A thesis entitled

The Regulation of E2F by 14-3-3 Proteins

Presented by

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To my wife and parents.

Abstract

The E2F family of transcription factors are key regulators of the mammalian cell cycle, integrating gene expression with cell cycle progression. Physiological E2F arises when a member of the E2F family complexes with a member of the DP family. The E2F/DP complex can be directly and indirectly regulated by a variety of proteins involved in cell cycle control including members of the RB family of tumour suppressers, members of the HAT (Histone Acetyl Transferase) family and the p53 tumour suppresser.

Previous work in the laboratory has now shown that the 14-3-3 family of molecules can influence the activity of E2F. This is dependent on the ability of 14-3-3 to bind to the DP3 partner. Using a DP3 mutant that was unable to bind to 14-3-3 but that retained its nuclear localisation, this study has further elucidated the role of 14-3-3 in influencing the activity of E2F. 14-3-3 can positively influence E2F-mediated cell cycle progression as well as negatively regulate the apoptotic ability of E2F. Specifically, 14-3-3 allows efficient S-phase entry as well as control over E2F-mediated apoptosis. This study suggests that this could be through the regulation of E2F/DP protein levels. The activity of 14-3-3 is dependent on its ability to bind DP3 since a mutant DP3, unable to bind 14-3-3, displays delayed S-phase entry and an enhanced ability to induce apoptosis. Interestingly, the interaction between DP3 and 14-3-3 was shown to be responsive to DNA damage, indicating that the interaction between these two proteins may be important in initiating a checkpoint control mechanism.

The results presented here point to a new role for 14-3-3 in the regulation of both the cell cycle and apoptosis. This regulation is carried out through the influence of 14-3-3 over E2F, mediated by the DP3 component.

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Abbreviations

A	Adenine
Ab	Antibody
AP	Ammonium persulphate
Apaf-1	Apoptotic protease activating factor-1
ARF	Alternative reading frame
ASK1	Apoptosis signal-regulating kinase 1
ATM	Ataxia-telengiectasia gene product
ATP	Adenosine tri-phosphate
ATR	Ataxia-telengiectasia related gene product
Bad	Bcl-2/Bcl-$_{XL}$-antagonist, causing cell death
Bcl-2	B-cell lymphoma-2
Bcl-$_{XL}$	B-cell lymphoma-$_{XL}$
β-GAL	β-Galactosidase
Brdu	5-bromo-2'-deoxyuridine
BRG1	IIuman brahma-related gene 1
BSA	Bovine serum albumin
C	Cytosine
cak	Cyclin activating kinase
CBP	CREB-binding protein
cdc2	Cell division cycle 2
cdc25C	Cell division cycle 25C
cdk	Cyclin dependent kinase
cdki	Cyclin dependent kinase inhibitor

ChIP	Chromatin immunoprecipitation
Chk1	Checkpoint kinase protein 1
Chk2	Checkpoint kinase protein 2
CMV	Cytomegalovirus
CRE	cAMP-responsive element
C-TAK1	Cdc25C-associated kinase 1
Cx.	Cycloheximide
DAPI	4,6-diamidino-2-phenylindole
ΔB	Basic region deletion protein
DHFR	Dihydrofolate reductase
DMEM	Dulbecco's modified eagles medium
DNA	Deoxyribonucleic acid
DP	DRTF1 protein
DRTF1	Differentiation-regulated transcription factor 1
DTT	Dithiothreitol
E1A	Adenovirus early protein 1A
E2F	E2 factor
EDTA	Ethylene diamine tetra-acetic acid
En.	Endogenous
Ex.	Exogenous
FACS	Fluorescence activated cell scanning
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
FKHRL1	Forkhead transcription factor 1

G	Guanine
G0	Gap 0
G1	Gap 1
G2	Gap 2
GST	Glutathione-S-transferase
HA	Hemagglutinin protein
HAT	Histone acetyltransferase
HBRM	Human brahma
HBS	HEPES-buffered saline
HDAC	Histone deacetylase
HDM2	Human double minute 2
HEPES	N- [2-Hydroxethyl] piperazine-N'-[2-ethanesulfonic acid]
HPV7	Human Papilloma Virus-7
HRP	Horseradish peroxidase
IGF-1	Insulin growth factor-1
IGF1-R	Insulin growth factor-1 receptor
IGF-2	Insulin growth factor-2
INK4	Inhibitors of cyclin-dependent kinase 4
IPTG	Isopropyl-β-D-thiogalactopyranoside
IVT	*In vitro* translated
LMB	Leptomycin B
Luc.	Luciferase
M	Molar
MDM2	Murine double minute 2

MEF	Mouse embryonic fibroblast
MPF	Maturation promotion factor
MRE11	Mre11/Rad50/NBS1 complex
NBS1	Nijmegen breakage syndrome 1
NES	Nuclear export signal
NLS	Nuclear localisation signal
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
P/CAF	p300/CBP-associated factor
PCR	Polymerase chain reaction
PKA	Protein kinase A
PMSF	Phenylmethysulfonyl fluoride
pRB	Retinoblastoma gene product
PS	Phosphatidyl serine
RFP	Red fluorescent protein
SCF	SKP1/CUL1/ F-Box protein complex
SDK	Sphingosine dependent kinase
SDS	Sodium dodecyl sulphate
S-phase	Synthesis phase
SWI/SNF	Switching defective/sucrose nonfermenter
T	Thymine
TAD	*Trans*activation domain
TK	Thymidine kinase
TNFα	Tumour necrosis factor α

TNF-R	Tumour necrosis factor receptor
TS	Thymidilate synthase
TUNEL	TdT-mediated dUTP-X nick end labelling
wt	Wild type
(-/-)	Homozygous mutant

Declaration

The work presented within this thesis was performed entirely by myself and in no way forms part of any other thesis. All work supported by others is acknowledged. The work was performed under the supervision of Professor Nicholas B. La Thangue at the Division of Biochemistry and Molecular Biology, Institute of Biomedical and Life Science, University of Glasgow, UK from September 1999 to October 2003.

Alasdair Milton

October 2003

Chapter 1: Introduction

1.1: The Biology of Cancer

1.1.1: Introduction

Cancer cells are defined by two heritable properties: they and their progeny (1) reproduce in defiance of the normal restraints and (2) invade and colonise territories that are normally reserved for other cells. An isolated abnormal cell that does not proliferate more than its neighbours does no significant damage, no matter what other disagreeable properties it may have. If however, its proliferation is out of control, it will give rise to a tumour, or *neoplasm*, a relentlessly growing mass of abnormal cells (Alberts *et al.*, 1994).

It is the genetic and epigenetic changes within a small set of genes within tumour cells that are responsible for neoplastic development. In this way, oncogenes and tumour suppresser genes play key roles in the development of cancer. Oncogenes are genes that are upregulated in tumour cells and whose protein products are required for cell cycle progression whilst tumour suppressor genes are genes that are disabled and whose protein products play a key role in halting cell cycle progression (Alberts *et al.*, 1994).

Normal cell cycle development can be divided into different sections. Gap1 (G1), Synthesis-phase (S-phase), Gap2 (G2) and Mitosis (M) are the traditional subdivisions of the cell cycle. G1, S-phase and G2 constitute the interphase portion of the cell cycle whilst mitosis involves separation of the duplicated DNA and finally, cytokinesis, or cell division (Figure 1.1A).

When the cell undergoes mitogenic stimulation to divide it will upregulate a set of genes involved in the progression through G1. In late G1 the cell reaches the restriction point, after which it is committed to entering S-phase where its DNA will be replicated. Cells in G1 that have not committed to enter S-phase enter a resting state called G0 where they remain until they decide to re-enter the cell cycle. Once past the restriction point the cell is no longer under the control of external signals and must divide unless cellular insults occur.

The G2 phase is a gap where the cell ensures that DNA replication is complete before mitosis, and finally cell division, is undertaken. If cellular insults such as DNA damage occur the cell initiates a checkpoint control mechanism whereby cell cycle arrest is initiated at either the G1/S or G2/M boundaries. The cell then either repairs the damaged DNA or commits to programmed cell death, or apoptosis. It is the mis-regulation of the genes involved in this intricate 'stop-go' mechanism of cell division that ultimately leads to the development of cancer.

1.1.2 The cdks and cdkis in Cancer

The mitogenic dependent progression through the G1 portion of the cell cycle and the entry into S-phase are regulated by the several types of cyclin dependent kinases (cdks) and their inhibitors, the cdkis. The cdks provide the catalytic portion of the cyclin/cdk complexes that are responsible for cell cycle progression whilst the cyclin portion of the complex provides the regulatory role, thereby activating the cdk.

The cyclin D- and E-dependent kinases control the progression through the G1 portion of the cell cycle. Following mitogenic stimulation the D-type cyclins (D1, D2 and D3) (Matsushime *et al.*, 1991; Xiong *et al.*, 1991; Motokura *et al.*, 1991) are upregulated and interact specifically with either cdk4 or cdk6 (Matsushime *et al.*, 1992; Meyerson *et al.*, 1994). The assembled cyclinD/cdk sub-unit then enters the nucleus where it must be phosphorylated by a cdk-activating kinase (cak) to be able to phosphorylate its target substrates (Sherr and Roberts, 1999). The phosphorylation of members of the pocket protein family by the cyclin/cdk complexes leads to the release of the E2F transcription factor, which induces genes involved in cell cycle progression and DNA synthesis.

One target for E2F is cyclinE, which forms a complex with cdk2 to produce a further level of phosphorylation of the pocket proteins and the complete induction of E2F and S-phase progression (Sherr, 2000). Up-regulation of cyclinD is under the control of mitogenic stimulus that, if withdrawn, results in removal from the

cell cycle. CyclinE induction by E2F correlates with the passing of the restriction point in late G1 that commits the cell to divide beyond the control of mitogenic signals.

Given its crucial role in initiating cell cycle progression, the cyclinD/cdk4/6 complex is frequently mutated in many human cancers. CyclinD itself is overexpressed in many human cancers as a result of gene amplification (Sherr, 1996). The cyclinD gene, located at position 13 on the long arm of chromosome 11 (11q13), is amplified in a variety of tumours including squamous cell carcinomas of the head and neck, esophageal carcinomas, bladder cancer and primary breast carcinomas (Hall and Peters, 1996). Work by Wang *et al.* (1994) showed that the targeted overexpression of cyclinD in mammary epithelial cells in mice led to tumour formation. This work suggests that cyclinD directly contributes to oncogenesis.

The cdks that cyclinD binds and activates, cdk4 and cdk6, are also misregulated in many types of human cancer. Cdk4 is amplified and overexpressed in a range of tumours including gliomas (Schmidt *et al.*, 1994; Sonoda *et al.*, 1995), sarcomas (Khatib *et al.*, 1993; Wunder *et al.*, 1999) and breast cancers (An *et al.*, 1999). In lymphoid tumours (Chilosi *et al.*, 1998), gliomas and squamous cell carcinomas (Costello *et al.*, 1997) the cdk6 gene is amplified, whilst point mutations in both cdk4 and cdk6 have been reported in melanomas (Zuo *et al.*, 1996) and neuroblastoma cell lines (Easton *et al.*, 1998). Such point mutations lead to the

inability of the cdks to bind to their inhibitors, the INK4 family (Ranade *et al.*, 1995).

CyclinE, and its catalytic partner, cdk2, have both been identified as having a role in tumourigenesis. Overexpression of cyclinE and cdk2 has been reported in colorectal carcinomas (Kitahara *et al.*, 1995) whilst cyclinE amplification has also been observed in bladder cancer (Richter *et al.*, 2000), esophageal adenocarcinomas (Lin *et al.*, 2000) and non-small cell lung cancers (Mishina *et al.*, 2000).

Whilst the cyclin/cdk complexes positively regulate cell cycle progression, the cyclin dependent kinase inhibitors (cdkis) act to prevent cell cycle progression. The cdkis comprise two families: the INK4 family (INhibitors of cyclin dependent Kinase 4) and the Cip/Kip family. The INK4 family consists of $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$ and $p19^{INK4d}$ (Sherr and Roberts, 1999). This group specifically inhibits the actions of the cyclinD dependent kinases, cdk4 and cdk6. The Cip/Kip family of cdkis are more general acting and are able to prevent the catalytic activity of the cyclinD-, E- and A-dependent kinases. This family consists of $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$ (Sherr and Roberts, 1999).

Not surprisingly, given their intricate role in the negative regulation of the cyclin/cdk complexes, the frequency of mutations within the cdkis is extremely high within the context of cancer. Homozygous deletions of the $p16^{INK4a}$ locus have been found in gliomas, mesotheliomas, acute lymphocytic leukemias and sarcomas

(Sherr, 1996). $p16^{INK4a}$ function is also lost in many sporadic cancers (Ruas and Peters, 1998). Surprisingly, work by Zindy *et al.* (2000) revealed that the disruption of both $p15^{INK4b}$ and $p19^{INK4d}$ did not result in tumourigenesis in mice however $p18^{INK4c}$ deletion in mice causes pituitary tumours (Franklin *et al.*, 1998).

In contrast to the INK4 family, the Cip/Kip family does not appear to be mutated to the same degree in human cancers. Since neither copy of the gene was found to be deleted in tumours, the Kip gene was not thought to act as a tumour suppresser. However, work by Fero *et al.* (1998) has shown that the gene need only be monoallelic to contribute to neoplasia. This is unlike most tumour suppressers, which are usually homozygously inactivated. Indeed, reduced $p27^{Kip1}$ levels are observed in primary breast cancer (Catzavelos *et al.*, 1997).

1.1.3 The Rb/E2F Pathway in Cancer

The E2F protein was the first cellular protein identified that bound to the retinoblastoma protein (pRB) (Bagchi *et al.*, 1991; Chittenden *et al.*, 1991; Bandara and La Thangue, 1991; Helin *et al.*, 1992; Kaelin *et al.*, 1992). There are six members of the E2F family (E2F1-6) and in a heterodimeric complex with a member of the DP family (DP1-3), E2F can transcriptionally activate genes with an E2F site. Such genes are essential for DNA synthesis and cell cycle progression.

E2F activity is primarily regulated by the pocket proteins. This family consists of pRB, p107 and p130. E2F/DP heterodimers guide the pocket proteins to E2F binding site-containing promoters where they form complexes with proteins that can induce the down-regulation of E2F transactivation by: (1) the direct shielding of the E2F transactivation domain; and (2) the modification of the chromatin environment (Muller and Helin, 2000).

As described above, the sequential phosphorylation of the pocket proteins by the cyclin/cdk complexes leads to the hyperphosphorylation of the pocket proteins and the release of E2F. It has now been firmly established that the $p16^{INK4a}$, cyclinD, cdk4, pRB pathway (Figure 1.1B) is frequently mutated in human cancers. The biochemical connection between these molecules as well as the fact that the deregulation of one component often alleviates the need for deregulation of another has led to the assumption that the mutation of this pathway is a prerequisite for oncogenesis (Muller and Helin, 2000). Interestingly, tumours carrying pRB mutations do not tend to exhibit mutations in any of the other components of this pathway.

Inactivation of pRB is frequently observed in tumour development. pRB mutation is commonly seen in small-cell carcinomas of the lung (Hall and Peters, 1996). Somatic mutations in pRB also contribute to osteosarcomas, renal cell carcinomas and bladder carcinomas (Hickman *et al.*, 2002). Although work by Suzuki *et al.* (1999) pointed to a role for E2F1 amplification in gastric and colorectal

carcinomas, E2F is not a frequent target for deletion or amplification in human cancers. Presumably, E2F deletion or amplification does not provide tumours with a significant proliferative advantage.

1.1.4 The ARF/p53 Pathway in Cancer

The INK4a gene encodes a second potent tumour suppresser named $p19^{ARF}$ ($p14^{ARF}$ in humans) (Quelle *et al.*, 1995). This gene is located on the short arm of chromosome 9 (9p21). The transcripts encoding these distinct genes are driven by two independent promoters and encompass a unique first exon (exon 1α and exon 1β) followed by two common exons, E2 and E3. These exons are translated using different open reading frames and hence, the complete lack of amino acid homology between $p16^{INK4a}$ and $p14^{ARF}$ (Figure 1.1C) (Ortega *et al.*, 2002).

ARF can block the actions of MDM2 (HDM2 in humans) by preventing the degradation of its primary target, the p53 tumour suppresser protein (Ortega *et al.*, 2002). p53 is a transcription factor that induces cell cycle arrest or apoptosis, depending on the biological setting (Sherr, 2000). MDM2 is a proto-oncogene product that neutralises the actions of p53 by binding to, and inhibiting, its transactivation domain (Momand *et al.*, 1992; Oliner *et al.*, 1992).

The introduction of ARF into cells causes cell cycle arrest in a p53-dependent fashion, suggesting that ARF acts upstream of p53 (Kamijo *et al.*, 1997). Cells lacking p53 have elevated levels of ARF and reintroduction of p53 lowers ARF levels (Kamijo *et al.*, 1998; Stott *et al.*, 1998). This suggests that p53 controls the level of ARF by a negative feedback mechanism. By blocking the effects of MDM2, ARF can allow p53 transcription (Kamijo *et al.*, 1998; Stott *et al.*, 1998), prevent p53 ubiquination (Honda and Yasuda, 1999) and block p53 export to the cytoplasm (Tao and Levine, 1999; Zhang and Xiong, 1999).

Work by Bates *et al.* (1998) showed that E2F1 could induce the expression of $p14^{ARF}$. Following the release of E2F activity from RB, E2F1 upregulates ARF expression which leads to the inhibition of MDM2 activity on p53. p53 stabilisation then leads to the targeting of genes involved in either cell cycle arrest or apoptosis. p21, a p53 target, can induce hypophosphorylated RB which then inhibits E2F activity. In this way, ARF links the RB/E2F pathway with p53 (Figure 1.1D).

The p53 gene is the most frequently mutated gene in human cancer (Nigro *et al.*, 1989; Levine *et al.*, 1991; Greenblatt *et al.*, 1994). The nature of these genetic changes in cancer cells is most commonly a mis-sense mutation in one allele, producing a faulty protein that is then observed at high concentrations in these cells, followed by a reduction to heterozygosity (Levine, 1997). Around one third of sarcomas inactivate p53 by the amplification of MDM2 (Oliner *et al.*, 1992). ARF mutation is also frequently observed in human cancers. Work by Kamijo *et al.*

(1999) showed that mice lacking ARF are prone to sarcomas, lymphomas, carcinomas and tumours of the central nervous system whilst inactivation of the ARF promoter by methylation occurs in carcinomas and adenomas of the colon (Robertson *et al.*, 1998; Esteller *et al.*, 2000).

1.1.5 The Apoptotic Pathway in Cancer

Apoptosis, or programmed cell death, is a physiological process for killing cells that is critical for the correct development of multicellular organisms. Apoptosis is subject to genetic control (Ellis and Horvitz, 1986; Vaux *et al.*, 1988) and abnormalities in the process can lead to a variety of diseases including cancer (Strasser *et al.*, 1990; McDonnell and Korsmeyer, 1991). Molecular changes induced during apoptosis include internucleosomal DNA cleavage and randomisation of the distribution of phosphatidyl serine (PS) between the inner and outer leaflets of the plasma membrane. Morphological changes include chromatin condensation, cytoplasmic shrinkage and plasma blebbing (Strasser *et al.*, 2000).

Distinct pathways that lead to apoptosis all converge on the activation of the caspases. These are a family of cysteine proteases that cleave substrates on the carboxyl side of an aspartate residue. The caspase family are essential for programmed cell death in all cells, regardless of their origin or the death stimulus (Strasser *et al.*, 2000). Initiator caspases such as -8 and -9 induce an increase in

caspase activity by activating the effector caspases-3, -6 and -7 (Nicholson and Thornberry, 1997). The effector caspases then cleave essential cellular proteins such as DNA repair enzymes, MDM2 and protein kinase δ (Nicholson and Thornberry, 1997; Thornberry and Lazebnik, 1998).

Cells can initiate apoptosis via two distinct mechanisms (Figure 1.1E). The first involves the receptor-mediated death-signalling pathway that triggers the activation of caspase-8 by cleaving its precursor, pro-caspase-8. This occurs through the interaction of members of the tumour necrosis factor (TNF) ligands with their receptors (TNF-R). CD95 (also called Fas or APO-1) as well as other members of this family are referred to as the death receptor family (Strasser *et al.*, 2000).

Activated caspase-8 then causes the cleavage, and activation, of the effector pro-caspases-3, -6 and -7. The second pathway is via the release of cytochrome c from the mitochondria via the actions of either caspase-8 or the Bcl-2 family. Cytochrome c interacts with both pro-caspase-9 and Apaf-1, creating the apoptosome. This causes the cleavage of pro-caspase-9 to caspase-9, which can then cleave, and activate, the effector caspases. Members of the Bcl-2 family heavily influence this second pathway (Rich *et al.*, 2000).

The Bcl-2 family consists of both pro-and anti-apoptotic members that focus much of their efforts at the mitochondria, and they play a pivotal role in deciding whether a cell will live or die (Gross *et al.*, 1999). The family consist of Bcl-xs, Bad, Bak and Bax that stimulate apoptosis and Bcl-2, MCL-1 and Bcl-x_L that inhibit the process (van Hemert., 2001). Both Bcl-2 and Bcl-x_L are bound, and inactivated, by Bad (Yang *et al.*, 1995; Mok *et al.*, 1999) which leads to cytochrome c release and cell death.

Indeed, work by Oltvai *et al.* (1993) suggested that the ratio between the pro-apoptotic and anti-apoptotic members of the family helped to determine the susceptibility of cells to death signals. The anti-apoptotic members of the family are found as integral membrane proteins, localised to the membranes of the mitochondria, endoplasmic reticulum and the nucleus (Krajewski *et al.*, 1993). In contrast, a substantial fraction of the pro-apoptotic members are found localised to the cytosol or the cytoskeleton prior to a death signal (Gross *et al.*, 1999).

Cell death signals lead to the activation of the pro-apoptotic members of the family (Zha *et al.*, 1996) which then inactivate the mitochondria-bound anti-apoptotic members. These death signals can be extra-cellular, such as interleukin-3 deprivation, or intra-cellular such as p53 transcription of pro-apoptotic target genes (Gross *et al.*, 1999). This leads to cytochrome c release, the formation of the apoptosome, the activation of the effector caspases and, ultimately, cell death.

