, sin incrementar su masa celular (figura 1).

Para asegurar que los correctos procesos del ciclo celular tienen lugar la célula dispone de una serie de puntos de control o 'checkpoints'. El primero tiene lugar al final de la fase G1 y se le conoce como punto de restricción R. Llegado a este punto, se examina que existe una correcta masa celular y que las condiciones externas son las adecuadas antes de permitir la continuación del ciclo celular. Un segundo punto de control está localizado antes de la fase M (punto de control G2-M) y asegura que el tamaño celular es correcto, unas buenas condiciones ambientales, y una correcta y completa replicación del DNA. Al final de la mitosis se encuentra el punto de control M, el cual asegura una buena alineación de los cromosomas antes de permitir la finalización de la división celular (figura 2).

La maquinaria de regulación básica del ciclo celular está basada en dos familias de proteínas principales: las proteínas quinasas dependientes de ciclinas (cdk) y las ciclinas. Las cdks inducen los procesos subordinados a través de la fosforilación de proteínas concretas en serinas o treoninas. Por otra parte, las ciclinas se unen a las cdks y controlan su capacidad de fosforilar proteínas diana. La formación y destrucción de los complejos ciclina-cdk son los procesos centrales que controlan el ciclo celular (figura 2). Además existe un control interno de estas dos proteínas como la regulación de la transcripción de los genes de ciclinas y su degradación. La modificación de las cdks mediante fosforilación e inhibición de su actividad es otro de los procesos regulados. También se ha demostrado la importancia de la localización intracelular de las ciclinas. A su vez, el complejo ciclina-cdk está regulado por su fosforilación por parte de la quinasa activadora de cdk (CAK) (la cual activa al complejo) y su inhibición por la unión de proteínas de la familia CIP/KIP y de la familia INK4.

Existen dos tipos principales de ciclinas: las ciclinas de la fase G1 las cuales se unen a las cdks durante la fase G1 y son importantes para el inicio de la fase S, y las ciclinas mitóticas las cuales se unen a las cdks durante la fase G2 y son esenciales para la entrada en mitosis.

La ciclinas de la fase G1 son del tipo D o E, tienen una vida muy corta y sus niveles están controlados por transcripción. La ciclinas D (D1, D2, D3) están implicadas en el control del punto de restricción R. Su síntesis está inducida por factores de crecimiento e inhibida por factores que reducen la proliferación. Las ciclinas D pueden unirse a diferentes cdks pero se unen principalmente a la cdk4 durante el final de la fase G1 y principio de la fase S dependiendo de las señales mitogénicas [Hitomi y Stacey 1999]. La función principal del complejo ciclina D-cdk4 es fosforilar a la proteína del retinoblastoma (pRB) y , en consecuencia, activar la expresión de genes necesarios para la entrada en la fase S. La ciclina E por su parte, forma un complejo con cdk2 y está involucrada en la replicación del DNA. Los complejos ciclina E-cdk2 también fosforilan a pRB.

Las ciclinas mitóticas A y B, regulan el punto de control G2-M. Son estables durante la interfase y son degradadas rápidamente durante la mitosis. Durante la fase S, el complejo ciclina A-cdk2 parece tener un papel en la elongación de la síntesis del DNA. El complejo ciclina B-cdk1 es importante para la finalización de la mitosis para la cual es necesaria la degradación de la ciclina B durante la transición metafase-anafase.

Como se ha mencionado anteriormente, el punto de restricción R es uno de los controles de los que la célula dispone para asegurar que el correcto progreso del ciclo celular tiene lugar. Está principalmente controlado por una familia de proteínas llamadas 'pocket proteins' y miembros de la familia de factores de transcipción E2F. La fosforilación de pRB elimina su capacidad de unión a los factores de transcripción E2F, liberándolos y permitiendo así el progreso a la fase S. El punto de transición entre la fase G1 y la fase S se considera un punto 'sin vuelta atrás' ya que a partir de dicha transición la célula se compromete a completar el ciclo celular con independencia de factores externos [Kondo et al., 2001].