With such potent mechanisms in existence to obliterate displaced cells, it is no surprise that suppression of apoptosis is high on the list of acquired attributes in cancer cells (Evan and Vousden, 2001). The survival signals, insulin-like growth factor (IGF)-1 and IGF-2, are known to be mutated in human cancers (Yu and Rohan, 2000) whilst both Bcl-2 and Bcl-x_L, members of the anti-apoptotic family of Bcl-2, are overexpressed in several tumour types (Evan and Vousden, 2001). Work by Soengas *et al.* (2001) also identified loss of Apaf-1 in malignant melanomas.

Figure 1.1A: The Mammalian Cell Cycle

Gap1 (G1), Synthesis-phase (S-phase), Gap2 (G2) and Mitosis (M) are the traditional subdivisions of the cell cycle. G1, S-phase and G2 constitute the interphase portion of the cell cycle whilst mitosis involves separation of the duplicated DNA and finally, cell division. G1 and G2 provide additional time for the cell to grow, S-phase involves duplication of the cells DNA whilst during mitosis the cells microtubules form the spindles that separate the duplicated chromosomes. Cells can enter a resting state called G0 if they have not committed to duplicating their DNA. They can remain here indefinitely until they decide to re-enter the cell cycle. Up until the restriction point the cell is under the control of mitogenic growth factors. After this point in late G1 the cell is committed to dividing unless cellular insults occur. If this happens the cell can initiate a cell cycle arrest at either the G1/S or G2/M boundaries. Red lines indicate an inhibitory effect. The figure is adapted from Alberts *et al.* (1994), Figure 17-3.

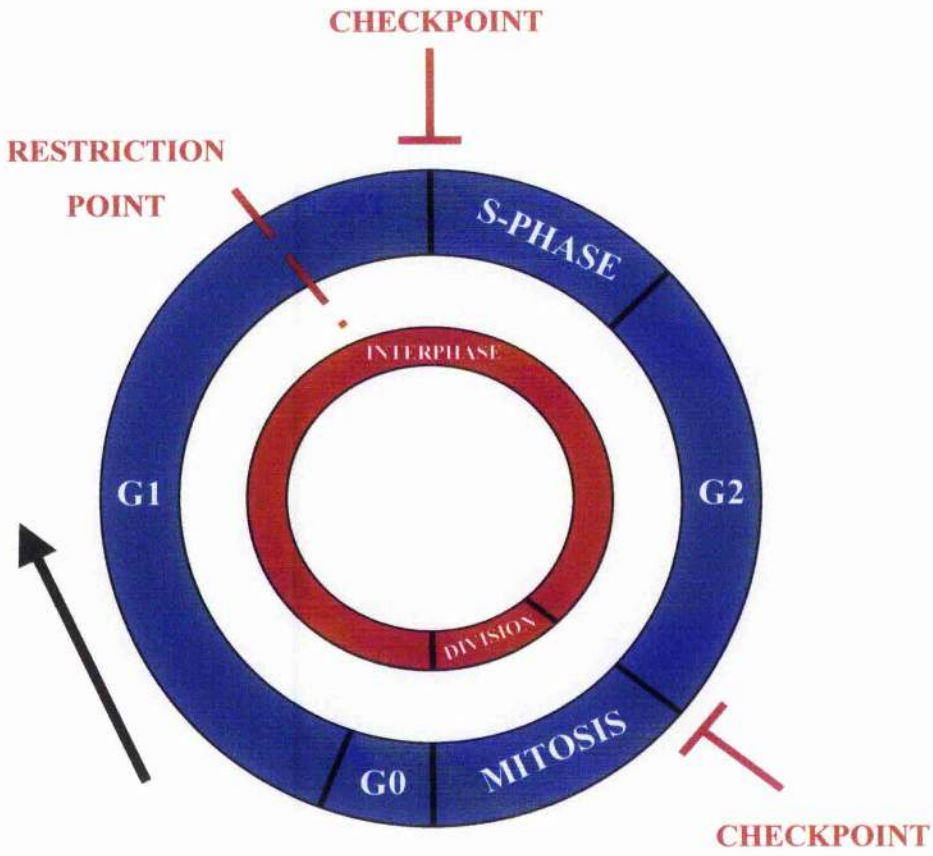

CHECKPOINT
RESTRICTION
POINT
S-PHASE
INTERPHASE
G1
G2
DIVISION
G0
MITOSIS
CHECKPOINT

Figure 1.1B: The pRB/E2F Pathway

Mitogenic stimulation induces the expression of the D-type cyclins which then complex with cdk4/6. This causes an induction in E2F activity, and subsequent S-phase entry, through the phosphorylation of pRB. The cyclin-dependent inhibitors (ckis) can block the induction of E2F by preventing the activity of the cdks. Analysis of human tumours has shown that deregulation of only one portion of the pathway is enough to confer neoplastic potential. Blue lines indicate stimulatory effect; red lines indicate inhibitory effect. The figure is adapted from Nevins, (2001), Figure 1.

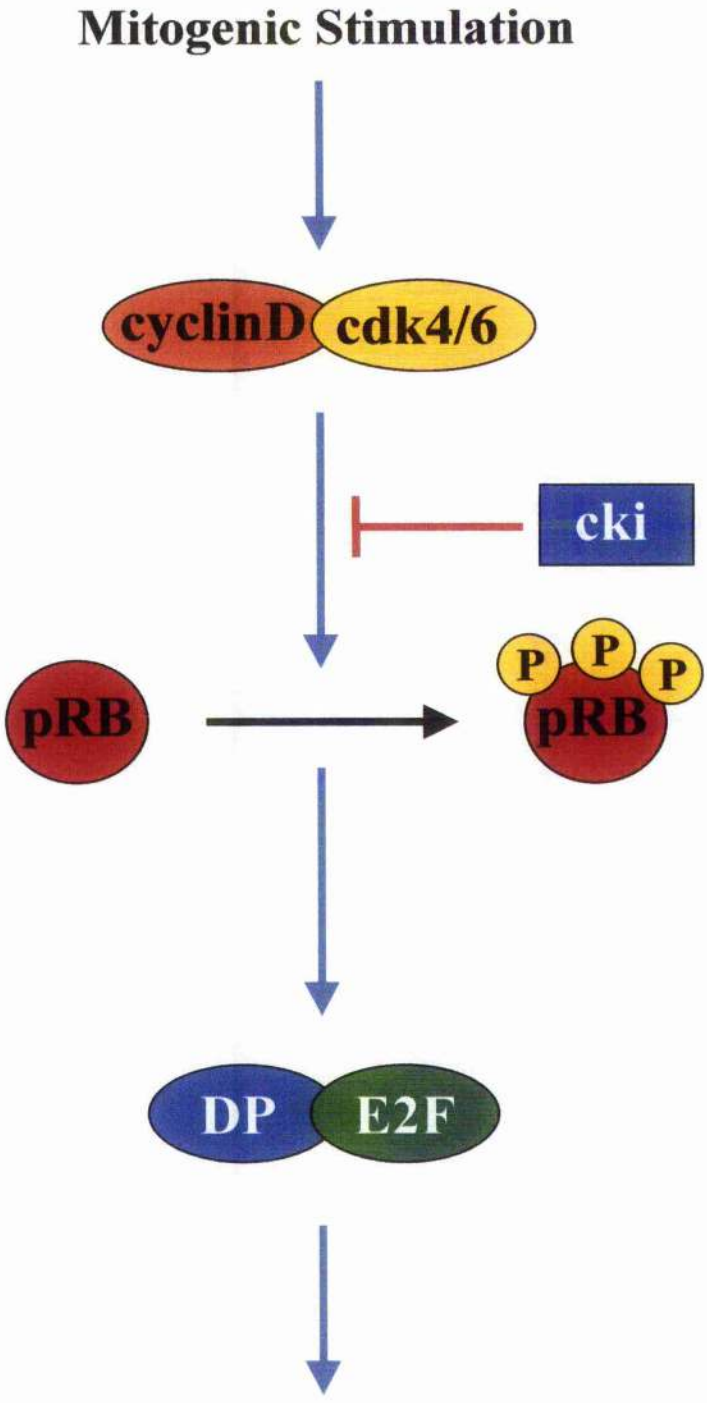

Mitogenic Stimulation
cyclinD
cdk4/6
cki
P
P
P
pRB
pRB
DP
E2F
S-Phase Induction

Figure 1.1C: The ARF-INK4a Gene

The transcripts encoding these distinct genes are driven by two independent promoters and encompass a unique first exon (exon 1α and exon 1β) followed by two common exons, E2 and E3. These exons are translated using different open reading frames and hence, the complete lack of amino acid homology between $p16^{INK4a}$ and $p14^{ARF}$. Common mutations in human cancers are indicated with red arrows whilst promoter methylation sites are shown as blue circles. The figure is adapted from Ortega *et al.* (2002), Figure 4.

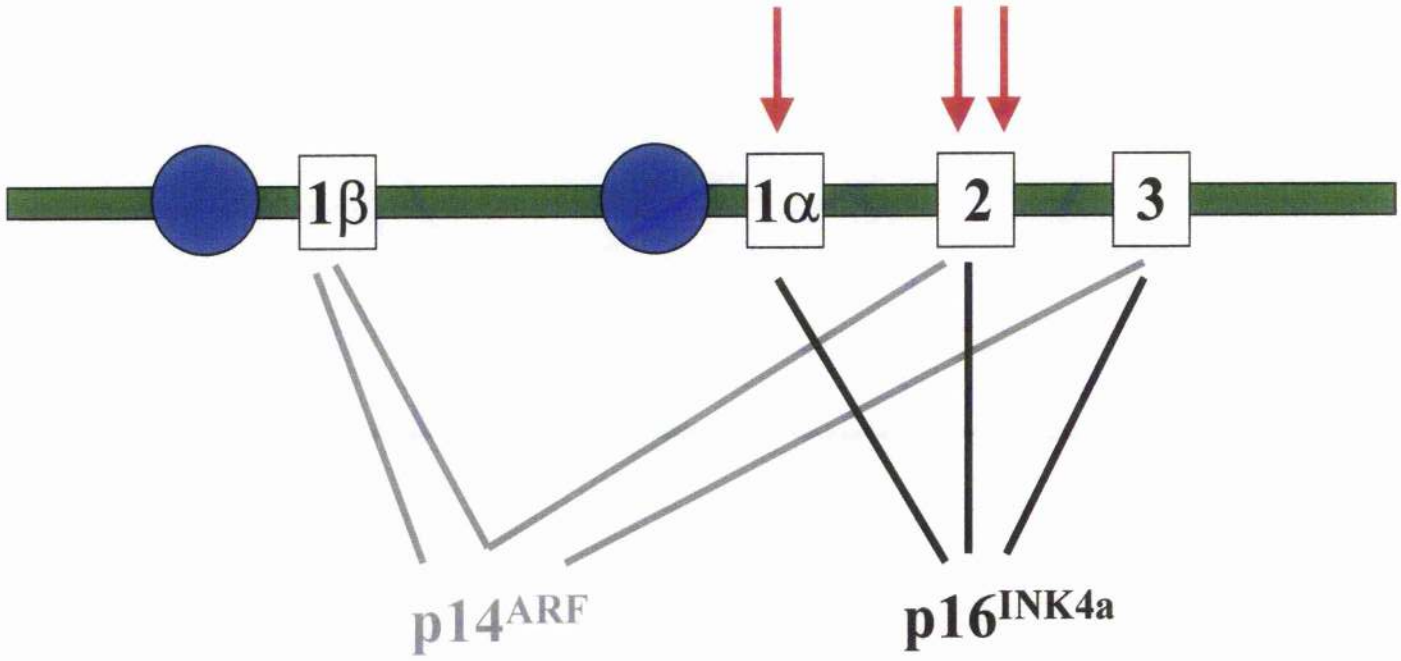

1β
1α
2
3
p14ARF
p16INK4a

Figure 1.1D: $\underline{p14^{ARF}\text{ Links E2F With p53}}$

Following the induction of E2F1, $p14^{ARF}$ is upregulated. $p14^{ARF}$ can subsequently block the negative activity of MDM2 on p53. This leads to the stabilisation of p53 and either an arrest in the cell cycle or the induction of apoptosis. The up-regulation of p21, a p53 target gene, leads to the inhibition of the cyclin/cdk complexes and the subsequent inhibition of E2F activity. Blue lines indicate stimulatory effect; red lines indicate inhibitory effect. The figure is adapted from Nevins, (2001), Figure 2.

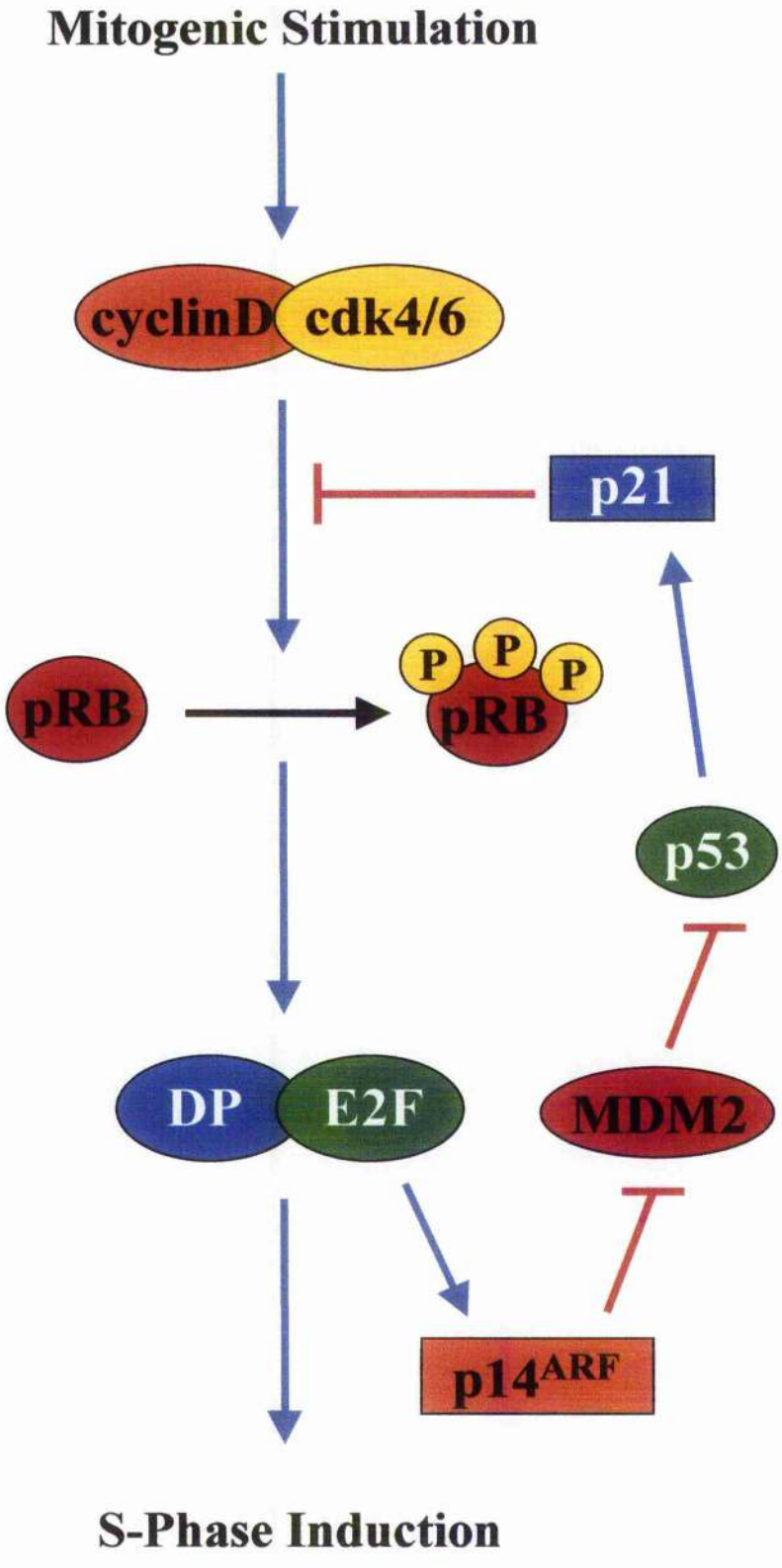
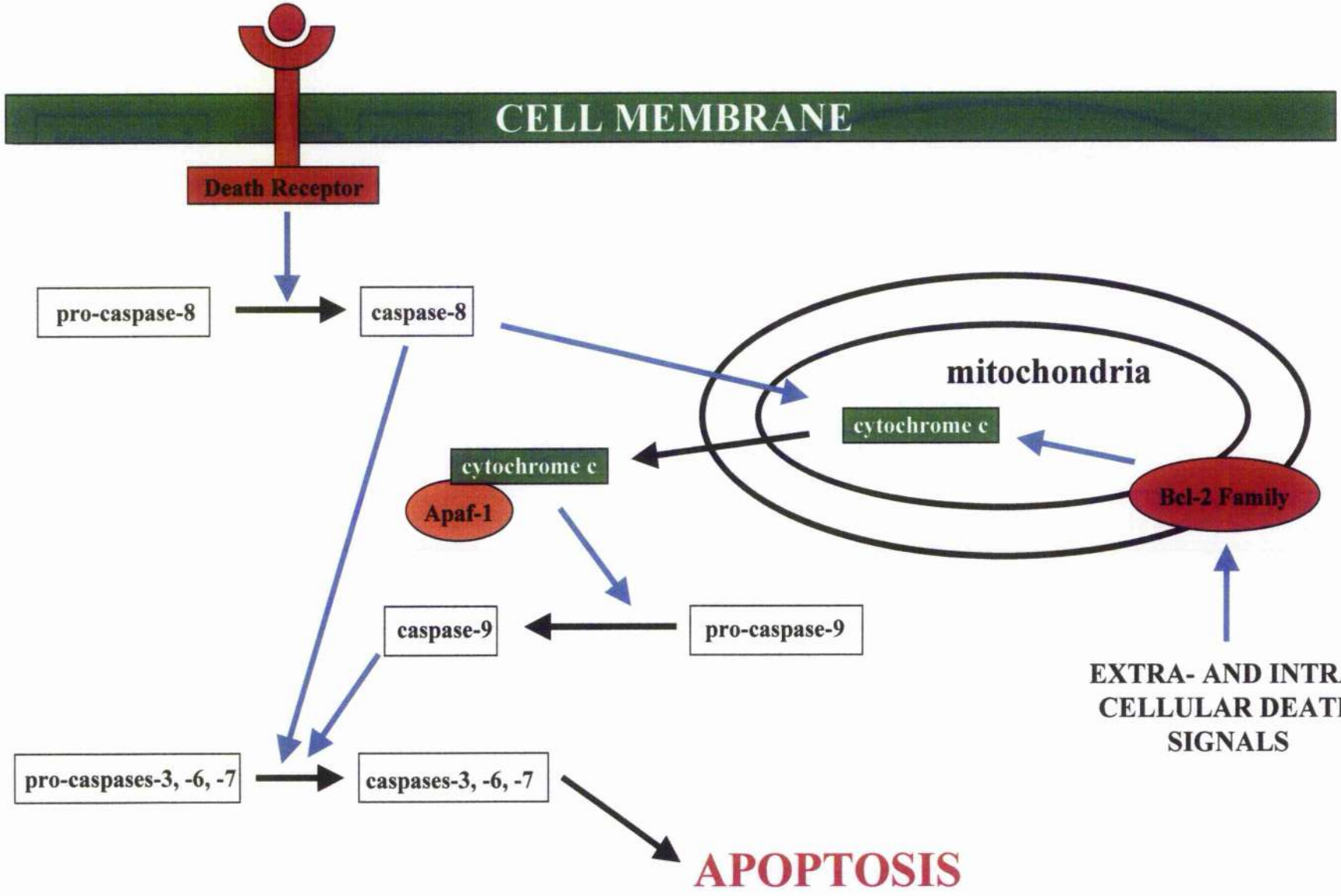

Mitogenic Stimulation
cyclinD
cdk4/6
p21
P
P
P
pRB
pRB
p53
DP
E2F
MDM2
p14ARF
S-Phase Induction

Figure 1.1E: Cells can initiate apoptosis via two distinct mechanisms

The initiation of the apoptotic response can occur through two different pathways. The first involves the receptor-mediated death-signalling pathway that triggers the activation of caspase-8 by the cleavage of its precursor, pro-caspase-8. Caspase-8 can then directly initiate apoptosis by the activation of effector caspases, caspase-3, -6 and -7. The mitochondrial pathway involves the release of cytochrome c by the effects of caspase-8 or the actions of the Bcl-2 family. Cytochrome c interacts with both pro-caspase-9 and Apaf-1, creating the apoptosome. This causes the cleavage of pro-caspase-9 to caspase-9, which then induces the activation of the effector caspases. Blue lines indicate a stimulatory effect. The figure is adapted from Gross *et al.* (1999), Figure 3.


CELL MEMBRANE
Death Receptor
pro-caspase-8
caspase-8
mitochondria
cytochrome c
cytochrome c
Apaf-1
Bcl-2 Family
caspase-9
pro-caspase-9
EXTRA- AND INTRA-CELLULAR DEATH SIGNALS
pro-caspases-3, -6, -7
caspases-3, -6, -7
APOPTOSIS

1.2: The E2F Proteins

1.2.1: Introduction

In mammalian cells the E2F family of transcription factors plays a crucial role in the regulation of genes that are involved in controlling cell proliferation, DNA synthesis and apoptosis (Figure 1.2A). Work by Kovesdi *et al.* (1986) identified E2F as a protein that was able to bind to, and activate, the adenoviral E2 gene promoter due to the actions of the adenoviral E1A product (DeGregori, 2002). La Thangue and Rigby, (1987) then identified a protein with the same consensus DNA-binding site as E2F that was down regulated in F9 (EC) stem cells during differentiation. They called this DRTF1 (differentiation-regulated transcription factor 1). DRTF1 and E2F were subsequently identified as being the same protein.