La fosforilación de pRB está controlada por dos reacciones enzimáticas opuestas: fosforilación por parte de proteínas quinasas y la reacción opuesta (defosforilación) , catalizada por fosfatasas las cuales reconstituyen la actividad de pRB. El estado de pRB fluctua a medida que el la célula pasa por las diferentes fases del ciclo celular (figura 3). En células ciclando, pRB se encuentra en su forma activa (hipofosforilado) sólo durante el inicio de la fase G1. Su fosforilación al final de la fase G1 por parte de ciclinaD-cdk4/6 y posteriormente por parte de ciclina E-cdk2, y su defosforilación al final de la fase M son considerados los dos elementos críticos que regulan la actividad pRB.

LA FAMILIA E2F

La familia E2F consiste en factores de transcripción que regulan la expresión de reguladores claves del ciclo celular. Como se ha mencionado anteriormente, la activación de E2F es suficiente para que las células repliquen su DNA de forma irreversible. Esto hace que esta familia de factores de transcripcion sean cruciales en el control de la proliferación celular tanto en células normales como tumorales. Estudios

posteriores también relacionen la familia E2F con mecanismos involucrados en la reparación del DNA, replicación, regulación de los puntos de control G2-M, entre otros [Ren et al., 2001]. Esta familia consta de dos subfamilias, E2F y DP. El complejo heterodimérico E2F (a excepción de E2F7) está formado por la unión de la subunidad E2F con una subunidad DP, para formar un factor de transcripción activo y capaz de regular genes que contengan lugares de unión a E2F en sus promotores.

La subfamilia de E2F se divide en cuatro grupos en base a su función principal y como la desarrollan. De está forma tenemos a los E2Fs 'activadores' que incluyen a E2F1, E2F2 y E2F3a y cuya función principal es la activación de genes esenciales para la correcta progresión del ciclo celular y para la inducción de apoptosis. Por contra los E2Fs 4 y 5 son considerados 'represores' y son principalmente nucleares durante las fases G0/G1 dónde están unidos a miembros de la familia de pRB. Sus principales funciones parecen ser la inducción de la salida del ciclo celular y la diferenciación [Lindeman et al., 1998; Gaubatz et al., 2000; Humbert et al., 2000; Rempel et al., 2000]. Los otros dos subgrupos, E2F6 y E2F7/8, también son considerados 'represores' de la transcripción [Morkel et al., 1997; Cartwright et al., 1998; Gaubatz et al., 1998; Trimarchi et al., 1998; de Bruin et al., 2003; Di Stefano et al., 2003]. El mecanismo preciso de actuación de estos dos subgrupos no está totalmente descrito hasta el momento aunque parece que E2F6 tendría su papel en la quiescencia.

REGULACIÓN DE LA ACTIVIDAD E2F.

Durante el ciclo celular, la actividad y la especificidad dentro de la familia de E2F está estrechamente regulada mediante varios mecanismos: interacción con las 'pocket proteins' (E2F1-5) o con las proteínas del 'Polycomb Group (PcG)' (E2F6), fosforilación o acetilación (E2F1-3).

Cuando las proteínas E2Fs están unidas a las 'pocket proteins' su actividad de transactivación es inhibida. Esta inhibición se revierte cuando la fosforilación de las 'pocket proteins' hace que se libere a E2F. Este es el modelo de regulación 'clásico' aceptado para E2F. En este modelo, E2F4-p130 es el complejo predominante asociado a

promotores en la fase G0 e inhibe la activación de genes diana involucrados en el ciclo celular. A medida que la célula se acerca a la transición G1/S, E2F-pRB reemplaza al complejo E2F4-p130. pRB es fosforilado por complejos ciclina-cdk, permitiendo la activación de genes diana. Como E2F6 y E2F7 no se unen a las 'pocket proteins' no son reguladas por este mecanismo. En el caso de E2F6 se ha visto que se une a las proteínas PcG pero no está claro que implicación tiene esta unión en su regulación [Trimarchi et al., 2001; Ogawa et al., 2002].

Por otra parte, la fosforilación de E2F1 regula su vida media y por tanto su efecto en la célula. La estabilización de E2F1 por fosforilación se ha observado en respuesta a daño del DNA. Las proteínas ATM, ATR o Chk2 son capaces de fosforilar E2F1 lo cual resulta en su inducción y estabilización respectivamente [Lin et al, 2001; Stevens et al., 2003]. También se han descrito fosforilaciones de E2F1 que implican un incremento en su degradación. Esto es el caso de las fosforilaciones por parte de cdk7 y p19ARF [Vandel and Kouzarides 1999; Martelli et al., 2001].

Recientemente se ha propuesto la acetilación como otro mecanismo de control de la actividad de E2F. La acetilación de las proteínas E2F implica una ganancia o pérdida de función dependiendo de dónde tenga lugar la acetilación. En el caso de E2F1, 2 y 3, se ha visto que la acetilación por parte de p300/CBP estimula sus uniones a DNA y por tanto su actividad transcripcional [Marzio et al., 2000]. E2F1 también es acetilado por P/CAF. Dicha acetiliación aumenta su actividad transcripcional así como su vida media.

Por último, otro nivel de regulación de E2F reside en su expresión génica y su localización.

FUNCIONES DE E2F.

Miembros de la familia de E2F juegan un papel principal en la mitosis, la segregación de los cromosomas, control del huso mitótico, reparación del DNA, la condensación de la cromatina, la apoptosis, la diferenciación y el desarollo. Existe redundancia de las

funciones de los diferentes E2Fs aunque también existen funciones específicas para algunos de los miembros.

De entre los miembros de la familia de E2F, E2F1 exhibe propiedades de oncogen (induciendo la proliferación) y de supresor de tumores (induciendo la apoptosis). Como consecuencia de ello, E2F1 ha sido estudiado en profundidad para poder dar una explicación de la existencia de estas dos funciones antagónicas. Parece ser que el nivel de expresión y los factores externos determinan cual de las dos funciones de E2F1 predomina (figura 7) [Pierce et al., 1999].

En cuanto a la regulación de la proliferación celular, E2F1 controla la transcripción de genes importantes para el ciclo celular como la ciclina A, ciclina E, pRB, Cdc6, entre otros. La acción represora de pRB regula su actividad y por tanto, pRB actúa cómo supresor del crecimiento en este caso.

Por el contrario, diferentes mecanismos han sido descritos relacionados con la función apoptótica de E2F1 (figura 8) [Ginsberg 2002; La Thangue 2003; Stevens and La Thangue 2003; Bell and Ryan 2004; Knezevic and Brash 2004]. Se ha comprobado que E2F1 puede inducir apoptosis mediante mecanismos dependientes o independientes de p53. Los primeros implican la estabilización de la proteína p53 la cual activa genes pro-apoptóticos. La vía independiente de p53 implica el incremento de proteínas apoptóticas como p73 [Irwin et al., 2000] y Apaf-1 [Moroni et al., 2001]. También se ha descrito que E2F1 puede inducir a apoptosis al impedir la translocación del factor de transcripción NF-κB al núcleo.

REGULACIÓN DE E2F A TRAVÉS DE DIFERENTES VÍAS DE TRANSDUCCIÓN DE SEÑALES.

E2F está principalmente regulado por su unión a proteínas pRB. Esta interacción está regulada por el complejo ciclina D-cdk4/6 que fosforila a pRB, permitiendo así la liberación de E2F. En consecuencia, muchos de los mecanismos que regulan la actividad transcripcional de E2F actúan sobre la actividad de ciclina D, cdk4/6 o ambos. Las proteínas cdks se activan inicialmente por su asociación con la subunidad de ciclina

y por fosforilación en un residuo de treonina localizado en una secuencia conservada. La fosforilación estabiliza a la cdk permitiendo la unión óptima de ATP y de substrato [Russo et al., 1996]. Por ello, la fosforilación y defosforilación de determinados aminoácidos modulan la activación de las cdks. La CAK (quinasa activadora de cdks) está involucrada en la fosforilación de las cdks, mientras que las fosfatasas de la familia Cdc25 son responsables de su desfosforilación.