The E2F family consists of six E2F members (E2Fs 1-6) that form heterodimers with a DP partner (DPs 1-3) (Helin *et al.*, 1992; Ivey-Hoyle *et al.*, 1993; Lees *et al.*, 1993; Beijersbergen *et al.*, 1994; Ormondroyd *et al.*, 1995; Zhang and Chellappan., 1995; Rogers *et al.*, 1996; Morkel *et al.*, 1997; Cartwright *et al.*, 1998; Trimarchi *et al.*, 1998) [Figure 1.2B(i)]. E2Fs 1-3 are traditionally seen as the 'activating' E2Fs with E2Fs 4-6 seen as being 'repressive' E2Fs [Figure 1.2B(ii)]. E2F6 is unique since it does not contain a transcriptional activation (*trans*-activation) domain (Trimarchi *et al.*, 1998). The E2F portion of the family recognises, and is able to bind to, the DNA promoter sequence -TTTCGCGC- (Lees *et al.*, 1993; Buck *et al.*, 1995; Zhang and Chellappan., 1995). The DP partner offers co-operative binding to the DNA (Dyson, 1998). In the literature DP2 refers to the human form and DP3 to

the mouse form of what is recognised as being the same protein. Work by Rogers *et al.* (1996) identified human DP2 as an E2F binding protein from a yeast two-hybrid screen of a HeLa cell library. DP3 was identified from a murine library. The protein consists of four splice variants of the same gene, termed α, β, γ and δ, determined by their various N-terminal organisations (Ormondroyd *et al.*, 1995) [Figure 1.2B(i)]. The α and δ splice variants of DP2/3 are nuclear due to an N-terminal bi-partite NLS whereas β and γ are cytoplasmic since they lack the NLS (de La Luna *et al.*, 1996). *In vitro* at least, any DP member can form a complex with any E2F, suggesting a potentially wide array of E2F/DP complexes *in vivo* (Dyson, 1998).

E2F can induce the expression of genes whose protein products are essential for DNA synthesis such as dihydrofolate reductase, thymidylate synthase and thymidine kinase (DeGregori, 2002). E2F is also able to transcriptionally activate a wide variety of genes involved in the cell cycle including cyclinE, cyclinA, cdc25A and the retinoblastoma protein pRB (DeGregori, 2002). Its association with pRB inhibits E2F activity.

1.2.2: The Role of E2F as a Transcription Factor

Although E2F and many other sequence-specific transcription factors are essential for the processing of DNA into mRNA, it requires many more protein factors for effective transcriptional control. Indeed, mRNA synthesis by RNA polymerase II (RNA pol II), is dependent on a wide array of additional factors including TFIIA,

B, D, E, F and H which, together, form the pre-initiation complex (PIC). These general transcription factors (GTFs) also assist in DNA unwinding as well in the transition from transcription initiation to RNA transcript elongation (Naar *et al.*, 2001).

This apparatus, referred to as the basal transcriptional machinery, is sufficient to mediate basal transcription. However, high levels of regulated, gene selective transcriptional activity additionally requires the action of sequence-specific activators such as E2F (Naar *et al.*, 2001). E2F is able to bind to the general transcription factor TFIID, which binds to the TATA nucleotide sequence found in many eukaryotic promoters (Horikoshi *et al.*, 1988).

TFIID is comprised of the TATA binding protein (TBP) and other TBP associated factors (TAFs) which act as adapters to bridge the gap between sequence-specific transcription factors such as E2F, and the basal transcriptional machinery. The multi-subunit TFIID works together with TFIIA and TFIIB to assemble the initiation complexes at eukaryotic promoters (Andel *et al.*, 1999). The GTFs and then RNA pol II can then bind to the DNA and the downstream initiator element. Figure 1.2C highlights the organisation of the PIC.

1.2.3: The pRb/E2F Association

pRB was the first tumour suppressor to be identified and it is absent or mutated in at least one third of human cancers (Trimarchi and Lees, 2002). pRB is the prototype member of a family of proteins, referred to as the pocket proteins, that includes p107 and p130 (Stevens and La Thangue, 2003). pRB preferentially binds E2Fs1-3 with p107 and p130 preferentially binding E2F4 and E2F5 respectively (Ginsberg *et al.*, 1994; Vairo *et al.*, 1995). Work by Qin *et al.* (1992) showed that the transcriptional activity of E2F was inhibited by its association with pRB and that this resulted in cell cycle arrest at G1.

The pocket domain of pRB (amino acids 395-876) interacts with an eighteen amino acid sequence within the C-terminal *trans*-activation domain of E2F, blocking E2F's transcriptional activity (Helin *et al*, 1993). This allows pRB to block E2F site-dependant *trans*-activation without affecting the DNA binding ability of E2F (Zamian and La Thangue, 1993; Helin *et al.*, 1993). Work by Hateboer *et al.* (1996) and Hofmann *et al.* (1996) showed that pRB binding protects E2F from degradation by masking potential ubiquination sites within the *trans*-activation domain of the protein.

Physiologically, three types of E2F complex can exist (Figure 1.2D). The first contains 'free' E2F as a result of hyperphosphorylated pocket proteins. This type of complex is a potent activator of target genes and cell cycle progression. The second type of E2F complex exists as an inhibitor complex. Hypophosphorylated pocket

proteins bind to, and prevent, E2F activity thereby acting as an inhibitor of E2F-responsive genes. Recent work by Brehm *et al.* (1998) and Dahiya *et al.* (2000) has now shown that pRB can also actively recruit HDACs (Histone DeACetylases). The HDACs are a family of enzymes that remove acetyl groups from lysine residues in the amino terminus of histones, thereby influencing the chromatin structure of target genes. The hypoacetylation of lysine residues by HDACs then leads to the methylation of these residues by histone methyltransferases (HTMases) such as SUV39H1. pRB-dependent transcriptional repression also involves recruitment of polycomb group (PcG) protein-containing complexes which can induce changes in chromatin packaging (DeGregori, 2002). pRB can also recruit BRG1 and HBRM, two ATPase components of the human SWI/SNF chromatin-remodelling complex. This results in nucleosome remodelling. This third type of E2F complex can therefore exist as an active repressor complex that influences long term gene silencing.

1.2.4: The Regulation of E2F Activity

During G0 and early G1 pRB is hypophosphorylated which results in E2F binding. Extra-cellular mitogenic growth factors then cause the activation of the cell-cycle-dependant kinase complexes cyclinD/cdk4/6 which phosphorylate pRB, causing the release of E2F. E2F can then induce the transcription of the cyclinE gene, which forms a complex with cdk2 causing a further increase in hyperphosphorylated pRB. This creates more 'free' E2F which drives the cell through the G1/S boundary, into

the DNA replication phase. Up until the expression of the cyclinE/cdk2 complex the cell is under the control of the mitogenic factors and can exit the cell cycle at any time. Upon the expression of the cyclinE/cdk2 complex the cell passes a restriction point in late G1 after which it is committed to undergo DNA replication and is no longer sensitive to growth factor signalling (Trimarchi and Lees, 2002). After this point the cell cycle can only be halted through cellular insults such as DNA damage. As mentioned above, the activity of the cyclin/cdk complexes can be blocked by the cyclin dependent kinase inhibitors (cdis), which results in hypophosphorylated pRB and inactive E2F. Figure 1.2E provides an overview of the actions of the cyclin/cdk complexes and cdkis on E2F function throughout the cell cycle.

The importance of pRB *in vivo* is highlighted by the fact that pRB-deficient mice die between embryonic day (E) 13.5 and 15.5 with defects in fetal liver haematopoiesis, neurogenesis and lens development that results from a combination of inappropriate cell cycle entry and apoptosis (Trimarchi and Lees, 2002). In contrast, $p107^{-/-}$ and $p130^{-/-}$ mice survive to term and show no increase in the incidence of tumour formation. This suggests that both p107 and p130 act independantly of pRB. However, $p107^{-/-}/p130^{-/-}$ mice are embryonic lethal which suggests functional redundancy between these two proteins (Stevens and La Thangue, 2003).

As well as the level of control that the pocket proteins exert over E2F, other factors can impinge on E2F activity. Whereas the cyclinD/cdk4 and cyclinE/cdk2 complexes have a positive influence on E2F activity, the cyclinA/cdk2 complex has a negative effect. During S-phase cyclinA/cdk2 binds to E2F1. This results in the phosphorylation of DP1 causing a loss of E2F DNA binding affinity (Krek *et al.*, 1995). Work by Xu *et al.* (1994) and Guida and Zhu, (1999) showed that the cyclinA/cdk2 complex could phosphorylate E2F1 at serine-375 resulting in a reduced DNA binding ability of E2F1.

To allow correct S-phase induction, E2F activity must be tightly regulated. This is achieved through the ubiquitin protein ligase SCF^{SKP2} (Marti *et al.*, 1999). During S-phase a component of the SCF complex, $p45^{SKP2}$, binds to E2F1 and promote its degradation via the ubiquination process (Marti *et al.*, 1999). Recent work by Lin *et al.* (2001) showed that this effect could be prevented by the DNA damage responsive elements ATM/ATR. These kinases can phosphorylate serine-31 in E2F1 which prevents the binding of $p45^{SKP2}$ and the subsequent degradation of E2F1 (Lin *et al.*, 2001).

Upon dissociation of pRB, E2F1 becomes the target of the p300/CBP family of coactivator proteins, which bind in the C-terminal of the protein (Trouche *et al.*, 1996). This family has an intrinsic histone acetyl transferase (HAT) activity. Work by Martinez-Balbas *et al.* (2000) showed that the p300/CBP-associated factor

(P/CAF) could acetylate E2F1 within its DNA binding domain. This activity enhanced the stability as well as the DNA binding activity of E2F1.

1.2.5: The p53/E2F Pathway

The p53 protein plays a key role in cellular decisions to either arrest the cell cycle, allowing the repair of damaged DNA, or to commit to cell death (Sears and Nevins, 2002). The accumulation of p53 is negatively regulated by the E3-ligase, MDM2 (Sherr and Weber, 2000). The activity of MDM2 is negatively regulated by $p14^{ARF}$ (Sherr and Weber, 2000) which is a direct target for E2F1 (DeGregori *et al.*, 1997). Stabilisation of p53 results in the transcriptional activation of p53 target genes involved in blocking cell cycle progression, including p21 (el-Deiry *et al.*, 1993). p21 is able to block the activity of cyclinD/cdk4, cyclinE/cdk2 and cyclinA/cdk2 (Levine, 1997). This then leads to hypophosphorylated pRB and inactivation of E2F activity. The induction of $p14^{ARF}$ by E2F1 and the subsequent expression of p21, via p53, highlight how the pRb/E2F pathway directly connects to p53.

1.2.6: The 'Activating' E2Fs

Recent work by Wu *et al.* (2001) has shown that E2Fs 1-3 are essential for cellular proliferation since the combined ablation of these proteins prevents S-phase entry. Work by Lukas *et al.* (1996) showed that overexpression of these E2Fs could overcome the growth inhibitory effects of p16.

E2F1 was originally cloned due to its ability to bind pRB (Helin *et al.*, 1992; Kaelin *et al.*, 1992; Shan *et al.*, 1992). E2F1 was subsequently shown to bind to DNA in a DP-dependant manner, and the resulting complex was shown to be a potent transcriptional activator of E2F-responsive promoters (Trimarchi and Lees, 2002). Subsequent work by Ivey-Hoyle *et al.* (1993) and Lees *et al.* (1993) identified E2F2 and E2F3. Both E2F2 and E2F3 are highly homologous to E2F1 within the area of the DNA binding, DP dimerisation and pRB binding domains. E2Fs 1-3 preferentially bind to pRB and all are able to potently induce the expression of genes involved in the cell cycle and apoptosis. As such, E2Fs 1-3 have been classified as 'activating' E2Fs.

In normal cells the 'activating' E2Fs are specifically targeted and inhibited by pRB but not by p107 or p130. The release of E2Fs 1-3 from pRB occurs at mid-late G1 and this activation correlates closely with the activation of E2F target genes and the potent induction of S-phase. Indeed the functional inactivation of pRB in either embryonic tissues or tumours induces inappropriate proliferation (Trimarchi and Lees, 2002).

The overexpression of E2F1, E2F2 or E2F3 is sufficient to cause quiescent cells to re-enter the cell cycle (Johnson *et al.*, 1993; Qin *et al.*, 1994; Lukas *et al.*, 1996). Work by Helin *et al.* (1992) and Lees *et al.* (1993) showed that E2Fs 1-3 were potent transcriptional activators of E2F-responsive promoters. As well as having a critical role in proliferation, E2Fs 1-3 have potent apoptotic effects as well, mediated through both p53-dependant and p53-independent pathways (Qin *et al.*, 1994; Shan and Lee, 1994; Hsieh *et al.*, 1997; Phillips *et al.*, 1999). The induction of apoptosis via E2F usually requires the transcriptional activity of the molecule however there is still considerable debate over the pathways that are responsible (Trimarchi and Lees, 2002).

With regards the p53-dependant pathway, the current model is that E2F1 induces cell death via the up-regulation of the ARF gene which is a known E2F target gene (DeGregori *et al.*, 1997; Bates *et al.*, 1998). $p14^{ARF}$ can target the inhibitory effects of MDM2 on p53 resulting in the stabilisation of p53 and the up-regulation of p53 pro-apoptotic genes such as Bax (Sherr and Weber, 2000). Recent work has now pointed to the fact that E2F1 can induce apoptosis in a p53-dependant manner in the absence of $p19^{ARF}$ (Tolbert *et al.*, 2002). This indicates alternative mechanisms for E2F1 induced apoptosis via p53.

Recent work by Irwin *et al.* (2000) has shown that E2F1 can induce the expression of the p53 homologue, p73. This points towards a role for E2F in p53-independent apoptosis. Indeed the Apaf1 gene, which is involved in cytochrome c release from

the mitochondria has also now been identified as a transcriptional target for E2F1 (Moroni *et al.*, 2001). As well as a transcriptional role for E2F induced apoptosis there may be active repression by E2F on survival signals such as the tumour-necrosis factor receptor (TNFR) (Phillips *et al.*, 1999).

Despite this there is still some debate as to whether E2Fs 2 and 3 play as significant a role in apoptosis as E2F1. Work by Degregori *et al.* (1997) suggested that the induction of apoptosis was a specific property of E2F1 however Ziebold *et al.* (2001) showed that mutation of E2F3 in pRB-deficient embryos was sufficient to suppress not only cellular proliferation but also p53-dependant and p53-independant apoptosis. Indeed this group showed that the degree of suppression exceeded that seen when E2F1 alone was lost, suggesting that E2F3 can induce apoptosis independently of E2F1. It therefore remains to be seen whether only E2F1 can induce apoptosis or whether indeed either, or both, E2F2 and E2F3 are equally as potent at inducing cell death.

Mutant mouse strains have also been used to investigate the role of the activating E2Fs in normal development (Trimarchi and Lees, 2002). Whereas a significant proportion of $E2F3^{-/-}$ mice die *in utero* (Humbert *et al.*, 2000), $E2F1^{-/-}$ mice are viable but display an excess of T cells (Garcia *et al.*, 2000) and develop tumours in older mice (Yamasaki *et al.*, 1996), suggesting that E2F1 plays a role in tumour suppression. At present many investigators believe that E2F1 acts as a tumour suppresser through its ability to induce apoptosis (Trimarchi and Lees, 2002).

1.2.7: The 'Repressive' E2Fs

The 'repressive' E2Fs include E2Fs 4-6. E2F4 and E2F5 were cloned by virtue of their association with pocket proteins or DP1 (Stevens and La Thangue, 2003). Since both E2F4 and E2F5 lack the intrinsic NLS seen in E2Fs 1-3 they require to be complexed with a pocket protein to enter the nucleus (Allen *et al.*, 1997; Verona *et al.*, 1997) and are seen as repressors of E2F-responsive genes. As well as their unique organisation (Figure 1.2B) E2Fs 4-5 differ remarkably from E2Fs 1-3 in their expression. Whereas the levels of E2Fs 1-3 peak at the G1/S boundary, both E2F4 and E2F5 are constitutively expressed throughout the cell cycle with the majority of E2F4/5 pocket protein complexes present in G0 and early G1 (Dyson, 1998). Since these complexes associate with HDACs *in vivo*, E2F4 and E2F5 are thought to be crucial for mediating the transcriptional repression of E2F-responsive genes (Trimarchi and Lees, 2002).

However, work by de la Luna *et al.* (1996) showed that E2F4 and E2F5 could be imported into the nucleus in a transcriptionally active state via their association with the NLS-containing α or δ splice variants of DP3. This suggests that the choice of DP partner in the E2F4/5 heterodimer can have an influence on the presence of transcriptionally active E2F4 or E2F5 in the nucleus (de la Luna *et al.*, 1996). Indeed, work by Loughran and La Thangue, (2000), showed that both E2F4 and E2F5 could induce apoptosis. This was dependent on both the nuclear accumulation and the transcriptional activity of the E2F complex.

Also, work by Morris *et al.* (2000) showed that the cyclinE/cdk2 complex could phosphorylate E2F5 within its *trans*-activation domain, which augmented the binding of the p300/CRE co-activator protein. This resulted in an increased stimulation of E2F-responsive genes in late G1. This study implies a positive autoregulatory mechanism for E2F-dependent transcription and supports the notion that both E2F4 and E2F5 play a role in the activation of target genes (Stevens and La Thangue, 2003).

The choice of pocket protein partner also differs significantly between the 'activating' and 'repressing' E2Fs. E2Fs 1-3 are specifically regulated by pRB whereas E2F4 associates with each of the pocket proteins at various point in the cell cycle and E2F5 is specifically regulated by p130 (Trimarchi and Lees, 2002). Subsequent analysis of E2F-responsive promoters such as cdc2 and E2F1 showed that the levels of transcription from these promoters rose significantly during G0/G1 when these sites were mutated (Dalton, 1992; Hsiao *et al.*, 1994).

Subsequent *in vivo* ChIP analysis by Takahashi *et al.* (2000) and Wells *et al.* (2000) has shown that the promoters of many E2F-responsive genes are occupied by E2F4/p107 and E2F4/p130 complexes during G0/G1. As cells progress towards late G1, the levels of the E2F4/p107 and E2F4/p130 complexes that are associated with the E2F-responsive promoters declines, and the 'activating' E2Fs seem to replace the 'repressing' complexes on these promoters. Significantly, the decrease in binding of E2F4 to E2F sites correlates with the dissociation of E2F4/DP

complexes, together with the relocalisation of E2F4/DP to the cytoplasm (Stevens and La Thangue, 2003).

E2F gene knockouts in mice have revealed distinct biological roles for the repressive E2F complexes. E2F4$^{-/-}$, E2F4$^{-/-}$ and E2F5$^{-/-}$, p107$^{-/-}$ or p130$^{-/-}$ MEFs all have defects in exiting the cell cycle in response to a variety of growth arrest signals such as p16 overexpression and contact inhibition (Trimarchi and Lees, 2002). Despite this none of these MEFs fail to respond to growth signals indicating that loss of the 'repressive' E2Fs impairs only the repression and not the activation of E2F-responsive genes, and therefore the ability to exit the cell cycle (Trimarchi and Lees, 2002).

The 'repressive' E2Fs also seem to play a critical role in differentiation. Work by Persengiev *et al.* (1999) showed that the differentiation of neuronal precursors could be induced by E2F4 overexpression. The primary defects in E2F4$^{-/-}$ mice are hematopoietic, craniofacial and intestinal defects whilst E2F5$^{-/-}$ mice are perinatal lethal with hydrocephalus (Humbert *et al.*, 2000; Rempel *et al.*, 2000; Lindeman *et al.*, 1998). In contrast to the defects in the initiation of differentiation seen with E2F4$^{-/-}$ and E2F5$^{-/-}$ mice, both p107$^{-/-}$ and p130$^{-/-}$ mice show defects in the development of long bones. This is due to the failure of the chondrocytes to exit the cell cycle, before finally undergoing differentiation after several rounds of inappropriate cell division (Trimarchi and Lees, 2002).

The most recently identified E2F is E2F6. This differs from the other E2Fs in that it lacks the C-terminal *trans*-activation domain and therefore the capacity to form a complex with the pocket proteins (Morkel *et al.*, 1997). Unsurprisingly this has lead to the belief that E2F6 acts as an active repressor of E2F-responsive genes by occupying E2F promoters. Indeed, work by Ogawa *et al.* (2002) showed that E2F6 can actively recruit chromatin-modifying proteins to E2F target genes, inducing gene silencing.

1.2.8: E2F and the DNA Damage Response

Several recent studies now point to a role for E2F in the DNA damage response pathway (Huang *et al.*, 1997; Lin *et al.*, 2001; Stevens *et al.*, 2003). Huang *et al.* (1997) showed that treatment of cells with ionizing radiation led to the up-regulation of E2F1 and subsequent cell death whilst most recently a report by Stevens *et al.* (2003) showed that the DNA damage responsive kinase, Chk2, could phosphorylate E2F1 on serine-364. This led to the stabilisation, increased half-life and transcriptional activation of E2F1 as well as enhanced sub-G1 population, induced by E2F1. Whilst previous work by Lin *et al.* (2001) had shown that E2F1 was phosphorylated by the ATM/ATR kinases, the work by Stevens *et al.* (2003) showed a further downstream effect by the Chk2 kinase, which is activated in response to DNA damage by ATM/ATR.

Interestingly, E2F1 has most recently been implicated in inducing an S-phase checkpoint by recruiting NBS1 and the MRE11 recombination/repair complex to origins of DNA replication after DNA damage during S-phase (Cam and Dynlacht, 2003). Also, recent studies have shown that E2F targets may include p53, Chk1, PCNA and FEN1, all of which are known to be intricately involved in the DNA damage response (Ishida *et al.*, 2001; Muller *et al.*, 2001; Polager *et al.*, 2002; Ren *et al.*, 2002).

Indeed not all the evidence for the role of E2F in the DNA damage response points to the role of the 'activating' E2Fs. Ren *et al.* (2002) have shown that E2F4 could induce the expression of a host of genes whose protein products are involved in DNA repair. All of these studies show a direct role for E2F in the DNA damage, DNA repair and apoptotic pathways.

1.2.9: E2F and Cancer

Given its central role in the control of proliferation, differentiation and apoptosis it is unsurprising that E2F is heavily implicated in tumourigenesis. Overexpression of the 'activator' E2Fs is sufficient to not only induce cell cycle entry but also to confer transforming potential to primary cells (Cam and Dynlacht, 2003). Whilst much is known about the role of the 'activator' E2Fs in cancer, much less is known about the role that the 'repressive' E2Fs play. Work by Humbert *et al.* (2000) and Rempel *et al.* (2000) suggested that E2F4 does not act as a tumour suppresser. The

role of E2F5 in tumour development is more difficult to assay since E2F5$^{-/-}$ mice die after six weeks. The compound loss of both E2F4 and E2F5 is difficult to study since the mice die as neonates (Cam and Dynlacht, 2003).