El incremento de los niveles de ciclina D está controlado por mecanismos dependientes de PI 3-quinasa y de ERK1/2 [Lukas et al., 1996a, Kummerle et al., 2004]. La vía de PI 3-quinasa regula la traducción de ciclina D a través de la activación de mTOR [Nicholson y Anderson 2002] e independientemente de mTOR en el caso de algunas líneas celulares [Takuwa et al., 1999]. La transcripción de ciclina D está controlada por la inactivación de factores de transcripción 'forkhead' por parte de la vía de PI 3-quinasa. También se ha descrito como GSK3β (effector de la vía de la PI 3-quinasa) fosforila a las proteínas ciclina D1 y a β-catenina (aumenta los niveles de ciclina D) incrementando la degradación de ambas. Los estímulos mitogénicos implican la amplitud y duración de la actividad de ERK que a su vez regula la transcripción de ciclina D.

La proteína Cdc25 se ve afectada por diferentes señales de transducción. Por ejemplo, se ha observado que TGF-β inactiva los complejos ciclina D-cdk4/6 reduciendo los niveles proteicos de Cdc25 [Ewen et al., 1993]. Chk1 fosforila a Cdc25 en respuesta a daño del DNA [Furnari et al., 1997]. Esta fosforilación implica la inactivación de Cdc25 y en última instancia, la fosforilación de cdk.

Estas y otras evidencias indican que la proteína E2F y sus efectos están regulados por más de una vía de transducción de señales. GSK3β regula E2F indirectamente a través de la degradación de ciclina D, pero nada hace pensar que no pueda haber un control directo de E2F1 por parte de GSK3β.

GLICÓGENO SINTASA QUINASA-3 (GSK3)

La glicógeno sintasa quinasa-3 (GSK3) recibió su nombre por su habilidad de fosforilar a la proteína glicógeno sintasa. No obstante, nuevos descubrimientos muestran que GSK3 juega un papel importante en muchos procesos celulares incluyendo la síntesis de glicogeno, la diferenciación, la proliferación y la transformación celular [Ferkey and Kimelman 2000; Diehl et al., 2000].

Hay dos isoformas de GSK3 de mamífero, codificadas por genes distintos: GSK3α y GSK3β [Woodgett 1990]. GSK3α tiene una masa de 51 KDa, mientras que la proteína GSK3β tiene una masa de 47 KDa. La diferencia de tamaño es debido a una extensión rica en glicinas en el extremo amino (N)-terminal de GSK3α. Las dos isoformas comparten secuencias prácticamente idénticas en sus dominios quinasa (98% identidad), pero se diferencian en los últimos 76 residuos del extremo carboxi (C)-terminal. Ambas isoformas comparten substratos aunque no siempre son funcionalmente idénticas y tienen patrones de expresión diferentes.

Más de 40 proteínas han sido descritas como substratos putativos de GSK3. No hay un motivo 'consensus' estricto de fosforilación por parte de GSK3 pero se ha visto que muchos substratos requieren una quinasa previa que genere el motivo –SXXXS(P) antes de poder ser fosforilados por GSK3.

REGULACIÓN DE LA ACTIVIDAD GSK3

La proteína GSK3 está sujeta a múltiples mecanismos de regulación. El mejor estudiado es la inhibición de la actividad GSK3 por la fosforilación de serina 9, en el caso de GSK3β, o serina 21, en caso de GSK3α. La formación de complejos proteicos, la localización intracelular y diferentes drogas tienen una influencia regulatoria sobre la actividad GSK3β.

La actividad GSK3 se reduce por fosforilación en serina 9 (GSK3β) o serina 21 (GSK3α) localizadas en su extremo N-terminal. Varias quinasas pueden fosforilar estas serinas: PKB, p90RSK y p70S6K. Se debe tener en cuenta que cada una de estas quinasa probablemente sólo afecte un 'pool' específico de GSK3 debido a la distribución subcelular de GSK3 y de la quinasa en particular. La fosforilación de estas serinas es inhibitoria de la actividad GSK3 y por tanto, en la mayoría de casos, activadora de sus substratos. Cuando estas posiciones están fosforiladas, el extremo N-terminal de GSK3 se transforma en un 'pseudosubstrato' que impide la unión del substrato.