Inactivation of pRB by viral oncoproteins such as E1A, HPV E7, the large T antigen , or mutations within pRB itself, are enough to prevent the formation of the E2F/pRB complex, resulting in 'free' E2F. Many upstream regulators of E2F are also inactivated or amplified in tumours. Work by Wolfel *et al.* (1995) showed that a mutant cdk4 in human melanomas was constitutively active due to its inability to complex with the cdki, p16. Mutations that prevent the degradation of cyclinD can also ensure that pRB is in a permanent state of hyperphosphorylation (Welcker *et al.*, 1996).

Figure 1.2A: E2F-Responsive genes

Tabular representation of a range of E2F-responsive genes involved in cell cycle regulation, DNA synthesis and apoptosis.

CELL CYCLE REGULATORS	NUCLEOTIDE SYNTHESIS	APOPTOSIS
Cyclin A	Ribonucleotide Reductatase 1, 2	Caspase 3, 7
Cyclin E		
E2F1/2/3	Thymidine Kinase (TK)	p73
DP1		
Cdk2	Thymidilate Synthase (TS)	Apaf1
Cdk7		
Cdc25A	Deoxycytidine Kinase	MAP3 Kinase 5
pRB		
p107	Dihydrofolate Reductase (DHFR)	ARF
Cdc2		

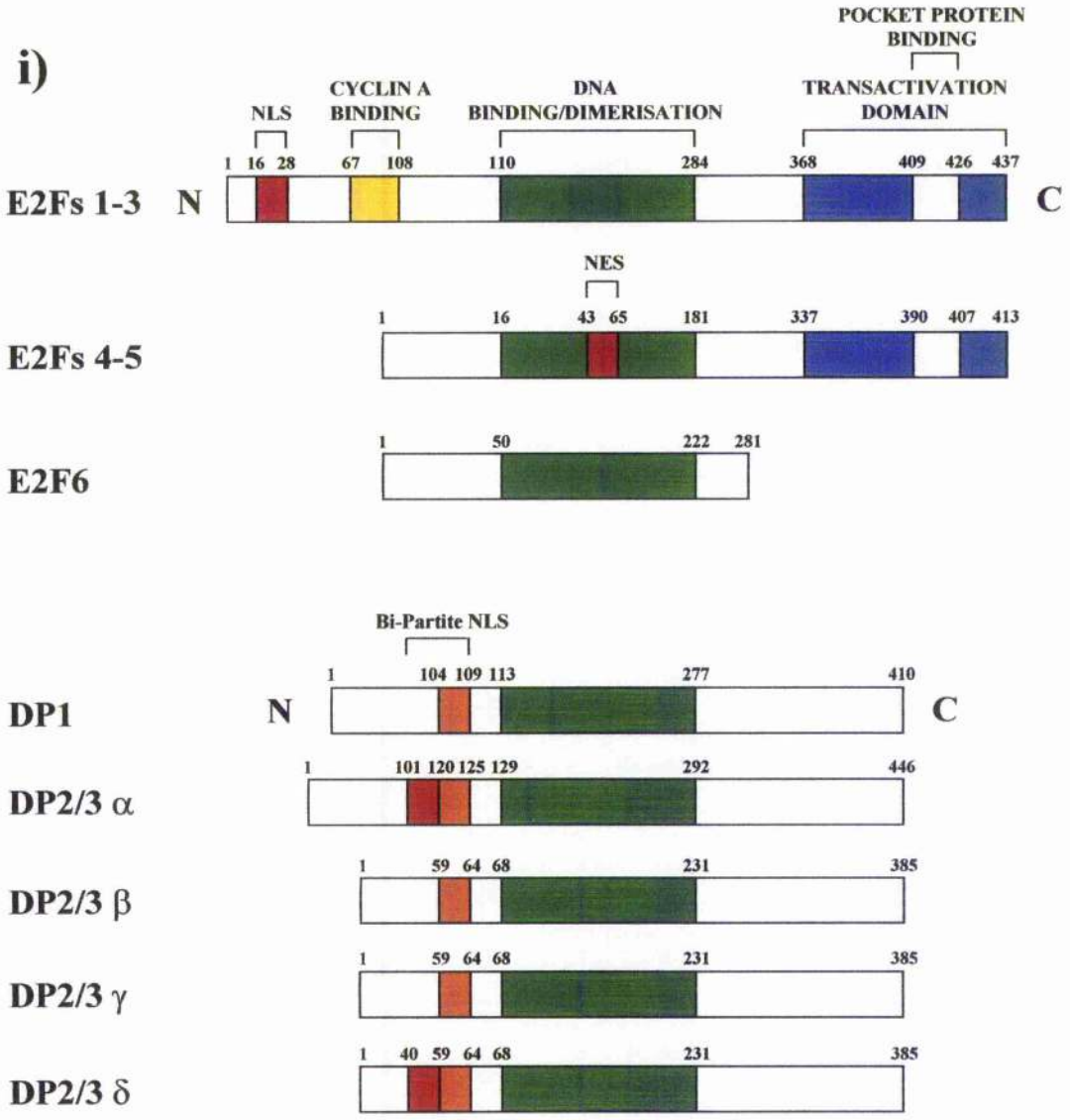
Figure 1.2B: The E2F and DP Families

i. Schematical representation of the E2F and DP family members. E2Fs 1-3 have an N-terminal cyclin A binding domain that allows the regulation of the DNA binding activity of these E2Fs during S-phase. E2Fs 4-6 have a truncated N-terminus and do not contain this domain. E2Fs 4-5 contain a nuclear export signal (NES) that is not present in E2Fs 1-3. E2Fs 1-5 contain a C-terminal *trans*-activation domain with a pocket protein binding domain located within this region. E2F6 does not contain the *trans*-activation domain. The numbers represent the amino acid positions of the various domains within the family members.

Three members of the DP family exist (DP 1-3). In the literature DP2 human and murine DP3 are the same protein. Four splice variants of DP2/3 exist: α, β, δ and γ. α and δ are nuclear due to an N-terminal NLS. β and γ are cytoplasmic, as is DP1, since they lack the NLS. The α splice variant of DP2/3 has an extended N-terminus. The numbers represent the amino acid positions of the various domains within the family members. The figure is adapted from Stevens and La Thangue, (2003), Figure 2.

ii. Tabular representation of the properties of members of the 'activating' and 'repressing' E2Fs as well as the DP family. The figure is adapted from Stevens and La Thangue, (2003), Table 1.

i)



ii)

CHARACTERISTIC	ACTIVATING E2Fs (E2F1/E2F2/E2F3)	REPRESSING E2Fs (E2F4/E2F5)
Pocket protein partner	pRB	p107: E2F4 p130: E2F4/E2F5
Nuclear Localisation Signal (NLS)	Yes	No
Nuclear Export Signal (NES)	No	Yes
Cyclin A Binding	Yes	No
Expression	G1/S	Constitutive
Ability to induce apoptosis upon overexpression	Only E2F1	No
Ability to override G1 arrest	Yes	No
Tumour suppressor function	E2F1 alone	No

Figure 1.2C: The Pre-Initiation Complex

The PIC forms when the TBP subunit of TFIID [which comprises TBP and TBP associated factors (TAFs)] binds to the TATA sequence in eukaryotic promoters. The GTFs (TFIIA, B, D, E, F and H) then assemble, followed by RNA pol II. E2F is able to form a complex with TFIID and initiate sequence-specific transcription of target genes.

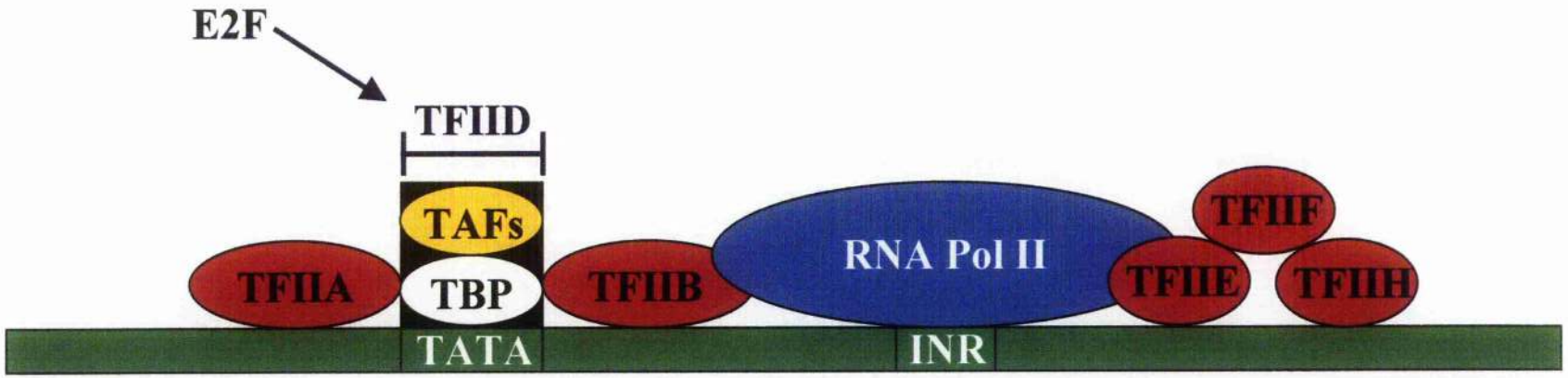

E2F
TFIID
TAFs
TBP
TFIIA
TFIIB
RNA Pol II
TFIIF
TFIIE
TFIIH
TATA
INR

Figure 1.2D: The Types of Generic E2F Complexes

Representation of the three types of physiological E2F complex.

i. 'Free' E2F results from the sequential phosphorylation of the pocket proteins. This allows E2F to transactivate its target genes and to induce cell cycle progression.

ii. Inhibitory E2F complexes result from the binding of the pocket proteins to E2F. By binding within the C-terminal domain of E2F the pocket proteins act to inhibit the transcriptional activation of E2F-responsive genes.

iii. Repressive E2F complexes arise when the pocket proteins actively recruit proteins such as the HDACs, PcG, HTMases and members of the SWI/SNF family. This results in chromatin remodelling and long term gene silencing.

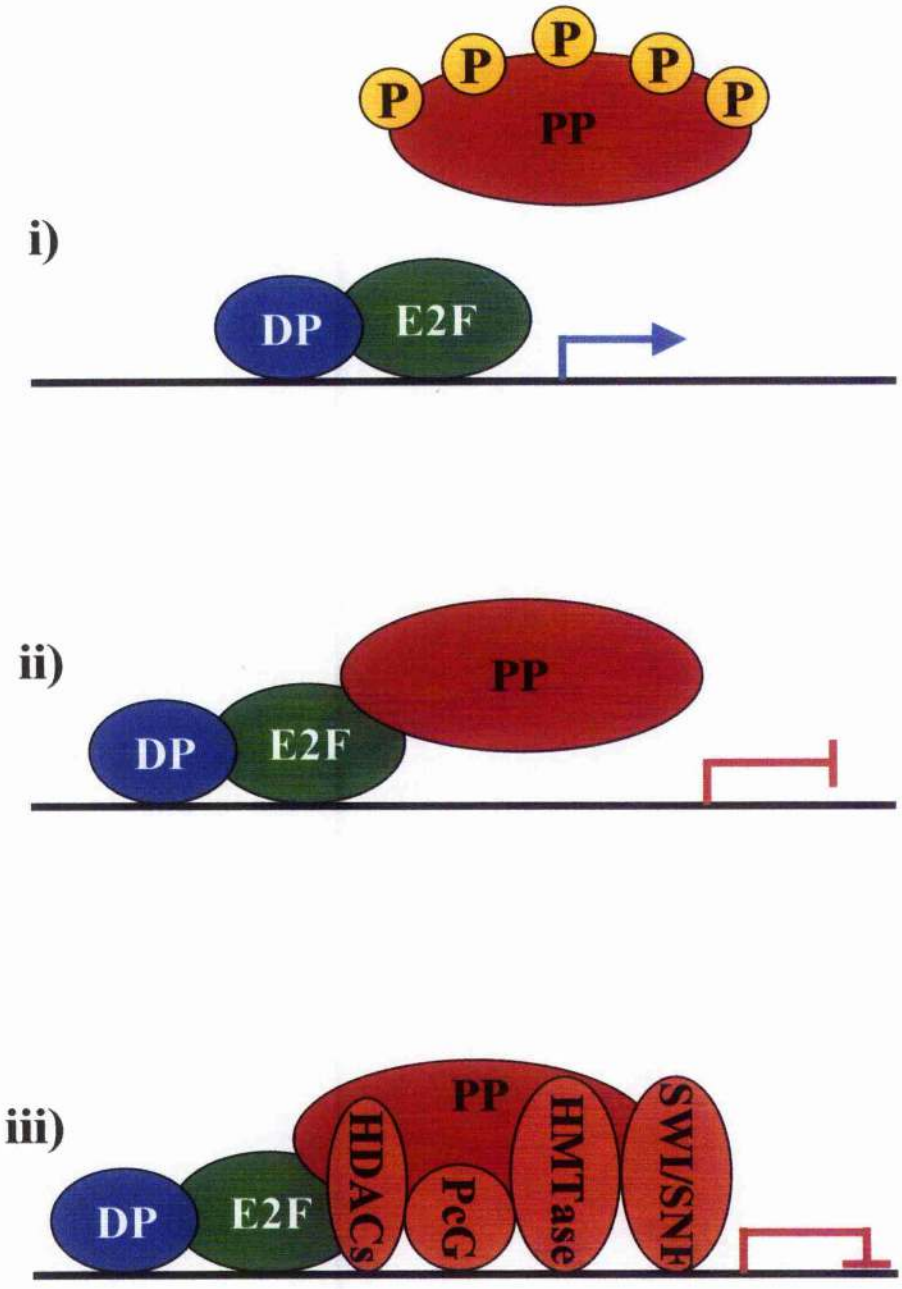
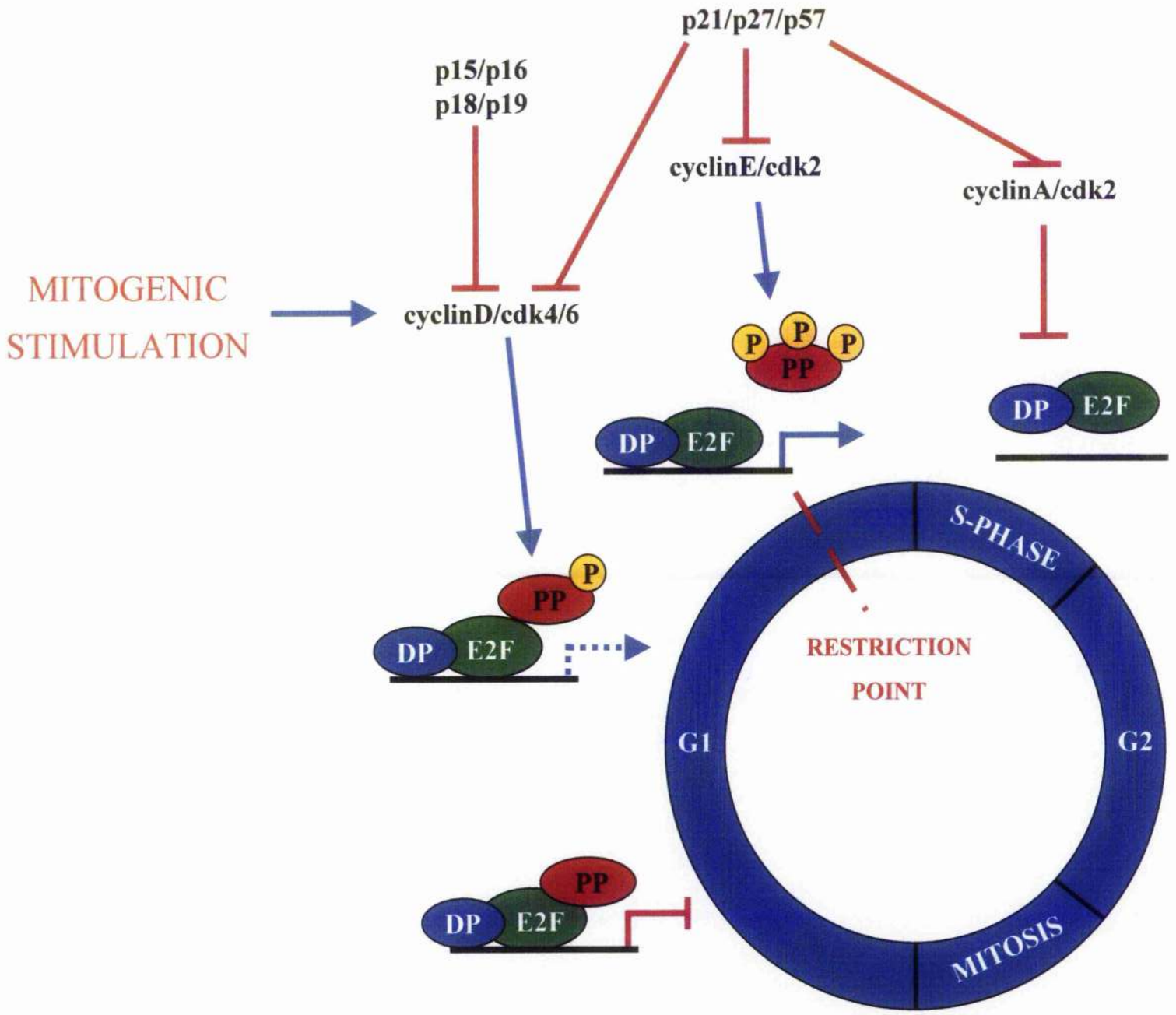

P
P
P
P
P
PP
i)
DP
E2F
ii)
PP
DP
E2F
iii)
PP
HDACs
PcG
HMTase
SWI/SNF
DP
E2F

Figure 1.2E: The Regulation of E2F by the cdks and cdkis

Overview of the regulation of E2F activity by the cyclin/cdk complexes and the cdkis during cell cycle progression. Up-regulation of cyclinD/cdk4/6 activity follows mitogenic stimulation. This results in low level phosphorylation of the pocket proteins, resulting in partial induction of E2F. CyclinE/cdk2 complexes then induce hyperphosphorylated pRB, resulting in 'free' E2F, which then drives the cell into S-phase. Once past the restriction point the cell is no longer under the control of mitogenic stimulation and must divide unless cellular stresses occur. During S-phase, phosphorylation of DP1 and E2F1 by cyclinA/cdk2 results in the removal of the DP/E2F complex from the DNA. The activity of the cyclin/cdk complexes can be blocked by the indicated cdkis. Red lines indicate an inhibitory effect on cell cycle progression. Blues lines indicate a stimulatory effect.


p21/p27/p57
p15/p16
p18/p19
cyclinE/cdk2
cyclinA/cdk2
MITOGENIC
STIMULATION
cyclinD/cdk4/6
P
P
P
PP
DP
E2F
DP
E2F
DP
E2F
P
PP
S-PHASE
RESTRICTION
POINT
G1
G2
MITOSIS
PP
DP
E2F

1.3: The 14-3-3 Proteins

1.3.1: Introduction

In 1967 Moore and Perez described an acidic, and highly abundant brain protein, which they termed 14-3-3, based on the fraction number after DEAE-cellulose chromatography and the position after starch gel electrophoresis (Moore and Perez, 1967). 14-3-3 proteins have now been identified in all eukaryotic organisms with seven isoforms in mammals, fifteen in *Arabadopsis Thaliana* and two in yeast (Rosenquist, 2003). In mammals the isoforms are named β, ε, η, γ, σ, τ and ζ [α and δ are the phosphorylated forms of β and ζ respectively (Aitken *et al.*, 1995)].

14-3-3s are involved in a wide range of biological processes including neuronal development, cell cycle control and apoptosis (Fu *et al.*, 2000) and bind a vast array of target substrates (Figure 1.3A). 14-3-3s can alter the activities of target proteins by sequestering them, relocalising them, conformationally altering their functional activity or promoting interaction with other protcins (Woodcock *et al.*, 2003).

14-3-3s exist as dimers with a monomeric molecular mass of 30 kilodaltons. Each monomer in the U-shaped dimer is composed of nine anti-parallel α-helices (van Hemert *et al.*, 2001), and can bind one target molecule, predominantly through phosphorylated serine residues in the target substrate (Fu *et al.*, 2000) [Figure 1.3B(i)].

This U-shape creates a negatively charged groove containing highly conserved amino acids between each isoform and more variable residues on the outside of the molecule. The N-terminus of 14-3-3 is involved in dimer formation [Figure 1.3B(ii)] (Liu *et al.*, 1995; Xiao *et al.*, 1995) with the dimer interface formed by the interaction of helix α1 from one monomer (residues 5-21) with helixes α3 and α4 from the other monomer (residues 58-89).

The sides of the channel are formed by the C-terminal domains (van Hemert *et al.*, 2001). Until recently it was thought that all 14-3-3 isoforms contained a nuclear export (NES) within this region and that this was responsible for the localisation of target ligands to the cytoplasm. However, recent work by Brunet *et al.* (2002) has now shown that this region is in fact required for ligand binding and does not act as an NES. By mutating lysine-49, which plays an essential role in ligand binding, the group were able to show that this 14-3-3 mutant, that could not now bind any of its ligands, homed to the nucleus. If the C-terminal domain did contain an NES then 14-3-3 would localise to the cytoplasm in this case. It seems now that 14-3-3 proteins influence the sub-cellular localisation of some of their targets by a process of molecular interference. By binding and masking either an NLS or an NES within the target, 14-3-3 is thought to be able to prevent these signals from complexing with either the nuclear import or export machinery. In this way 14-3-3 is thought to facilitate either nuclear or cytoplasmic localisation.

By binding two ligands simultaneously 14-3-3s are also able to promote a more stable interaction with weak interacting motifs or to bring together two different proteins that modulate each other's activity (Fu *et al.*, 2000).

Phosphopeptide library screening for potential consensus sites for 14-3-3 binding revealed two motifs: RSXpSXP and RX(Y/F)XpSXP (Yaffe *et al.*, 1997) where X is any amino acid and pS denotes phosphorylated serine. However 14-3-3s have also been shown to bind non-phosphorylated substrates such as 5-phosphatase, exoenzyme S, A20 and the platelet glycoprotein IB-IX-V complex (Masters *et al.*, 1999; Vincenz and Dixit. 1996; Du *et al.*, 1996; Campbell *et al.*, 1997).