La fosforilación de la treonina 216 de GSK3β se ha descrito como una fosforilación activadora. Algunos autores defienden una autofosforilación de este residuo [Wang et al., 1994] mientras que otros involucran al sistema de señalización intracelular [Murai et al., 1996]. No obstante el significado fisiológico de esta fosforilación no está claro por el momento.

Otra forma de regulación de GSK3 es su localización celular que determina el acceso a sus substratos. GSK3 es predominantemente citosólica pero también está presente en núcleo y mitocondria. En estos dos últimos compartimentos se ha visto que la GSK3 es de cinco a diez veces más activa que en el compartimento citoplasmático. Los niveles nucleares de GSK3β no son estáticos sino que fluctuan a lo largo del ciclo y en respuesta a diferentes estímulos. Además los diferentes 'pools' de GSK3β pueden ser regulados en tiempo y formas distintas.

La unión de GSK3 a otras proteínas como las proteínas de la familia de GBP o la familia de la Axina inhiben o activan a la GSK3 respectivamente. La unión de GSK3 también puede afectar la actividad de la otra proteína como es el caso de la unión de GSK3 a la proteína *cubitus interruptus* (Ci) [Price y Kalderon 2002; Jia et al., 2002]. Complejos proteicos específicos de orgánulos también han sido descritos para GSK3. La inclusión de GSK3 en diferentes complejos proteicos podría determinar su especificidad o facilitar el acceso a determinados substratos.

FUNCIONES DE GSK3

GSK3 fosforila *in vitro* e *in vivo* a un gran número de proteínas regulando en la mayoría de los casos la estabilidad de la proteína [Frame y Cohen 2001; Doble y Woodgett 2003, Jope y Johnson 2004] pero también la activación transcripcional [Turenne y Price 2001] y la localización subcelular [Zhou et al., 2004]. Proteínas descritas como substratos putativos de GSK3β se muestran en la Tabla 1.

Considerando la variedad de posibles substratos de GSK3 esta quinasa queda involucrada de una u otra manera en los siguientes aspectos celulares: metabolismo (fosforilación de eIF2B, glicógeno sintasa, etc), arquitectura celular y movilidad (fosforilación de tau, MAPs, etc), supervivencia celular y apoptosis (participación en la salida de citocromo *c* y regulación de p53).

RESULTADOS

1. Estudio de la fosforilación *in vitro* e *in vivo* de E2F.

Para evaluar los posibles lugares de fosforilación de GSK3β en el factor de transcripción E2F1 utilizamos el programa Scansite que predice los posibles aminoácidos fosforilados considerando el motivo –SXXXS(P)- [Obenauer et al., 2003]. Considerando los tres niveles de astringencia que ofrece el programa, diez fueron los aminoácidos predichos (figura 1.1).

Con el fin de examinar la fosforilación de los residuos predichos por el programa Scansite realizamos un ensayo quinasa *in vitro* utilizando diferentes proteínas fusionadas a GST (glutation S transferasa). El resultado demostró que GSK3β fosforila a E2F1 *in vitro*. La señal obtenida era mayor que la obtenida para otros substratos de GSK3 ya descritos como ciclina D1 y p53. Esto sugiere que la fosforilación de E2F1 por parte de GSK3β es específica (figura 1.2).

Para determinar si una fosforilación previa de E2F1 aumentaba la fosforilación por parte de GSK3β (demostrado para otros substratos de GSK3β) preincubamos la proteína E2F1 junto con p38 y ATP. Los resultado indicaban que una preincubación con p38 no suponía un efecto aditivo en la fosforilación por parte de GSK3β. Por tanto, los resultados indican que en el caso de E2F1 no se requiere una fosforilación previa para que GSK3β pueda fosforilarla *in vitro* (figura 1.3).

A continuación realizamos una espectrofotometría de masas para determinar qué aminoácidos estaban siendo fosforilados *in vitro*. Tres fosfopéptidos fueron detectados aunque sólo dos residuos fueron identificados: serina 403 y treonina 433. Estos dos residuos ya han sido descritos como dianas de fosforilación de la quinasa cdk7 [Vandel y Kouzarides 1999], siendo el resultado el aumento de la degradación de la proteína E2F1.

Para comprobar los resultados de la espectrofotometría de masas repetimos el ensayo quinasa *in vitro* utilizando GSK3β y diferentes construcciones de E2F1. Comprobamos que la mutación de uno de los residuos reducía la señal mientras que la mutación de ambos aminoácidos revertía totalmente la fosforilación (figura 1.8).

No obstante, en el estudio posterior de la fosforilacíon *in vivo* de E2F1 utilizando diferentes construcciones víricas e inhibidores químicos no pudimos detectar la fosforilación de E2F1 por parte de GSK3β (figura 1.14).

2. Ensayos de unión entre GSK3β y E2F1.

Con el objetivo de determinar la unión entre las proteínas GSK3β y E2F1 realizamos diversos experimentos de coinmunoprecipitación en diferentes líneas celulares. En un primer ensayo, infectamos células HeLa con la forma ‘wildtype’ de GSK3β e inmunoprecipitamos E2F1 endógeno utilizando un anticuerpo específico para E2F1. Mediante Western Blot de los productos inmunoprecipitados detectamos la presencia de

GSK3β, demostrando así su coinmunoprecipitación con E2F1 y por tanto su unión a E2F1 (figura 2.1).

Para determinar si esta unión es exclusiva de la línea celular HeLa repetimos el ensayo en otra línea celular humana: las células HEK293T. En este caso no podíamos utilizar el adenovirus con la construcción 'wildtype' utilizada anteriormente ya que las células HEK293T contienen la proteína E1A la cual permitiría al adenovirus completar su ciclo lítico. Por tanto las diferentes construcciones de GSK3β de las que disponíamos, 'wildtype' (WT), no inhibible (S9A) y catalíticamente inactiva (KM) fueron extraídas del adenovirus y clonadas en vectores adecuados. Una vez obtuvimos las construcciones las células HEK293T fueron transfectadas con las construcciones HA-E2F1 y WT-GSK3β, en un primer momento. Los resultados obtenidos son totalmente comparables a los descritos para HeLA. GSK3β, tanto endógena como sobreexpresada, coinmunoprecipita con E2F1 (figura 2.2).

A continuación abordamos la cuestión de si la actividad GSK3β podría influir en su unión a E2F1. Realizamos transfecciones transitorias de células HEK293T con HA-E2F1 y diferentes formas de GSK3 (WT, S9A, KM). Esta vez la proteína HA-E2F1 fue inmunoprecipitada utilizando anticuerpos (policlonal y monoclonal) contra su epítopo HA. En ambos casos el resultado fue el mismo: todas las construcciones de GSK3β utilizadas coinmunoprecipitaron con E2F1 con la misma eficacia lo que indica que la actividad quinasa no afecta la unión entre GSK3β y E2F1 (figuras 2.3.a y 2.3.b). De igual forma se demuestra que la utilización de diferentes anticuerpos (contra E2F1 o contra HA) no afecta a la coinmunoprecipitación de GSK3β.

Por último se comprobó el hecho de que la proteína E2F1 coinmunoprecipita cuando se inmunoprecipita GSK3β (figura 2.4)..

3. Efecto de GSK3β sobre la actividad transcripcional de E2F1.

Considerando los resultado anteriores es razonable pensar que GSK3β pudiera participar directamente en el control de la actividad transcripcional de E2F1. El modelo de estudio escogido fue la línea celular HEK293T ya que una de las características de esta línea celular es el hecho de que expresan la proteína E1A la cual secuestra a pRB. Esto evita la interacción pRB-E2F1 lo que hace posible el estudio del efecto directo de GSK3β sobre E2F1 evitando el control indirecto a través del control de la degradación de ciclina D.