1.3.2: 14-3-3s and Cell Cycle Control

Numerous studies have now shown 14-3-3 to play a pivotal role in control of the cell cycle. The phosphatase cdc25 is crucial in allowing onset of mitosis. Human cdc25C can dephosphorylate tyrosine-15 on the protein kinase cdc2, resulting in active cdc2 (Peng *et al.*, 1997). Cdc2 is part of the cdc2/cycB complex that causes G2/M progression. Cdc25C is phosphorylated at serine-216 during interphase (Peng *et al.*, 1997) which creates a 14-3-3 binding site, leading to the sequestration of cdc25C in the cytoplasm [Figure 1.3C(i)]. Several kinases that phosphorylate serine-216 have been described including C-TAK1 (Peng *et al.*, 1998), Chk1 (Blasina *et al.*, 1999) and Cds1/Chk2 (Blasina *et al.*, 1999). Substitution of serine-216 with alanine leads to abolition of the association. 14-3-3 does not alter the

catalytic state of cdc25 (Conklin *et al.*, 1995; Peng *et al.*, 1997) and this suggests that 14-3-3 negatively regulates cdc25C by altering its sub-cellular localisation.

Indeed, cdc25C contains an NLS (Ogg *et al.*, 1994) located near serine-216 (Peng *et al.*, 1997). This suggests that 14-3-3 binding can regulate the sub-cellular localisation of cdc25C, perhaps by disrupting this signal. Indeed, 14-3-3 has been shown to tether *Xenopus* cdc25C in the cytoplasm by blocking the interaction between the NLS in cdc25C and the importin α/β complex (Zeng and Piwnica-Worms, 1999; Kumagai and Dunphy, 1999).

However, recent studies by Graves *et al.* (2001) have shown that 14-3-3 binding is not required for the nuclear export of cdc25C. A mutant of cdc25C that could not bind to 14-3-3 was seen to accumulate in the nucleus when the cells were treated with leptomycin B, which blocks nuclear export. This allowed the conclusion that this mutant was still able to enter and exit the nucleus and therefore the complete nuclear export of cdc25C required **both** an intact 14-3-3 binding site and the intrinsic NES within cdc25C which is located close to serine-216. Interestingly, Lopez-Girona *et al.* (1999) showed that elimination of a 14-3-3 homologue, *rad24+*, in *Schizosaccharomyces pombe* causes the nuclear accumulation of cdc25 whilst work by Dalal *et al.* (1999) showed that the cytoplasmic localisation of human cdc25C required an intact 14-3-3 binding site.

As well as being involved in preventing G2/M progression, 14-3-3 can block S-phase entry by associating with cdk2 (Laronga *et al.*, 2000). Cdk2 is active when complexed with the cyclin proteins, which then leads to the phosphorylation of Rb family members. This causes cell cycle progression since Rb is no longer able to negatively regulate E2F activity. By blocking cdk2 activity 14-3-3σ can prevent a G1-to-S transition.

Recent work by Grozinger and Schreiber, (2000), showed that 14-3-3s could also indirectly regulate the activity of both HDAC4 and HDAC5. The HDACs are nuclear proteins involved in repression of transcription. It was found that HDAC4 and –5 were phosphorylated, which resulted in 14-3-3 binding. This binding caused the cytoplasmic localisation of the HDAC molecules, which prevented them from contributing to gene silencing. As in the case with cdc25C, 14-3-3 binding was found to prevent the association of an NLS within HDAC4 with the importin α/β heterodimer (Grozinger and Schreiber, 2000).

1.3.3: 14-3-3s and DNA Damage Response

DNA damage induces cell cycle arrest providing time for DNA repair before cells continue cycling (van Hemert *et al.*, 2001). DNA damage induces the activation of both Chk1 and Chk2 (Chehab *et al.*, 2000) which leads to the phosphorylation of serine-216 on cdc25C. As described above this creates a 14-3-3 binding site on cdc25C leading to nuclear export of this complex, which prevents mitotic onset. 14-

3-3ζ has recently been shown to associate with cdc25C in irradiated cells, resulting in G2 checkpoint activation (Qi and Martinez, 2003).

The direct target of cdc25C is cdc2, which binds to cyclin B to form the MPF (Maturation Promoting Complex). 14-3-3 can also regulate the sub-cellular localisation of cdc2 after DNA damage. After damage the activity of cdc2 is suppressed through phosphorylation by the Wee1 and Myt/Mik1 kinases (van Hemert *et al.*, 2001). In response to cellular stresses p53 upregulates 14-3-3σ (Hermeking *et al.*, 1997) which then binds the phosphorylated cdc2 and localises it to the cytoplasm thus preventing mitotic onset (Chan *et al.*, 1999). Recent work by Bulavin *et al.* (2003) has now shown that phosphorylation of serine-214 in cdc25C prevents phosphorylation of serine-216 during mitosis. Activation of the IR-induced DNA damage checkpoint in mitotic cells did not induce serine-216 phosphorylation. These findings suggest that mitotic cells lose their ability to phosphorylate cdc25C on serine-216 and inactivate cdc2 after DNA damage due to the phosphorylation of serine-214 in cdc25C.

Waterman *et al.* (1998) have now shown that p53 itself is regulated by 14-3-3σ [Figure 1.3C(ii)]. Normally p53 is phosphorylated at serines-376 and 378. After DNA damage serine-376 is dephosphorylated thereby creating a 14-3-3 binding site at serine-378. 14-3-3σ then associates with p53 causing an enhancement of p53 DNA binding activity. As was described above this then leads to up-regulation of 14-3-3σ and subsequent cell cycle arrest.

14-3-3 proteins can also regulate both Chk1 and Wee1. The *S. pombe* 14-3-3 homologues, Rad24 and Rad25 bind to Chk1 in response to DNA damage (Chen *et al.*, 1999). Although the nature of this association is unknown it may target Chk1 to either a specific part of the cell or target protein. Recent findings by Honda *et al.* 1997) now point to both 14-3-3β and 14-3-3ζ being able to bind to Wee1. Phosphorylation of Wee1 creates a 14-3-3 binding site on the molecule and work by Wang *et al.* (2000) showed that this association causes an increase in Wee1 stability and kinase activity. This causes an increase in the G2/M cell population.

1.3.4: 14-3-3s and Apoptosis

Apoptosis is the process of programmed cell death that plays a crucial role in the normal development of tissues as well as in the pathophysiology of a variety of diseases including cancer (Fu *et al.*, 2000). 14-3-3s play a pivotal role in control of apoptosis. The Bcl-2 family of pro- and anti-apoptotic molecules are involved in the cell death response. As described above, the family consist of Bcl-xs, Bad, Bak and Bax that stimulate apoptosis and Bcl-2, MCL-1 and Bcl-x_L that inhibit the process (van Hemert., 2001). Both Bcl-2 and Bcl-x_L are bound, and inactivated, by Bad (Yang *et al.*, 1995; Mok *et al.*, 1999) which leads to cell death.

However, 14-3-3 proteins can bind Bad, which prevents it from interfering with the function of both Bcl-2 and Bcl-x_L (Xing *et al.*, 2000) [Figure 1.3C(iii)]. Phosphorylation of Bad on serine-136 by Akt1 (Datta *et al.*, 1997) and on serines-

112 and 155 by RSK1 (Tan *et al.*, 2000) and PKA (Lizcano *et al.*, 2000), in response to survival signals, creates three 14-3-3 binding sites within the molecule. 14-3-3 then sequesters Bad in the cytoplasm, away from the mitochondrial bound Bcl-2 and Bcl-x_L. Dephosphorylation of Bad by the calcium activated protein phosphatase, calcineurin, allows Bad to associate with its targets which leads to caspase release from the mitochondria and subsequent cell death.

The activation of transcription factors is an important process in maintaining the balance between agonists and antagonists of apoptosis (Rosenquist, 2003). The Forkhead Transcription Factor 1 (FKHRL1) is involved in transcribing apoptosis-promoting genes (Rosenquist, 2003). It is regulated by Akt1 kinase, which is involved in promoting cell survival. Activated Akt1 translocates to the nucleus where it phosphorylates FKHRL1 (Brunet *et al.*, 1999). This allows 14-3-3 to bind FKHRL1 and to retain it in the cytoplasm. Dephosphorylation of FKHRL1 due to removal of survival signals leads to dissociation of 14-3-3 from FKHRL1 and translocation of the molecule to the nucleus where it can induce apoptosis.

ASK1 (Apoptosis Signal-Regulating Kinase1) is stimulated by pro-apoptotic elements such as TNFα and cisplatin. When ASK1 becomes phosphorylated, 14-3-3 can bind and inhibit the pro-apoptotic effects of ASK1 (Zhang *et al.*, 1999). An ASK1 mutant that has an abrogated ability to bind 14-3-3 has an enhanced pro-apoptotic effect (Zhang *et al.*, 1999).

1.3.5: Regulation of the 14-3-3s

Possible mechanisms that regulate the activities of the 14-3-3s include isoform specificity and post-translational modifications such as phosphorylation (Fu *et al.*, 2000). The various isoforms of 14-3-3 all contain the same key residues involved in ligand binding (Yaffe *et al.*, 1997; Rittinger *et al.*, 1999). This would suggest that isoform specificity is not important in the activity of these molecules and work by Muslin *et al.* (1996) showed that different 14-3-3 isoforms could bind peptides with similar affinities. Some 14-3-3s do have specificity to target ligands. 14-3-3η is the only member of the family that can bind the zinc finger protein A20 (Vincenz *et al.*, 1996).

Post-translational modifications may regulate activity. 14-3-3ζ is phosphorylated on serine-185, which lies within a proline-directed kinase consensus. This phosphorylation gives rise to the δ isoform (Aitken *et al.*, 1995). Work by Liu *et al.* (1995) showed that this site is near the N-terminus of helix 8, close to the ligand-binding groove, suggesting that it may play a role in ligand binding. Work by Dubois *et al.* (1997) also showed that 14-3-3ζ was phosphorylated on threonine-233 by Casein Kinase I. This negatively regulates the binding to c-Raf, presumably since this site is close to residues involved in ligand binding. Interestingly, recent work by Woodcock *et al.* (2003) has shown that phosphorylation of serine-58 in 14-3-3ζ by a sphingosine-dependent kinase (SDK) led to the disruption of the dimeric status of 14-3-3ζ since serine-58 is located near to the dimer interface. Monomeric 14-3-3ζ could still bind to a phospho-peptide target. Indeed, recent

findings by Hamaguchi *et al.* (2003) have now shown that this SDK is in fact identical to the C-terminal half kinase domain of PKCδ. Cleavage of PKCδ by caspase-3 releases the kinase domain which then confers SDK1 activity, leading to phosphorylation of 14-3-3ζ on serine-58 (Hamaguchi *et al.*, 2003). These findings could have profound implications for the understanding of the ways that 14-3-3s regulate their targets *in vivo*.

1.3.6: 14-3-3 and Cancer

Given that a significant number of 14-3-3 ligands are proto-oncogenes or oncogenic products this suggests that 14-3-3s may be involved in neoplastic transformation (Fu *et al.*, 2000).

Recent work by Craparo *et al.* (1997) showed that 14-3-3 interacts with the insulin-like growth factor-1 receptor (IGF1R). The binding site within IGF1R is near a quartet of serines that are essential for cell transformation mediated by IGF1R. This would suggest that 14-3-3 proteins are involved in tumorogenesis in this case. Indeed, in many major types of lung cancer, both IGF1R and 14-3-3 proteins have elevated levels (Fu *et al.*, 2000). In contrast, work by McDonald *et al.* (1994) showed that 14-3-3ε, located on chromosome 17, was in a region frequently mutated in several cancers. This suggests that this isoform, and perhaps others, are important in the suppression of neoplastic transformation.

Figure 1.3A: 14-3-3 Binding Ligands

Tabular representation of a range of 14-3-3 binding ligands showing the recognition sequence within the ligand and the effect of 14-3-3 binding. The figure is adapted from Aitken. (2002), Table 1.

LIGAND	BINDING SEQUENCE(S)	EFFECT OF 14-3-3 BINDING
cdc25C	R S P pS(216) M P	Cytoplasmic retention/Block Mitosis
cdc25A	R S P pS(290) M P	Prevent binding to cyclinB1/Block Mitosis
Bad	R H S pS(112) Y P, R S R pS(136) A P	Cytoplasmic retention/Inhibit pro-apoptotic effect
FKHRL1	R S C pT(32) W P, R A V pS(253) M D	Cytoplasmic retention/Inhibit pro-apoptotic effect
ASK1	R S I pS(967) L P	Inhibit pro-apoptotic effect
p53	K G Q S T pS(378) R H	Enhance DNA binding
Middle T Antigen	R S H pS(257) Y P	Cause neoplastic transformation in some tissues
RAF-1	R S T pS(259) T P, R S A pS(621) E P	Maintain both inactive and active states
SLOB	R S N pS(54) A I, R S A pS(79) S E	Modulate voltage sensitivity of associated slowpoke potassium channels
CBL	R H pS(619) L P F pS(623) R L G pS(639) T F pS(642)	Unknown
cdk2	G V T pS(229) M P, Y K P pS(236) F P	Cytoplasmic retention/Cause G1/S block
HDAC4	R K T A pS(246) E P R T Q pS(467) A P R A Q pS(632) S P	Cytoplasmic retention/Prevent cell cycle progression
HDAC5	R K T A pS(259) W P R T Q pS(498) S P	Cytoplasmic retention/Prevent cell cycle progression
5-Phosphatase	L V L R S E S E E K V V(371)	Stimulate phosphatase activity
Exoenzyme-S	D A L D L(428)	Stimulate ADP-ribosylation activity

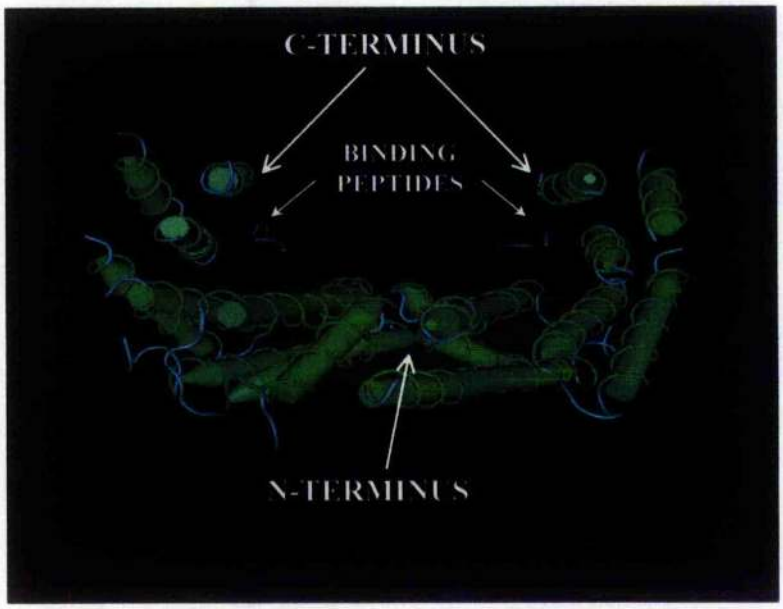
Figure 1.3B: Diagram of 14-3-3

i. Diagrammatical representation of the 14-3-3 binding cleft. This U-shaped binding region consists of highly conserved amino acids, which forms an amphipathic groove. Each monomer can bind one target molecule (as shown by representation by the synthetic peptides).

ii. Diagrammatical representation of the N-terminal interaction of the 14-3-3 dimer. The dimer is formed by the interaction of helix α1 from one monomer with helixes α3 and α4 from the other monomer.

i and ii: The representations were adapted from the structure of 14-3-3ζ bound to R18 peptide (Protein Data Bank accession number 1A38) (Petosa *et al.*, 1998).

i)



ii)

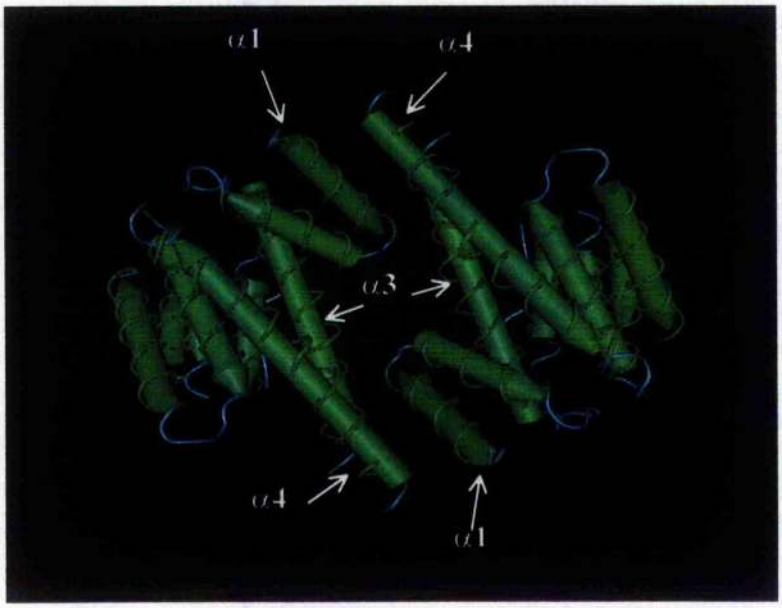
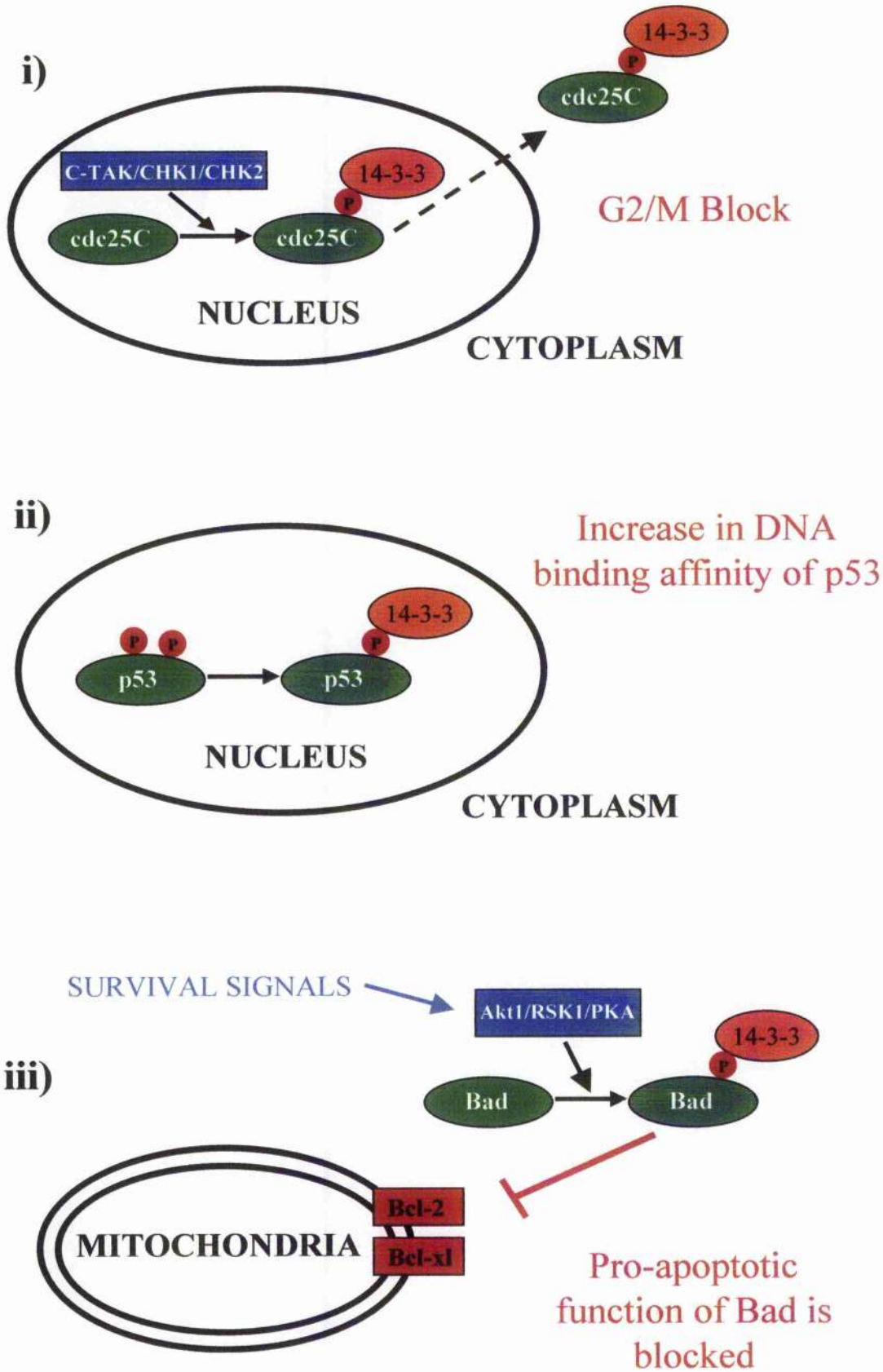


Figure 1.3C: Effects of 14-3-3 Binding on Different Ligands

i. 14-3-3 sequesters cdc25C in the cytoplasm. C-TAK and both Chk1 and Chk2 can phosphorylate cdc25C, allowing 14-3-3 to form a complex with and sequester cdc25C in the cytoplasm. This creates a G2/M block.

ii. 14-3-3 can increase the DNA binding affinity of p53. After DNA damage p53 becomes dephosphorylated at serine-376 which creates a 14-3-3 binding site at serine-378. This results in an enhancement of the ability of p53 to bind to DNA.

iii. 14-3-3 can block Bad induced cell death. Following survival signals, Bad becomes phosphorylated by Akt1, RSK1 and PKA. This allows 14-3-3 to bind and sequester Bad away from its target substrates in the mitochondria. This promotes cell survival. Blue lines indicate stimulatory effect; red lines indicate inhibitory effect.


i)
14-3-3
P
cdc25C
C-TAK/CHK1/CHK2
14-3-3
P
cdc25C
cdc25C
G2/M Block
NUCLEUS
CYTOPLASM
ii)
Increase in DNA
binding affinity of p53
14-3-3
P
P
P
p53
p53
NUCLEUS
CYTOPLASM
SURVIVAL SIGNALS
iii)
Akt1/RSK1/PKA
14-3-3
P
Bad
Bad
MITOCHONDRIA
Bcl-2
Bcl-xl
Pro-apoptotic
function of Bad is
blocked

1.4: Objectives

Recent work in the laboratory has shown that 14-3-3 can influence E2F, via the DP3 sub-unit (Cruickshank, 2000). This interaction is dependent on the basic portion of the bi-partite NLS of DP3. Removal of this region results in a loss of 14-3-3 binding as well as an accumulation of the E2F/DP3 complex in the cytoplasm. Since 14-3-3s are known to be able to influence the sub-cellular localisation of certain target substrates this result raised the question of whether 14-3-3 was influencing the sub-cellular localisation of the E2F/DP3 heterodimer.