En una primera aproximación para medir la actividad transcripcional de E2F1, se utilizó el gen de la proteína luciferasa como gen 'reporter' bajo el control de un promotor artificial con tres copias de la secuencia consensus de unión de E2F ($[E2F]_3$-LUC). Transfectamos células HEK293T transitoriamente con esta construcción y tratamos las células con diferentes inhibidores: LY 294002 (inhibidor de PI 3-quinasa), SB 216763 y SB 415286 (inhibidores de GSK3β). Un grupo de células fueron además transfectadas con la construcción HA-E2F1. Los resultados mostraron una reducción de la actividad transcripcional de E2F1 cuando las células en que E2F1 estaba sobreexpresada eran tratadas con el inhibidor de PI 3-quinasa, LY 294002. La inhibición de la actividad GSK3β no tenía efecto sobre la actividad transcripcional de E2F1. Todo ello implica que la actividad transcripcional de E2F1 está bajo el control de la vía de la PI 3-quinasa pero es independiente de la actividad quinasa de GSK3β (figura 3.1). No obstante, los experimentos de coinmunoprecipitación demuestran la unión entre E2F1 y GSK3β lo cual sugiere que la modulación de la actividad transcripcional de E2F1 sí depende de la concentración de proteína GSK3β.

Procedimos a depleccionar la proteína GSK3β endógena en las células HEK293T mediante la técnica de RNA interference (RNAi) y analizamos la actividad transcripcional de E2F1 utilizando la construcción $[E2F]_3$-LUC de nuevo. La actividad transcripcional de E2F1 se redujo en proporción a la deplección de la proteína GSK3β (figura 3.3). Por tanto la presencia de la proteína GSK3β es necesaria para la modulación de la actividad transcripcional de E2F1.

A continuación sobreexpresamos distintas formas de GSK3β (no inhibible y catalíticamente inactiva) y analizamos el efecto de dicha sobreexpresión sobre la actividad transcripcional de E2F1. En concordancia con los resultados obtenidos anteriormente la actividad transcripcional de E2F1 se vio a afectada por el aumento de la cantidad de proteína GSK3β total y no por su actividad quinasa (figuras 3.4 y 3.5).

El direccionamiento de las construcciones descritas anteriormente hacia el núcleo mediante la introducción de una señal de localización nuclear (NLS) no incrementó de forma significativa el efecto observado anteriormente (figura 3.8). Una posible explicación es que la sobreexpresión de la proteína GSK3β ya fuese suficiente para translocar la cantidad de proteína necesaria para modular la actividad transcripcional de E2F1, al núcleo.

Por último, comprobamos mediante transfecciones transitorias con construcciones parciales de E2F1 que la presencia del dominio transactivador de E2F1 es suficiente para que GSK3β module la actividad transcripcional de dicho factor de transcripción. Mutaciones de los residuos serina 403 y treonina 433, fosforilados *in vitro*, no afectaban la modulación.

DISCUSIÓN

Los datos obtenidos en esta Tesis demuestran la implicación de la proteína GSK3β en la regulación de la actividad transcripcional de E2F1. GSK3β fosforila a E2F1 en los residuos serina 403 y treonina 433 *in vitro* en un mecanismo independiente del requisito de una fosforilación previa. La presencia de prolinas contiguas a ambos residuos sugieren que la fosforilación por parte de GSK3β sea una fosforilación prolina-dirigida, mecanismo ya sugerido para la fosforilación de ciclina D por parte de GSK3β.

La no necesidad de una fosforilación previa de E2F1, requisito indispensable de mucho substratos de GSK3β, hace pensar en la posibilidad de que la unión de E2F1 a GSK3β se produzca en otro dominio de GSK3β diferente al utilizado por los substratos prefosforilados. Al igual que las proteínas Axina y FRAT, E2F1 podría unirse a GSK3β

por su extremo carboxi-terminal (C-terminal). La fosforilación de las serinas 9 (GSK3β) o 21 (GSK3α) no afectaría esta unión ya que la conversión del extremo amino-terminal (N-terminal) no bloquearía el dominio de unión de E2F1.

Curiosamente, los residuos 403 y 433 de E2F1 también son fosforilados por el factor de transcripción TFIIH y concretamente por uno de sus componentes: cdk7 [Vandel y Kouzarides 1999]. El hecho de que E2F1 sea substrato de cdk7 implica una actividad adicional de esta quinasa en la regulación del progreso del ciclo celular. TFIIH se une a E2F1 específicamente en la fase S del ciclo celular y dicha unión resulta en un incremento de la fosforilación de E2F1 y como consecuencia de su degradación vía el proteosoma [Campanero et al., 1997].