This project firstly attempted to answer whether 14-3-3 was influencing the ability of E2F/DP3 to accumulate in the nucleus. To answer this question, a mutant of DP3 was generated that failed to bind 14-3-3 but still retained the ability to localise to the nucleus. The generation of this mutant therefore showed that 14-3-3 was not influencing the nuclear accumulation of DP3.

The ability of E2F to *trans*-activate its target genes is dependent on its nuclear accumulation. It was therefore important to generate this DP3 mutant, that was able to localise to the nucleus, form a complex with E2F and yet be defective in its ability to bind 14-3-3. This would then allow the further elucidation of the role of 14-3-3 over E2F activity, via DP3.

The project then analysed whether 14-3-3 could influence the cell cycle, via E2F. Both E2F and 14-3-3 have key roles to play in the control of cell cycle progression.

It was postulated that 14-3-3 may influence the cell cycle, via E2F, as it has been shown to do for a wide array of target substrates. Subsequent analysis revealed a role for 14-3-3 in the control of the cell cycle, specifically S-phase entry. This was dependent on the ability of 14-3-3 to bind to DP3.

14-3-3s have been shown to play a pivotal role in the control of apoptosis. Recent work has now pointed to a role for E2F in the apoptotic response. Given this, it was of interest to analyse whether 14-3-3 could influence E2F-mediated apoptosis. Further investigation revealed a role for 14-3-3 in the control of E2F-mediated apoptosis. Again, this was dependent on the ability of 14-3-3 to bind DP3.

Finally, further analysis revealed a role for DNA damage in the interaction between DP3 and 14-3-3. DNA damage caused an abrogation in binding between 14-3-3 and DP3. Given that 14-3-3 is intricately involved in checkpoint control, this result may point to a role for the DP3/14-3-3 interaction in a similar checkpoint control mechanism.

This study has now shown that an intricate relationship exists between E2F and 14-3-3 and that this relationship is dependent on the DP3 portion of the E2F/DP heterodimer. These results now point to a role for 14-3-3 in the control of E2F-mediated cell cycle progression, apoptosis and DNA damage response.

Chapter 2: Materials and Methods

2.1: PLASMIDS

The following plasmids have previously been described; pCMV-HA-E2F5 and pCMV-TAD-HA-E2F5 (Allen *et al.*, 1997), pG-DP3δ (de la Luna *et al.*, 1996), pCE-luc (Botz *et al.*, 1996) and pSG5 (Green *et al.*, 1988). The vector pAS-RFP was kindly supplied by the Milligan laboratory and was purchased from CLONTECH Laboratories (cat no. K6100-1).

Both pCMV-14-3-3ε-myc encoding mammalian expression myc-tagged 14-3-3ε and pGEX-14-3-3ε encoding bacterial expression GST-14-3-3ε were kind gifts from Prof. A. Aitken.

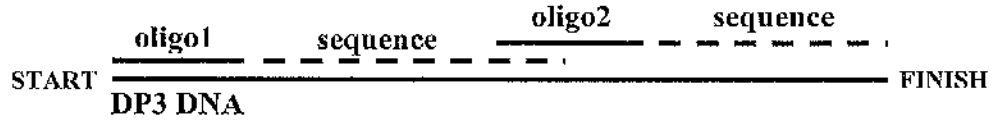
Both pGEX-DP3 and pGEX-DP3 5S encoding bacterial expression GST-DP3 and GST-DP3 5S were constructed by amplifying DP3 wt and DP3 5S by PCR from the mammalian expression plasmids pG-DP3 and pG-DP3 5S respectively using the following oligonucleotides containing the indicated restriction enzyme sites.

SmaI (5′-3′): TA**CCCGGG**AATGGTCACTCAGACTC

XhoI (3′-5′): GGGAGGAGGGGTCTTATT**GAGCTC**AT

PCR amplification was carried out using Taq polymerase (Promega). PCR was carried out using the manufacturers incubation buffer for 30 cycles as follows: 5 minutes denaturation at 94°C, followed by 30 cycles of denaturation at 94°C for

30 seconds; annealing at 51°C for 30 seconds; extension at 72°C for 1 minute (last cycle 5 minutes). These fragments were then sub-cloned into the pGEX backbone vector by digesting the backbone and the fragments with the indicated restriction enzymes and using DNA ligase to glue the fragments into the pGEX vector. The products were sequenced in the 5′ to 3′ direction using the following sequencing oligonucleotides to ensure that no random mutations had been introduced during the PCR process; oligo1: 5′-GCTGGCAAGCCACGTTTGGTGGTGGCGACC-3′ and oligo2: 5′-CCATTCAGCTGCCATTTATAATC-3′. The sequencing process was carried out by MWG-Biotech Ltd. The oligos were arranged as follows:



2.2: ANTIBODIES

Primary Antibodies were as follows:

14-3-3ε	Rabbit polyclonal T16 (Santa Cruz) (Blocking peptide was T16P)
DP2	Mouse monoclonal G12 (Santa Cruz)
DP3 (7.2)	Rabbit polyclonal raised against the N-terminal sequence in DP3 coded by: E V A L A T G Q L P A S N S H Q (de la Luna *et al.*, 1996)
HA	Mouse monoclonal HA11 (Babco)
myc	Mouse monoclonal 9E10 (Santa Cruz)

Secondary Antibodies were as follows:

Anti-Ig-AP	Alkaline phosphatase conjugated (Promega)
Anti-Ig-HRP	Horseradish peroxidase conjugated (Amersham)
Anti-Ig-Alexa Fluor 488	Fluorescein conjugated (Molecular Probes)
Anti-Ig-Alexa Fluor 594	Fluorescein conjugated (Molecular Probes)
Anti-CD20-FITC	Fluorescein conjugated (Becton Dickinson)

2.3: SITE-DIRECTED MUTAGENESIS

In vitro mutagenesis was carried out using the QuikChange Single Site-Directed Mutagenesis Kit (Stratagene). To obtain the 2S, 3S, 4S and 5S mutants it was necessary to create multiple rounds of individual mutations using wild type DP3δ as the initial template and the new mutant as the template thereafter. This was done by the use of the following oligonucleotide primers (Sigma-Genosys) which carried the relevant mutation in one or more bases which thereby created a product which carried the relevant serine to alanine change:

1. DP3 2S (S56A/S58A):

(5′-3′): GACTCTGATTTT**GCA**GAA**GCT**AAACGAAGCAAAAAAGGAG

(3′-5′): CTGAGACTAAAA**CGT**CTT**CGA**TTTGCTTCGTTTTTTCCTC

2. DP3 3S (S61A):

(5′-3′): GACTCTGATTTTGCAGAAGCTAAACGA**GCC**AAAAAAGGAG

(3′-5′): CTGAGACTAAAACGTCTTCGATTTGCT**CGG**TTTTTTCCTC

3. DP3 4S (S53A):

(5′-3′): GCTAGAGAATTTATAGAC**GCT**GATTTTGCAGAAGC

(3′-5′): CGATCTCTTAAATATCTG**CGA**CTAAAACGTCTTCG

4. DP3 5S (S42A):

(5′-3′): GCTGCTGGCTGGGTTCCC**GCT**GATAGAAAACG

(3′-5′): CGACGACCGACCCAAGGG**CGA**CTATCTTTTGC

After each round of mutations was complete the product was sequenced in the 5′ to 3′ direction using **both** the T7 promoter to create a sequencing oligonucleotide and the following oligonucleotide: 5′-CCATTCAGCTGCCATTTATAATC-3′ which was located further downstream. This process was carried out by MWG-Biotech Ltd. Throughout the work presented in this thesis DP3 corresponds to DP3δ since the DP3 constructs made above were constructed from the mammalian expression vector containing the full length coding sequence of DP3δ (de la Luna *et al.*, 1996).

2.4: TRANSFECTION

Cells were maintained in Dulbeccos Modified Eagle Medium (DMEM) (Gibco BRL), supplemented with 10% (v/v) Foetal Calf Serum (FCS) and antibiotics (10mg/ml streptomycin and 100U/ml penicillin) (Gibco BRL) at 37°C in a 5% CO_2/H_20 atmosphere. All cell culture and transfection work was carried out in a class II microbiological safety cabinet.

Around 24 hours before transfection the cells were gently removed from the storage flasks by addition of trypsin-EDTA (Gibco). Trypsinisation was stopped by the addition of DMEM. Cells were then plated out at a concentration of $1x10^5$ per 6-well plate (p6), $2x10^5$ per 6cm plate (p60), $5x10^5$ per 10cm plate (p100) and $2x10^6$ per 15cm plate (p150). Final DNA concentrations were made up with pSG5 or pcDNA3 empty vectors, with final volumes of DNA for each size of plate as follows: (six well plate: 8μg), (p60: 15μg), (p100: 30μg) and (p150: 90μg).

For all assays the cells were transfected using the calcium phosphate-DNA precipitate method. Based on a 15cm plate (p150), 1.35ml of distilled water was added to 90μg of total DNA. 150μl of 2.5M $CaCl_2$ was then added to the DNA/water mix and then this was then added drop-wise to 1.5ml of 2X Hepes Buffered Saline (HBS) to give a final volume of 3ml. This mix was then vortexed briefly and then left at room temperature for 30 minutes, during which time the cells were washed twice in sterile PBS and fresh media was added.

After 30 minutes the transfection mix was vortexed briefly again and was then added drop-wise to the cells. The cells were then returned to the incubators. Around 17-20 hours post-transfection the cells were washed three times in sterile PBS to remove the precipitate that had formed, 30ml of fresh DMEM containing serum and antibiotics was added and the cells were returned to the incubators to allow appropriate gene expression of the transfected plasmids. After a further 48 hours the cells were harvested for the appropriate assays. Transfections for other

sizes of plates (six well plate, p60 and p100) involved scaling of the reagents in the appropriate manner.

2.5: PURIFICATION OF RECOMBINANT GST PROTEINS

Transformed BL21s were grown overnight in a 37°C shaker in a 50ml culture. This culture was diluted in a further 450ml until bacteria had reached mid-logarithmic stage. Protein expression was induced by the addition of 0.5mM (final concentration) of IPTG (isopropyl-β-D-thiogalactopyranoside) (Melford Laboratories Ltd) and bacteria were incubated in 37°C shaker for 3 hours. Bacterial pellet was lysed in 10ml PBS with protease inhibitors then sonicated three times for 10 seconds at 4°C. Triton X-100 was then added (1% v/v) and the bacteria were left on a roller at 4°C for 30 minutes. Cells were centrifuged at 13000 rpm for 30 minutes and supernatant was removed.

300µl of glutathione-agarose beads (33% v/v) in suspension (0.05% NaN_3 in PBS) was washed in PBS (3 x 5 minutes) and the bacterial supernatant was added to the beads, which were placed on a roller at 4°C for 1 hour. The beads were washed twice with 20ml of PBS/Triton X-100 (1% v/v) then three times in 20ml PBS alone after which they were transferred to a 1.5ml eppendorf, and resuspended in GST storage buffer (PBS/PMSF (1mM final concentration)/Glycerol (10% v/v). Protein expression was measured by coomassie after SDS-PAGE.

2.6: IMMUNOPRECIPITATION

COS-7 cells were plated out onto p150 plates at a concentration of $2x10^6$ per plate. Around 65 hours post-transfection the media was removed and the cells were washed twice in non-sterile PBS. The cells were trypsinised and the trypsinisation was stopped by the addition of complete DMEM, after which the cells were transferred to 50ml falcon tubes and pelleted in a Sorvall centrifuge for 5 minutes at 1000 rpm. The media was removed and the cells were washed in PBS, transferred to a 1.5ml eppendorf and pelleted once more for 15 minutes at 13000 rpm at 4°C.

The supernatant was removed and the cells were resuspended in 1ml of micro-extraction (ME) buffer (20mM HEPES, 100mM NaCl, 25% Glycerol, 50mM NaF, 0.2mM EDTA, 0.5mM PMSF, 60mM β-Glycero-phosphate, 1mM Na_3VO_4, 0.1% NP-40 and protease inhibitor cocktail) and left on ice for 30 minutes. Cells were then centrifuged for 15 minutes at 13000 rpm, the supernatant was removed and the appropriate β-Galactosidase (β-Gal) and protein concentration assays were carried out where necessary. The cell extract was pre-cleared for 20 minutes on a rotating wheel at 4°C by adding the extract to 50µl of protein-G beads (Sigma) which had previously been equilibrated by washing in ME buffer.

The beads were centrifuged for 3 minutes at 4500 rpm at 4°C and the pre-cleared cell extract was removed to a clean 1.5ml eppendorf. After this, 5% of the extract was removed for input and the rest of the extract was added to the immunoprecipitating antibody, which had previously been coupled to protein-A

beads (Sigma). This was achieved by equilibrating 50µl of protein-A in ME buffer then adding 5µl's of the immunoprecipitation antibody to the beads in a total volume of 500µl of ME buffer. The beads and antibody were placed on a rotating wheel at 4°C for 5 hours to allow efficient binding.

Once the cell extract had been added to the protein-A/antibody complex, the samples were placed on a rotating wheel at 4°C overnight. The samples were washed three times in 1ml of ME buffer, the supernatant was removed and loading buffer was added to the beads. The samples were boiled for 3 minutes to dissociate the antigen/antibody complex from the beads then the samples were loaded onto an SDS-polyacrylamide gel, the proteins were blotted onto nitrocellulose membrane (Inverclyde Biologicals Ltd) then detected using the appropriate antibodies.

For immunoprecipitation of 14-3-3 and DP2 from ML-1 cells protocol was repeated as above except $10x10^6$ cells were collected and lysed in immunoprecipitation buffer. Immunoprecipitation from cells treated with etoposide (Sigma) was as above except a final concentration of 10µM etoposide was added to the cells 15 hours before harvesting. Immunoprecipitation assays were carried out three times unless otherwise stated.

2.7: *IN VITRO* PROTEIN EXPRESSION

In vitro transcription and translation of cDNA plasmids was carried out using the TNT T7 coupled reticulocyte lysate system from Promega. The protocol was carried out as per the manufacturer guidelines, in the presence and absence of ^{35}S methionine. To ensure expression, 10% of the extract was used in SDS-PAGE and then subjected to autoradiography overnight.

2.8: *IN VITRO* PULL-DOWN ASSAY

The cold *in vitro* translated product was firstly pre-cleared overnight. An equivalent bed volume of glutathione-agarose beads, which had been washed twice in LDB pull-down buffer (20mM HEPES, 100mM KCl, 12mM $MgCl_2$, 2mM EDTA, 17% Glycerol, 0.2mM NaF, 0.5mM PMSF, 60mM β-Glycero-phosphate, 1mM Na_3VO_4 and protease inhibitor cocktail), was added to the amount of *in vitro* translated product to be used in the assay. The *in vitro* translated product was pre-cleared in 0.5ml LDB buffer overnight on the rotating wheel at 4°C.

The beads were centrifuged at 4500 rpm for 2 minutes at 4°C and the pre-cleared supernatant was removed to a fresh 1.5ml eppendorf. The appropriate amount of GST (used as a negative control) and GST-fusion protein, bound to glutathione-agarose beads, was washed twice in LDB buffer and the pre-cleared extract was added to the beads for 1 hour on the rotating wheel at 4°C. The beads were washed 6 times in LDB buffer, SDS-sample buffer was added and the samples

were boiled for 5 minutes, electrophoresed and then subjected to SDS-PAGE electrophoreses and western blotting. *In vitro* pull-down assays were carried out three times unless otherwise stated.

2.9: PULL-DOWN FROM CELL EXTRACTS

For pull-downs from cell extracts, COS-7 cells were seeded out onto p150 plates at a concentration of $2x10^6$ per plate. Around 65 hours post-transfection the cells were removed from the plates by trypsinisation. Complete media was then added and the cells were transferred to 50ml falcon tubes and centrifuged at 1000 rpm for 5 minutes. The media was removed and the cells were washed in PBS, transferred to 1.5ml eppendorfs and centrifuged at 13000 rpm for 20 minutes at 4°C.

The supernatant was removed and the cells were resuspended in 1ml of micro-extraction buffer (ME) buffer (20mM HEPES, 100mM NaCl, 25% Glycerol, 50mM NaF, 0.2mM EDTA, 0.5mM PMSF, 60mM β-Glycero-phosphate, 1mM Na_3VO_4, 0.1% NP-40 and protease inhibitor cocktail). After lysing on ice for 30 minutes the cells were centrifuged at 13000 rpm for 20 minutes at 4°C. The supernatant was removed and the appropriate β-Galactosidase (β-Gal) and protein concentration assays were then carried out.

GST or the appropriate GST-fusion proteins coupled to glutathione-agarose beads were washed twice in ME buffer to allow equilibration with the cell extract.

Around 1mg of cell extract was added to the beads and the samples were placed on the rotator wheel at 4°C for 3 hours. The beads were washed three times in 1ml of ME buffer. SDS loading buffer was added to the samples, which were then subjected to SDS-PAGE electrophoreses and western blotting. Pull-down assays from cells were carried out three times unless otherwise stated.

2.10: IMMUNOFLUORESCENCE

COS-7 cells were plated out onto glass coverslips in six-well plates at a concentration of $1x10^5$ per plate. Around 65 hours post-transfection the cells were fixed onto coverslips using 4% (w/v) paraformaldehyde (Sigma). The cells were then permeabilised using 1ml of 1% (v/v) Triton X-100 (Sigma).

After 15 minutes the cells were blocked in 10% (v/v) FCS in PBS. The appropriate primary antibodies, diluted to 1 in 500 in 1% FCS (v/v) in PBS, were added to the coverslips and left at room temperature for 30 minutes. The primary antibodies were removed and the cells were subjected to 3x10 minute washes in PBS with 10% (v/v) FCS, followed by a 1x10 minute wash in PBS alone.

The coverslips were incubated for 30 minutes in the appropriate secondary antibodies; Alexa Fluor 488 goat anti-mouse or Alexa Fluor 594 goat anti-rabbit IgG conjugated to fluorescein succinimidyl (Molecular Probes), diluted to 1 in 400 in 10% FCS (v/v) in PBS. The secondary antibodies were removed and the cells were given 5x10 minute washes in PBS alone.

To allow detection of the cells on the coverslips the cell nuclei were stained with a final concentration of 5μM of DAPI (4',6-Diamidino-2-phenylindole dihydrochloride). The coverslips were dried and finally mounted onto glass slides using citifluor (Citifluor Ltd) before being viewed using a fluorescence microscope (Olympus).

2.11: LEPTOMYCIN-B TREATMENT

This assay was carried out as per the immunofluorescence protocol except that, 16 hours before harvesting the cells, leptomycin-B (Novartis) was added to a final concentration of 1μM.

2.12: FLUORESCENCE ACTIVATED CELL SORTING (FACS)

For FACS analysis, COS-7 cells were plated out onto p100 plates at a concentration of $5x10^5$ per plate. Around 65 hours post-transfection the cells were dissociated from the dishes using 1ml of cell dissociation buffer (Sigma).

The cells were scraped on ice into polypropylene tubes, centrifuged at 1000 rpm for 3 minutes, the supernatant was removed and the cells were washed in PBS. 200μl of DMEM containing 20μl of anti-CD20 antibody leu-16 (Becton Dickinson) coupled to fluorescein isothiocyanate (FITC) was added to the cells. The cells were washed again then fixed overnight by the addition, dropwise, of a 50% ethanol/50% PBS solution.

The following day the cells were washed then resuspended in 400µl of propidium iodide (20µg/ml) and 200µl RNase (125U/ml) in PBS and incubated in the dark, on ice for 20 minutes. Flow cytometry was performed using a Becton Dickinson fluorescence activated cell sorter. The cell cycle profile of the transfected population of cells was determined by analysing the intensity of the propidium iodide staining in cell populations that were positive for FITC staining. At least 10,000 events were counted in each sample.

2.13: LUCIFERASE REPORTER ASSAY

For reporter assays, COS-7 cells were plated out onto p60 plates at a concentration of $2x10^5$ per plate. Around 65 hours post-transfection the cells were removed from the dishes using 300µl of reporter lysis buffer (25mM TRIS-H_3PO_4 pH 7.8, 2mM 1,2-diaminocyclohexane tetra-acetic acid, 2mM DTT, 10% glycerol and 1% Triton X-100). The cells were collected into1.5ml eppendorf tubes and centrifuged at 13,000 rpm for 20 minutes.

The supernatant was removed and 50µl's was taken from each sample and added to 50µl of β-Galactosidase substrate buffer (200mM sodium phosphate buffer pH 7.3, 2mM $MgCl_2$, 100mM β-mercaptoethanol and 1.33mg/ml O-nitrophenyl-β-D-galactopyranoside) to determine transfection efficiency. To assay for luciferase activity 50µl was removed from each sample and added to an 80 well plate which was then placed into a Microlumat Plus luminometer (Berthold Technologies) which injected 100µl of luciferase assay reagent (Promega) into each sample.

2.14: CELL PROLIFERATION (Brdu) ASSAY

COS-7 cells were seeded out onto coverslips in six-well plates at a concentration of $1x10^5$ per well. Around 65 hours post-transfection 10μM (final concentration) Brdu (5-bromo-2'-deoxyuridine) (Roche) was added to the cells in culture and left for 1 hour. The cells were washed twice in non-sterile PBS and fixed for 45 minutes in ethanol (70%) in 50mM glycine buffer, pH 2.

The coverslips were washed twice in PBS and one coverslip was removed and used in an immunofluorescence assay to ensure efficient expression of the exogenous protein. The remaining coverslips were simultaneously incubated with 5U/ml DNAse and the appropriate dilution of anti-Brdu-FLUOS antibody for 1 hour at 37°C. The coverslips were washed three times in PBS, stained with DAPI and mounted onto glass slides using citifluor. The samples were viewed using a fluorescence microscope (Olympus).

2.15: TUNEL ASSAY

COS-7 cells were seeded out onto coverslips in six-well plates at a concentration of $1x10^5$ per well. Around 65 hours post-transfection the cell were fixed onto coverslips by the addition of 4% (w/v) paraformaldehyde for 1 hour. One coverslip was removed and used in an immunofluorescence assay to ensure expression of the exogenous proteins. The coverslips were washed twice in PBS and permeabilised on ice for 2 minutes by the addition of 1% (v/v) Triton X-100 in PBS. The coverslips were washed again in PBS and the TUNEL (TdT-

mediated dUTP-X nick end labelling) reaction mixture (50% terminal transferase/50% fluorescein-dUTP) was added for 1 hour at 37°C.

The samples were washed twice in PBS and rinsed in DAPI stain to enable detection of the cells. The coverslips were mounted on glass slides using citifluor and analysed using a fluorescence microscope.

2.16: CYCLOHEXIMIDE TREATMENT

COS-7 cells were seeded out onto p150 plates at a concentration of $2x10^6$ per plate. After washing off the DNA precipitate, the cells on each p150 were trypsinised and split into the relevant number of p100 plates depending on the number of time points. This method ensured that transfection efficiency was maintained between each time point.

Around 48 hours later a final concentration of 0.01μg/μl cycloheximide (Promega) was added to the relevant plates and each plate was harvested thereafter at the correct time point. The cell extracts were lysed in ME buffer and the supernatant was removed after which the samples were subjected to SDS-PAGE and finally western blotting.

2.17: WESTERN BLOTTING

To allow measurement of protein expression in cells the extracts were lysed in ME buffer, left on ice for 30 minutes and centrifuged at 13000 rpm for 20 minutes at 4°C. The supernatant was removed and protein concentration was measured by use of Bradford reagent (Biorad). 100μg of total protein was loaded onto a SDS polyacrylamide gel and the samples were subjected to western blotting onto nitrocellulose membrane (Inverclyde Biochemicals Ltd), then blocked in PBS/10% (w/v) powdered milk.

The membrane was washed three times for 10 minutes in PBS/0.1% Tween-20 (Sigma) and the primary antibody was added overnight. The membrane was washed three times in PBS/Tween and the appropriate secondary antibodies were added [either alkaline phosphatase-conjugated goat anti-rabbit or anti-mouse IgG (1:5000, Promega) for chemi-luminescent detection or horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG (1:5000, Amersham) for ECL].

Chapter 3: A nuclear DP3 mutant that cannot bind to 14-3-3

3.1: Introduction

Recent work in this laboratory (Cruickshank, 2000) has shown that DP3 interacts with the epsilon isoform of 14-3-3 (14-3-3ε). This interaction is mediated via the region containing the bi-partite nuclear localisation signal (NLS) of DP3. Furthermore it has been shown that this interaction is dependent on the basic region of the NLS [Figure 3.1(i) and (ii)]. Removal of this region (creating the DP3 ΔB construct) abrogates the ability of 14-3-3ε to bind DP3, and to thereby influence E2F function (Cruickshank, 2000).

However, despite providing a useful insight into the nature of the relationship between 14-3-3 and the DP3/E2F heterodimer, the DP3 ΔB mutant has its limitations as an effective biochemical tool since it becomes localised to the cytoplasm. Given the role that 14-3-3 proteins are known to play in mediating the sub-cellular localisation of many of their target substrates, this raised the question of whether the cytoplasmic localisation was due to disruption of the NLS or whether 14-3-3 plays a role in mediating the nuclear localisation of DP3. The cytoplasmic localisation of DP3 ΔB also complicates the studies aiming to understand the role of a nuclear transcription factor complex.

The work described in this chapter aimed to identify a DP3 mutant that was unable to bind 14-3-3 but continued to localise to the nucleus. Such a mutant would allow the further elucidation of the nature of the E2F/DP3/14-3-3 interaction.

3.2: RESULTS

3.2A: Identification of a DP3 mutant that cannot bind 14-3-3 *in vitro*

In order to identify a mutant of DP3 that was unable to bind to 14-3-3 but retained both the basic region and its nuclear localisation, a series of mutants were made that contained amino acid residue changes within the NLS of the protein [Figure 3.1(i)]. It is known that the various isoforms of 14-3-3 predominantly bind to their target ligands via phosphorylated serine residues (Muslin *et al.*, 1996; Yaffe *et al.*, 1997). For that reason point mutations were generated in the DP3 plasmid construct. These created serine to alanine substitutions within the NLS of DP3 [Figure 3.1(i)].

In order to test the ability of each mutant to bind to 14-3-3 *in vitro* both wt DP3 and each one of the mutant (2S, 3S, 4S and 5S) were cold *in vitro* translated [Figure 3.2A(ii)] and then incubated with either purified GST alone (negative control) or purified GST-14-3-3ε [Figure 3.2A(i)]. As can be seen from Figure 3.2A(iii), the amount of the 2S, 3S and 4S mutants binding to 14-3-3 is reduced compared to the amount of wt (compare lanes 1, 3, 5 and 7). Despite this, each of these mutants was seen to bind to 14-3-3 whereas the 5S mutant completely lost its ability to bind to 14-3-3 (lane 9). None of the DP3 proteins were seen to bind GST alone (compare lanes 2, 4, 6, 8 and 10). This indicated that the binding of the DP3 proteins to 14-3-3 was specific. It should be noted that over-expression of DP3 in cells results in a doublet that runs at approximately 45KD (see Figure

3.2C) however it can be seen from Figure 3.2A that *in vitro* translation results in a single band. The identity of both DP3 bands is unknown at this time however it is postulated that they represent either proteolytic digestion of the upper band or that the lower band represents translation from an internal initiating methionine within the cDNA. Although this is unlikely, it is possible if leaky translation occurs from the first methionine.

It is known that both of these bands do represent DP3 since previous work in the laboratory has shown that both bands are competed out with peptide competition of the DP3 antibody. The upper band does not represent a phosphorylated form of DP3 since this band does not disappear upon phosphatase treatment. The presence of only one band in Figure 3.2A indicates that, whatever the reason for the DP3 doublet *in vivo*, this is not reproduced *in vitro*. It is known however that this single band does represent DP3 since this band is not produced in an *in vitro* control translation.

3.2B: The DP3 5S mutant is unable to bind 14-3-3 *in vitro*

In order to confirm the result from Figure 3.2A, that the DP3 5S mutant was unable to bind 14-3-3, it was decided to use a pull-down system from cell extracts. Both the wt DP3 and the 5S mutant were sub-cloned into a GST tagged bacterial expression vector (pGEXKG) and the GST tagged DP3 wt and 5S mutant proteins were purified [Figure 3.2B(i)]. Transiently transfected COS-7 cells expressing myc-tagged 14-3-3 were harvested and lysed in microextraction buffer (as described in Materials and Methods).

The cellular extracts were incubated with either GST alone (negative control), GST-DP3 wt or GST-DP3 5S mutant. As can be seen from Figure 3.2B(ii) the 14-3-3 was seen to bind to only the wt GST DP3 construct (lane 3) and not the 5S mutant (lane 4), with no 14-3-3 being seen to bind to GST alone (lane 2). This indicated that the interaction between DP3 and 14-3-3 was specific. This result suggested that *in vitro* at least the DP3 5S mutant was unable to effectively complex with 14-3-3 compared to the wt DP3.

3.2C: The DP3 5S mutant is unable to bind 14-3-3 *in vivo*

To provide evidence that the 5S mutant had lost its ability to bind to 14-3-3 *in vivo* the interaction between those two proteins was assayed by immunoprecipitation. Transiently transfected COS-7 cells, expressing myc-tagged 14-3-3ε and either DP3 wt or DP3 5S were harvested, lysed in microextraction buffer (as described in Materials and Methods) and the cellular extracts were

immunoprecipitated with either the rabbit polyclonal DP3 antibody, 7.2 [Figure 3.2C(i)] or the rabbit polyclonal antibody directed against the epsilon isoform of 14-3-3 [Figure 3.2C(ii)].

Figure 3.2C(i) shows that equal amounts of wt and 5S DP3 were immunoprecipitated (lanes 5 and 6 respectively) and these amounts were equivalent to the amount of protein in the input (lanes 2 and 3). The amount of 14-3-3 present in either complex was remarkably different however. A significant amount of 14-3-3 co-precipitated with the wt (lane 5) however no 14-3-3 was present in the sample expressing DP3 5S (lane 6). No detectable 14-3-3 co-precipitated with the DP3 antibody alone (lane 4), suggesting that the 14-3-3 seen in lane 5 forms a specific complex with wt DP3.

The immunoprecipitation experiment was repeated using the antibody directed against 14-3-3ε. Again, similar amounts of 14-3-3 were immunoprecipitated (lanes 7 and 8) when compared to the input lanes (lanes 3 and 4) however the amount of DP3 co-immunoprecipitating was significantly different. Comparing DP3 levels in lanes 7 and 8 it can be seen that wt DP3 was present in the complex with 14-3-3 but that the 5S mutant was not. The control immunoprecipitation experiments using the 14-3-3 antibody incubated with either DP3 wt (lane 5) or the mutant (lane 6) proved negative, implying that the co-immunoprecipitation of wt DP3 with 14-3-3 was specific.

3.2D: Endogenous 14-3-3 and DP2 form a complex

In the literature DP3 and DP2 are the same protein, DP3 being the murine form of human DP2. It was of interest to investigate whether DP3 and 14-3-3 could interact at an endogenous level however the DP3 antibody, 7.2, had certain limitations in that it could not recognise the endogenous DP3 protein. To overcome this it was decided to look at the interaction between DP2 and 14-3-3 in the human myeloid leukaemia cell line, ML-1. Despite not being able to assay the 5S mutant for its ability to bind 14-3-3 at an endogenous level, this experiment would provide some idea of how physiologically relevant the interaction between DP2 and 14-3-3 was.

To test this, the ML-1 cell line was lysed in microextraction buffer and the cell extract was subjected to immunoprecipitation with a polyclonal antibody specifically directed against the epsilon isoform of 14-3-3. Detection of DP2 was carried out using a monoclonal antibody specific for DP2. Figure 3.2D, lane 4 shows that DP2 (top track) is forming a complex with 14-3-3 (bottom track) when the extract is immunoprecipitated with the 14-3-3ε antibody. To ensure that the band seen in lane 4 was 14-3-3ε, peptide competition was carried out. The 14-3-3ε antibody was incubated with the peptide that it was raised against and this was then incubated with the right half of lane 4. It can be seen that this band disappears when the antibody is incubated with the peptide and the membrane, indicating that this band is 14-3-3ε. No detectable amounts of either DP2 or 14-3-3ε were seen to form a complex with a control antibody, implying that the

interaction between DP2 and 14-3-3ε was specific (lane 3, lower and upper tracks respectively).

Since no peptide was available to show that the band observed was DP2, it was necessary to run the input lane from the endogenous IP against extract with over-expressed DP3 and 14-3-3ε from COS-7 cells. This showed that endogenous DP2 in ML-1 cells runs at the same size as the upper form of over-expressed DP3 in COS-7 cells (compare top track, lanes 1 and 2). Endogenous 14-3-3ε in ML-1 cells runs just below over-expressed 14-3-3ε, and at the same size as endogenous 14-3-3ε, in COS-7 cells (compare bottom track, lanes 1 and 2).

3.2E: The DP3 5S mutant retains nuclear localisation

It has been reported that DP3 is localised in the nucleus due to the fact that it contains a bi-partite NLS (de la Luna *et al.*, 1996). Nuclear localisation thereby allows a further level of control over E2F (Allen *et al.*, 1997). Since the 5S mutant contained changes within the NLS it was necessary to ensure that, before using the mutant in further studies, that it retained the ability to localise in the nucleus.

To address this question COS-7 cells were transiently transfected with either the wt DP3 protein or the 5S mutant. The cells were then stained with the polyclonal 7.2 antibody and subjected to immunofluorescence. Figure 3.2E shows that both the wt DP3 protein and the 5S mutant were able to effectively localise to the nucleus (compare C and D). This result showed that, despite having multiple point

mutations within its NLS region, the DP3 5S mutant retained the ability to localise in the nucleus.

3.3 CONCLUSIONS

It has recently been shown that effective nuclear localisation of DP3 is dependent on its N-terminal bi-partite NLS domain (de la Luna *et al.*, 1996). Subsequent work identified this region of DP3 as important in the complex formation with the 14-3-3 family of proteins (Cruickshank, 2000).

Deletion of the basic portion of the NLS resulted in the abrogation of the ability of the DP3 protein to bind 14-3-3. However this also resulted in the cytoplasmic localisation of DP3 ΔB. This raised the question of whether 14-3-3 played a role in the nuclear localisation of the DP3/E2F complex. Also, to allow further investigation of the relationship between DP3 and 14-3-3 it was necessary to generate a mutant DP3 that was unable to bind 14-3-3 but still retained the ability to accumulate in the nucleus.

Given that 14-3-3 proteins have been shown to bind to their target ligands via phosphorylated serine residues (Muslin *et al.*, 1996; Yaffe *et al.*, 1997), it was queried whether this was the case with DP3 since the NLS of this molecule contained five serines in total. Consequent generation and analysis of a series of mutants containing serine to alanine changes within the NLS resulted in the identification of a mutant which bears changes to all serines within the known 14-3-3 binding region.

Subsequent biochemical analysis of this mutant showed that it was unable to bind to 14-3-3 and that, despite the multiple changes made within the bi-partite NLS

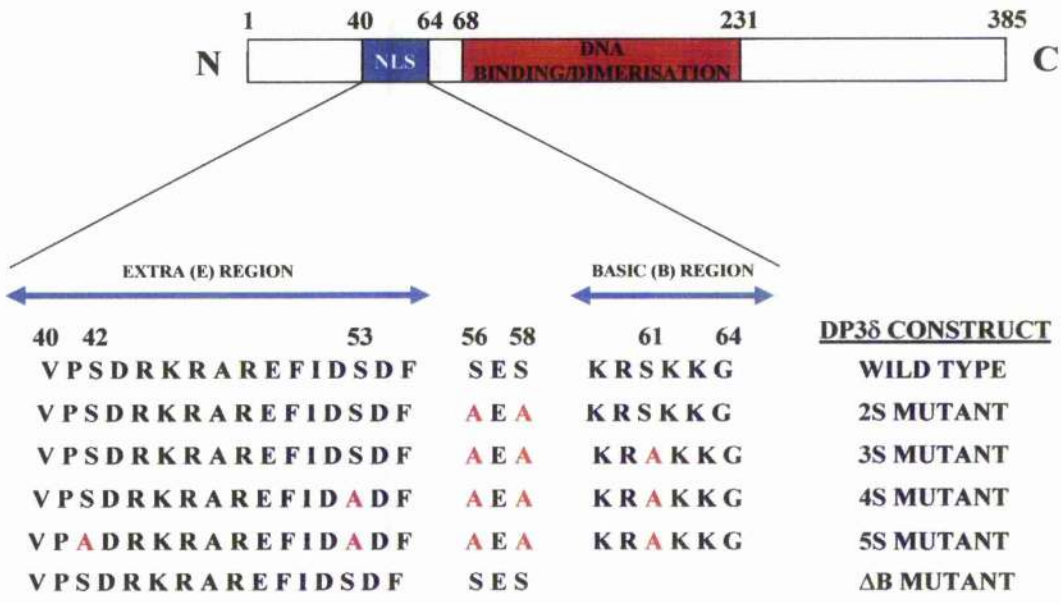
domain of the molecule, it still retained the ability to localise in the nucleus. This result provides evidence that one of the roles for 14-3-3 is not in the nuclear localisation or retention of DP3. The generation of a nuclear DP3 that is unable to bind to 14-3-3 also provides a powerful tool in the understanding of the influence that 14-3-3 exerts on the activity of E2F, via the interaction with DP3. Furthermore, Figure 3.2D showed that DP2 and 14-3-3 could interact at an endogenous level. This was an important result since it showed that this interaction is physiologically relevant. Figure 3.1(ii) provides a tabular overview of the properties of the various DP3 proteins used in the study.

Figure 3.1: The DP3δ Proteins

i. Diagrammatical representation of the DP3δ proteins used in the study. The DP3ΔB mutant used in the previous study is also shown (Cruickshank, 2000).

ii. Tabular overview showing the sub-cellular localisation and the ability to bind 14-3-3 of each of the DP3δ proteins.

i)



ii)

DP3δ PROTEIN	SUBCELLULAR LOCALISATION	14-3-3 BINDING
Wild Type	Nucleus	Yes
2S Mutant	Nucleus	Yes
3S Mutant	Nucleus	Yes
4S Mutant	Nucleus	Yes
5S Mutant	Nucleus	No
ΔB	Cytoplasm	No

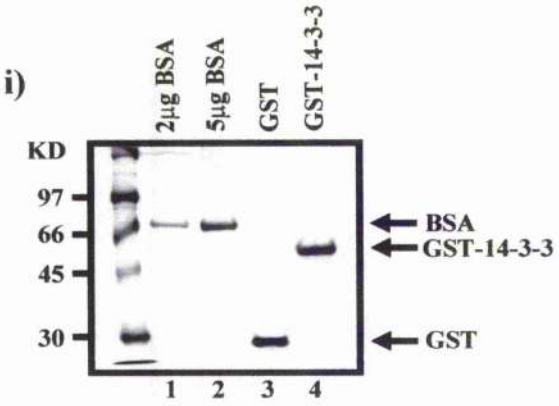
Figure 3.2A: Identification of a DP3 mutant that cannot bind 14-3-3 *in vitro*

i) Coomassie stain showing bacterially expressed GST (lane 3), GST-14-3-3 (lane 4) and 2μg and 5μg BSA (lanes 1 and 2 respectively) as protein standards.

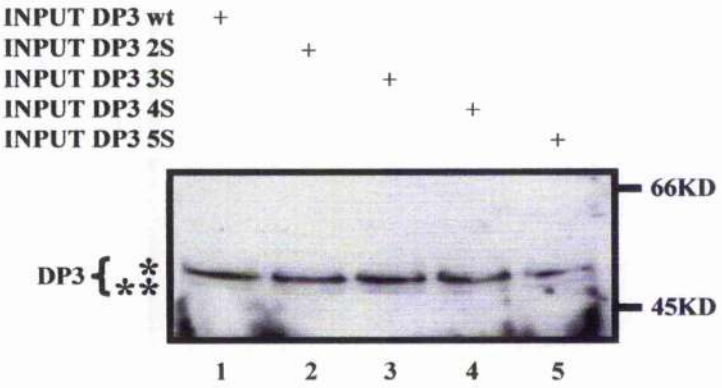
ii) 1μg of wt DP3, DP3 2S, 3S, 4S and 5S mutants were cold *in vitro* translated. Input levels of the various DP3 constructs are shown. DP3 was detected using the anti-DP3 antibody, 7.2. The * and ** indicate the upper and lower bands of DP3 respectively.

iii) Equal amounts of each extract were used in a pull-down assay. The samples were incubated with either 5μg GST (as a negative control) or 5μg of GST-14-3-3. DP3 was detected using the anti-DP3 antibody, 7.2. The * indicates the upper band of DP3.

i)


2μg BSA
5μg BSA
GST
GST-14-3-3
KD
97
66
45
30
BSA
GST-14-3-3
GST
1
2
3
4

ii)


INPUT DP3 wt +
INPUT DP3 2S +
INPUT DP3 3S +
INPUT DP3 4S +
INPUT DP3 5S +
66KD
45KD
DP3 {***
1
2
3
4
5

iii)

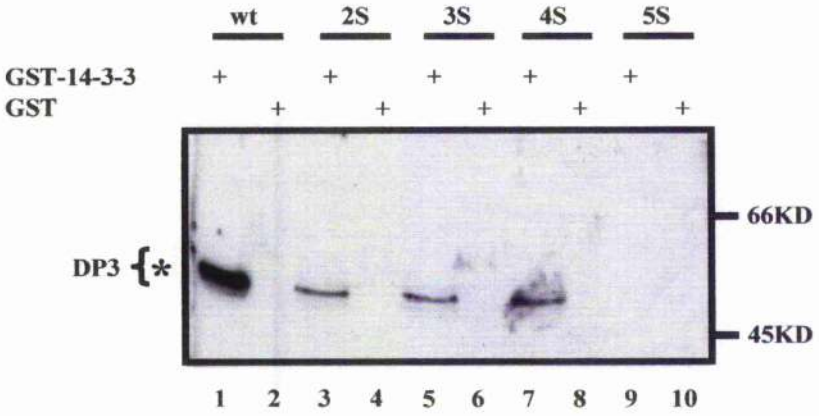
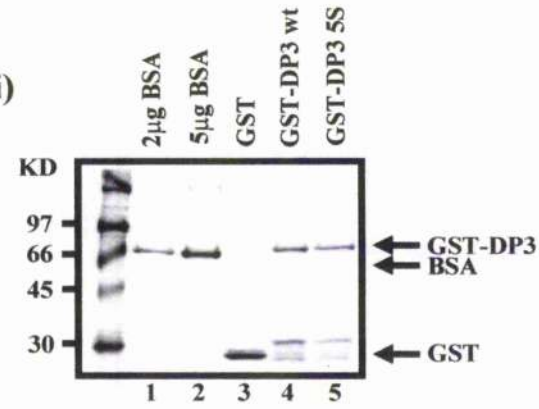

wt
2S
3S
4S
5S
GST-14-3-3 + + + + +
GST + + + + +
66KD
45KD
DP3 {*
1
2
3
4
5
6
7
8
9
10

Figure 3.2B: <u>The DP3 5S mutant is unable to bind 14-3-3 *in vitro*</u>

i) Coomassie stain showing bacterially expressed GST (lane 3), GST-DP3 wt (lane 4), GST-DP3 5S mutant (lane 5) and 2μg and 5μg BSA (lanes 1 and 2 respectively) as protein standards.

ii) COS-7 cells were transiently transfected with the following amount of the indicated plasmid: 50μg myc-tagged 14-3-3. Around 65 hours post-transfection the samples were harvested and used in a pull-down assay. The extracts were incubated with either 5μg of GST, 5μg of GST-DP3 wt or 5μg of GST-DP3 5S mutant. The exogenous (Ex.) 14-3-3 was detected using the anti-myc antibody.

i)


2μg BSA
5μg BSA
GST
GST-DP3 wt
GST-DP3 5S
KD
97
66
45
30
GST-DP3
BSA
GST
1
2
3
4
5

ii)