Por tanto, quien fosforila a E2F1 *in vivo*? Existen incongruencias entre el patrón de unión de cdk7 a E2F1 y la fosforilación de esta última. La fosforilación de E2F1 tiene lugar durante la fase G1 y la fase G2 en células HeLa pero este patrón de fosforilación no concuerda con la unión de TFIIH a E2F1. Además todos los sistemas de deplección de cdk7 utilizados para demostrar la implicación de esta quinasa en la fosforilación de E2F1, mostraban una fosforilación remante del 30 al 40%. Entonces, GSK3β es responsable de esta fosforilación restante?

El hecho de que GSK3β y E2F1 puedan compartir substrato no es sorprendente. De hecho ya se ha dado el caso. TFIIH y GSK3β fosforilan a p53 incluso en el mismo residuo *in vitro* e *in vivo* sin el requerimiento de una fosforilación previa para el caso de GSK3β. Todos los estudios apuntan a una interacción física entre p53 y TFIIH y GSK3β y una modulación directa, o indirecta, de la actividad transcripcional de p53. De igual forma GSK3β podría compartir la regulación de E2F1 con cdk7. En el caso de GSK3β, su interacción física con E2F1 podría afectar la unión de éste a otras proteínas (pRB, factores de transcripción) y regular su actividad. El hecho de que los residuos serina 403 y treonina 433 se localizan en el dominio transactivador de E2F1 apoyan este modelo. También podría darse el caso que GSK3β fosforilase a E2F1 *in vivo* pero que nosotros no hubiésemos podido detectar dicha fosforilación debido a la sensibilidad del ensayo o a una especificidad del tipo celular.

Los experimentos realizados en esta Tesis han sido enfocados para determinar si GSK3β (proteína o actividad) puede regular la actividad transcripcional de E2F1. No obstante cabe la posibilidad de que E2F1 pudiese regular a su vez, la especificidad de substrato de GSK3β.

CONCLUSIONES

1. GSK3β fosforila a E2F1 *in vitro*. Esta fosforilación es específica y tiene lugar sin el requerimiento de una prefosforilación de E2F1. Fosforilación previa por parte de p38 no mostró ninguna actividad sinérgica.

2. Estudios de LC-MS indican que serina 403 y treonina 433 son los aminoácidos de la proteína E2F1 humana fosforilados por GSK3β *in vitro*. Esta fosforilación se pierde cuando ambos aminoácidos se mutan, confirmando así los resultados obtenidos con espectrofotometría de masas.

3. Ensayos quinasa demuestran que E2F1 es fosforilada *in vivo*. No obstante la inhibición de la actividad GSK3β no afecta la fosforilación *in vivo* de E2F1 endógeno.

4. Experimentos de coinmunoprecipitación revelan la interacción entre GSK3β y E2F1 en células HeLa y HEK293T in vivo. Esta interacción se detecta al inmunoprecipitar cualquiera de las dos proteínas. Está unión no se ve afectada por la actividad GSK3β, como se evidencia con las coinmunoprecipitaciones utilizando las formas no inhibible (S9A), catalíticamente inactiva (KM) y 'salvaje', que dan lugar al mismo resultado.

5. La utilización de inhibidores químicos para modular la actividad GSK3b endógena en células HEK293T sobreexpresando E2F1, no afecta la actividad transcripcional del promotor de respuesta a E2F, $[E2F]_3$-LUC. Por contra, la actividad transcripcional se vió reducida de manera dosis-dependiente cuando se redujo la cantidad de proteína GSK3β mediante RNAi.

6. Concentraciones iguales de las formas no inhibible (S9A) y catalíticamente inactiva (KM) de GSK3β modulan la actividad transcripcional de E2F1 endógeno y sobreexpresado sobre un promoter de respuesta a E2F de forma similar. Esto sugiere que la actividad quinasa de GSK3β no es requerida para la regulación de la habilidad de E2F1 para activar la transcripción.

7. Estudios funcionales utilizando construcciones de E2F1 que expresan su dominio transactivador (AD) indican que este dominio es suficiente para la modulación por parte de GSK3β. Mutación de los residuos 403 y 433 no evitan esta modulación por lo que el estado de fosforilación de estos aminoácidos se presenta como irrelevante en este caso.