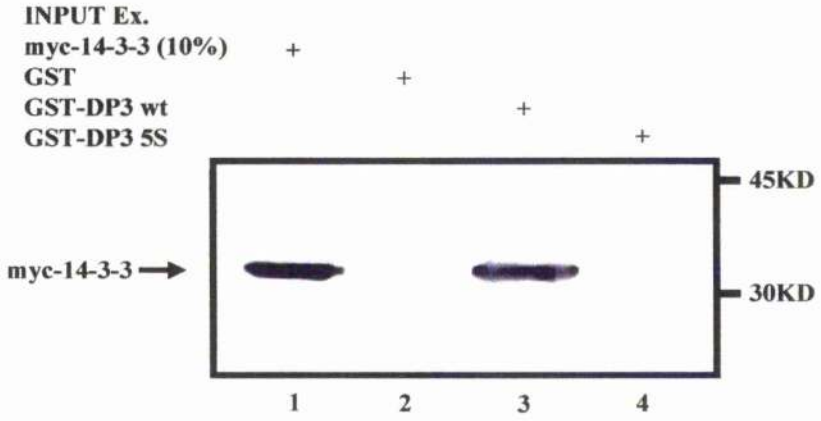
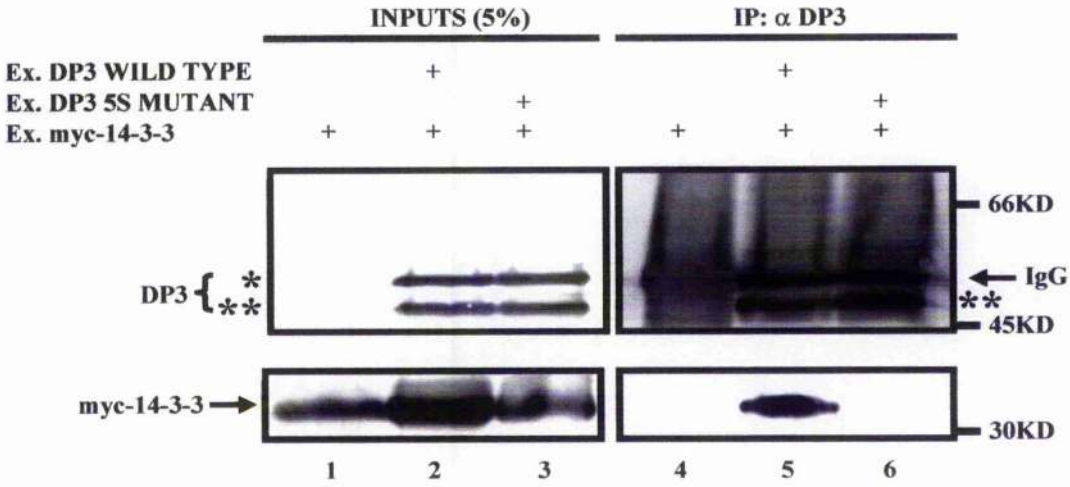

INPUT Ex.
myc-14-3-3 (10%)
GST
GST-DP3 wt
GST-DP3 5S
+
+
+
+
45KD
30KD
myc-14-3-3
1
2
3
4

Figure 3.2C: <u>The DP3 5S mutant is unable to bind 14-3-3 *in vivo*</u>

i) COS-7 cells were transiently transfected with the following amounts of the indicated plasmids: 20μg DP3 wt, 20μg DP3 5S mutant, 40μg myc-tagged 14-3-3 as well as 5μg β-Gal as an internal transfection control (Ex.=Exogenous). Around 65 hours post-transfection the samples were harvested and subjected to immunoprecipitation with the specific anti-DP3 antibody, 7.2. DP3 was detected using the anti-DP3 antibody, 7.2 whilst 14-3-3 was detected using the anti-myc antibody. The * and ** indicate the upper and lower bands of DP3 respectively and the position of the IgG is indicated.

ii) COS-7 cells were transiently transfected with the following amounts of the indicated plasmids: 20μg DP3 wt, 20μg DP3 5S mutant, 40μg myc-tagged 14-3-3 as well as 5μg β-Gal as an internal transfection control (Ex.=Exogenous). Around 65 hours post-transfection the samples were harvested, and subjected to immunoprecipitation with the specific anti-14-3-3ε antibody, T16. DP3 was detected using the anti-DP3 antibody, 7.2 whilst 14-3-3 was detected using the anti-myc antibody. The * and ** indicate the upper and lower bands of DP3 respectively and the position of the IgG is indicated.

i)


INPUTS (5%)
IP: α DP3
Ex. DP3 WILD TYPE
Ex. DP3 5S MUTANT
Ex. myc-14-3-3
66KD
IgG
45KD
30KD
DP3
myc-14-3-3
1 2 3 4 5 6

ii)

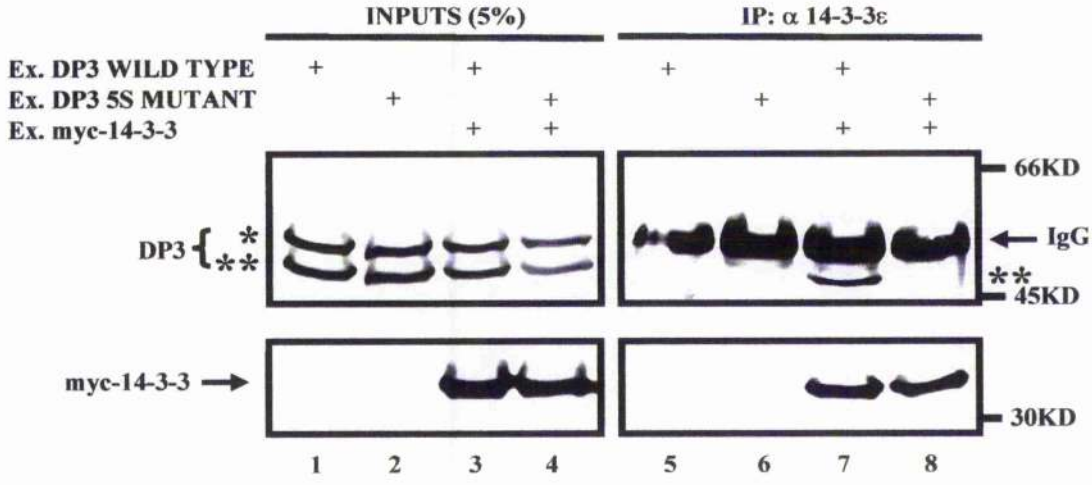

INPUTS (5%)
IP: α 14-3-3ε
Ex. DP3 WILD TYPE
Ex. DP3 5S MUTANT
Ex. myc-14-3-3
66KD
IgG
45KD
30KD
DP3
myc-14-3-3
1 2 3 4 5 6 7 8

Figure 3.2D: Endogenous 14-3-3 and DP2 form a complex

ML-1 cells were harvested and subjected to immunoprecipitation with the antibody T16, which is specific for 14-3-3ε. Detection of DP2 was carried out using the monoclonal anti-DP2 antibody, G12, whilst detection of 14-3-3 was carried out using T16 (Ex.=Exogenous and En.=Endogenous). Control immunoprecipitation was carried out using alkaline phosphatase conjugated secondary antibody (Promega) (lane 3). Peptide competition between the T16 antibody and the peptide that it is raised against was carried out in the right half of lane 4 (bottom panel) to ensure that the band seen in the left half of lane 4 (bottom panel) was endogenous 14-3-3ε. To show that the bands seen in lane 2 (upper and lower panels) were indeed endogenous DP2 and 14-3-3ε, cell extract from COS-7 cells with over-expressed DP3 and myc-14-3-3ε was ran next to the endogenous extract from the ML-1 cells. This shows that the bands seen in lane 2 line up with the bands seen in lane 1. The • indicates an unidentified band picked up by the 14-3-3ε antibody that may be another isoform of 14-3-3 that cross-reacts with the antibody. The * and ** indicate the upper and lower bands of DP3 respectively.

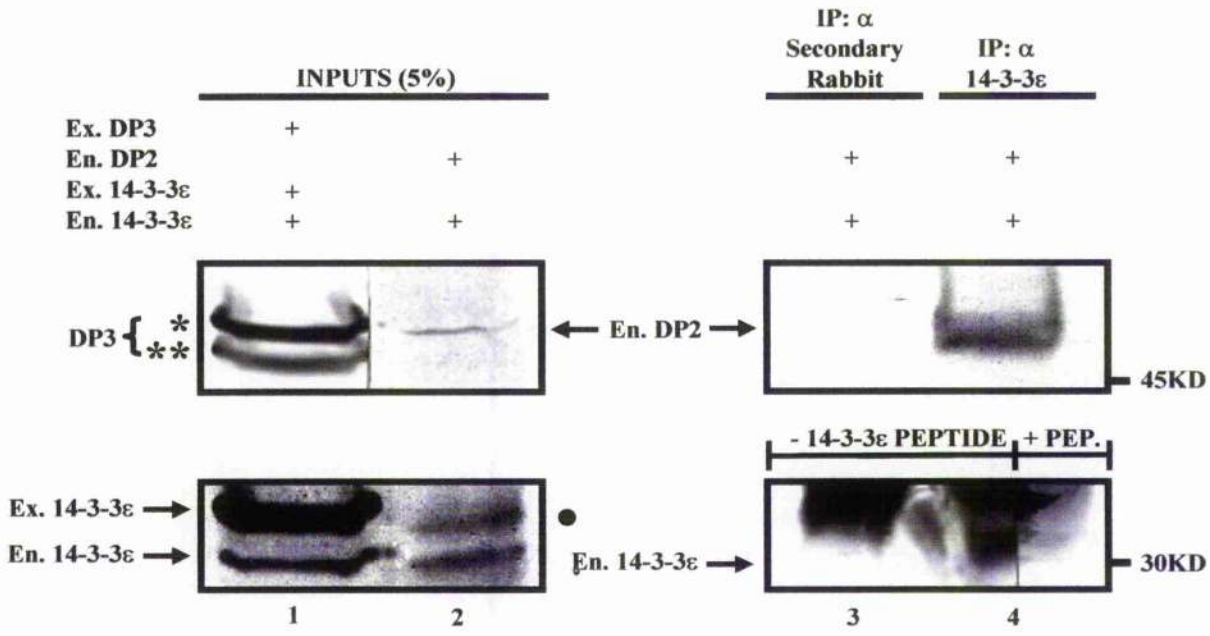
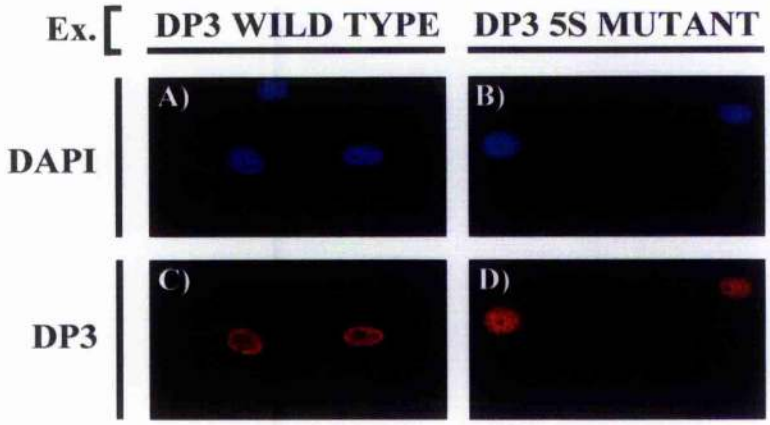

IP: α
Secondary
Rabbit
IP: α
14-3-3ε
INPUTS (5%)
Ex. DP3 +
En. DP2 + + +
Ex. 14-3-3ε +
En. 14-3-3ε + + + +
DP3 *
**
En. DP2
45KD
- 14-3-3ε PEPTIDE
+ PEP.
Ex. 14-3-3ε
En. 14-3-3ε
En. 14-3-3ε
30KD
1
2
3
4

Figure 3.2E: The DP3 5S mutant retains nuclear localisation

COS-7 cells were transiently transfected with the following amounts of the indicated plasmids: 2μg DP3 wt or 2μg DP3 5S mutant. Around 65 hours post-transfection the cells were subjected to immunofluorescence. Cellular DNA was stained with DAPI (parts A and B) whilst detection of the exogenously (Ex.) expressed DP3 was carried out using the polyclonal antibody, 7.2. The assay was carried out blind and the data shown is representative of three independent experiments. DP3 wt (part C); DP3 5S (part D).


Ex.
DP3 WILD TYPE
DP3 5S MUTANT
DAPI
DP3
A)
B)
C)
D)

Chapter 4: Characterisation of the DP3 5S mutant

4.1: Introduction

The DP3 gene gives rise to at least four distinct splice variants. The α and δ protein products contain the bi-partite NLS whilst the β and γ protein products contain only the basic portion of this signal, thus rendering them cytoplasmic (de la Luna *et al.*, 1996) [Figure 1.2B(i)]. The NLS spans amino acid residues 40-64 with the DNA binding/dimerisation domains spanning amino acid residues 56-146 [Figure 3.1(i)].

With the close proximity of these regions it was therefore deemed necessary to further characterise the DP3 5S mutant in a series of biochemical assays. This would ensure that it possessed properties like the wild type protein in terms of its abilities to interact with an E2F partner and to drive an NLS deficient E2F into the nucleus. It was therefore necessary to ensure that the only function that the DP3 5S mutant lacked was its ability to bind to 14-3-3 and that it was not defective in any other way that could prejudice the study of the relationship between E2F and 14-3-3, mediated via DP3.

4.2 RESULTS

4.2A: The DP3 5S mutant binds E2F5 *in vitro*

For effective transcriptional activation, E2F exists as a complex between E2F and a DP partner (Bandara *et al.*, 1993). It was therefore important to ensure that the DP3 5S mutant still retained its ability to complex efficiently with E2F. Previous work in the laboratory, exploring the relationship between DP3 and E2F had largely involved using E2F5 in the studies. This was because E2F5 is constitutively expressed in cells and lacks an NLS. This allowed the investigation of the effects of an NLS-containing DP partner on E2F. It was therefore decided to use E2F5 in all of the following studies.

We first addressed the interaction between DP3 wt or the 5S mutant and E2F5 *in vitro* using the GST-fusion DP3 wt and 5S mutant previously described [Chapter 3, Figure 3.2(B)]. Transiently transfected COS-7 cells expressing HA-tagged E2F5 were harvested, lysed in microextraction buffer and equal amounts of the extract were incubated with either GST alone, GST-DP3 wt or GST-DP3 5S.

As can be seen from Figure 4.2A(ii), although no E2F5 was in complex with GST alone (lane 2) equal amounts of the protein were present in a complex with both the wt DP3 (lane 3) and the 5S mutant (lane 4). These amounts are roughly equal to the amount of input protein (10% of the extract used in each point). From these *in vitro* studies it can be concluded that the 5S mutant is able to bind to E2F5 as effectively as wt DP3.

4.2B: The DP3 5S mutant binds E2F5 *in vivo*

In order to confirm that the 5S mutant retained its ability to bind to E2F5 *in vivo*, COS-7 cells were transiently transfected with E2F5 expression vector, wt or 5S DP3 and β-Gal as an internal control. An immunoprecipitation assay was performed using the polyclonal DP3 antibody, 7.2.

Figure 4.2B shows that equal amounts of DP3 wt (lower track, lane 5) or 5S mutant (lower track, lane 6) were immunoprecipitated. The amount of E2F5 co-immunoprecipitating with both the wt DP3 and the mutant DP3 was the same (compare upper track, lanes 5 and 6 respectively). The interaction between DP3 wt or 5S mutant with E2F5 was specific since there was no detectable E2F5 binding to 7.2 Ab alone (upper track, lane 4).

The expression levels of DP3 wt and DP3 5S mutant were the same (compare lower track, lanes 2 and 3 respectively), as were the E2F5 levels in these samples (compare upper track, lanes 2 and 3). This result allowed the conclusion that the 5S mutant is able to form a complex with E2F5 *in vivo* to a similar level as the wt DP3.

4.2C: The 5S mutant localises E2F5 to the nucleus

The sub-cellular localisation of both E2F4 and E2F5 during the cell cycle differs remarkably from that of the other members of the E2F family. E2F4 and E2F5 are present in the nucleus predominantly in G0 and early G1. During mid-G1, they localise in the cytoplasm (Dyson, 1998). The fact that both of these E2F's lack an intrinsic NLS, which DP3 possesses, points to a level of control that DP3 can exert over E2F4 and E2F5, and therefore, gene expression.

In order to ensure that the 5S mutant could still efficiently drive an NLS-deficient E2F into the nucleus, COS-7 cells were transiently transfected with E2F5 alone or E2F5 and either wt DP3 or the 5S mutant. Immunofluorescence studies were then carried out. Figure 4.2C shows that both wt DP3 and the 5S mutant localise to the nucleus (compare E and F), a result in accordance with Figure 3.2E.

When expressed alone, E2F5 displayed a cytoplasmic localisation (part G). Nuclear localisation of E2F5 could be seen when E2F5 was co-expressed with either the wt DP3 or the 5S mutant (compare II and I). This result allowed the conclusion that, despite the multiple changes within the bi-partite NLS region of the DP3 5S mutant, this protein was still able to drive an NLS deficient E2F partner into the nucleus.

4.2D: E2F5 has no effect on the ability of DP3 to bind to 14-3-3

Taken together, experimental work pursued towards characterising the 5S mutant showed that this mutant was still able to act like the wt DP3 in its ability to bind to E2F5 and to drive E2F5 into the nucleus. In order to address the question whether E2F5 had an effect on the ability of DP3 to bind 14-3-3, COS-7 cells were transiently transfected with wt DP3 and 14-3-3 in the presence or absence of E2F5 expression vector. The cells were harvested and subjected to immunoprecipitation with the anti-DP3 antibody, 7.2.

As can be seen from Figure 4.2D, the amount of 14-3-3 being co-immunoprecipitated with the wt DP3 is similar irrespective of the presence of E2F5 (compare lanes 5 and 6, bottom track). The amount of DP3 immunoprecipitated in the presence or absence of E2F5 is unchanged (compare lanes 5 and 6, top track). Lanes 1, 2 and 3 show the levels of the input protein. Lane 4 shows that no 14-3-3 was present in a complex with the DP3 antibody alone, suggesting that the complex formations seen in lanes 5 and 6 were specific. This result allowed the conclusion that the ability of DP3 and 14-3-3 to form a complex is not dependent on the presence or absence of E2F5.

4.3 CONCLUSIONS

In this chapter a series of experiments were undertaken in order to elucidate that the DP3 5S mutant was the proper biochemical tool towards analysing in detail the functional role of the E2F/DP3/14-3-3 complex. E2F5 was chosen since it is highly expressed throughout the cell cycle and lacks an NLS.

The results of this work firstly showed that the DP3 5S mutant could bind to E2F5 both *in vitro* and *in vivo* to a comparable level to that of the wt DP3 protein (Figures 4.2A and 4.2B). Importantly this mutant still retained the ability to drive the NLS-deficient E2F5 into the nucleus, the same way as the wt DP3 (Figure 4.2C). Finally E2F5 had no effect on the ability of DP3 to form a complex with 14-3-3 (Figure 4.2D).

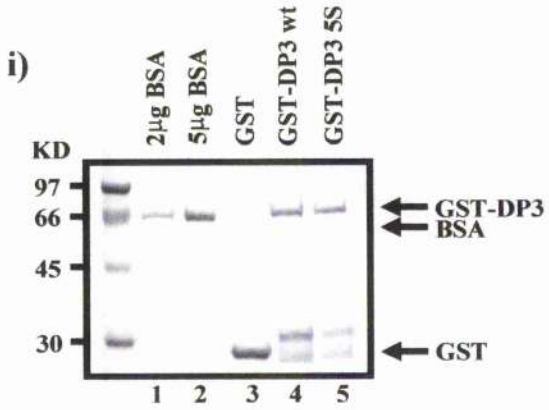
The only function that the DP3 5S mutant was proved to be defective in comparison with the wt protein was its ability to bind 14-3-3. These results were important since they showed that no other property that the DP3 5S mutant possessed could prejudice the study of the nature of the relationship between E2F and 14-3-3, mediated by DP3.

Figure 4.2A: The DP3 5S mutant binds E2F5 *in vitro*

i) Coomassie stain showing bacterially expressed and purified GST alone (lane 3), GST-DP3 wt (lane 4), GST-DP3 5S mutant (lane 5) proteins and 2μg and 5μg BSA (lanes 1 and 2 respectively).

ii) COS-7 cells were transiently transfected with the following amount of the indicated plasmid: 50μg HA-tagged E2F5. Around 65 hours post-transfection the samples were harvested and used in a pull-down assay. The extracts were incubated with either 5μg of GST, 5μg of GST-DP3 wt or 5μg of GST-DP3 5S. The exogenous (Ex.) E2F5 was detected using an anti-HA antibody.

i)


2μg BSA
5μg BSA
GST
GST-DP3 wt
GST-DP3 5S
KD
97
66
45
30
GST-DP3
BSA
GST
1 2 3 4 5

ii)

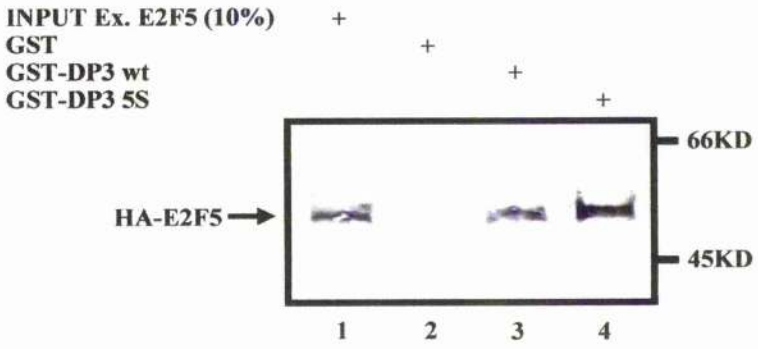

INPUT Ex. E2F5 (10%)
GST
GST-DP3 wt
GST-DP3 5S
+
+
+
+
66KD
45KD
HA-E2F5
1 2 3 4

Figure 4.2B: <u>The DP3 5S mutant binds E2F5 *in vivo*</u>

COS-7 cells were transiently transfected with the following amounts of the indicated plasmids: 20μg HA-tagged E2F5, 20μg DP3 wt, 20μg DP3 5S mutant as well as 5μg of β-Gal as an internal transfection control (Ex.=Exogenous). Around 65 hours post-transfection the samples were harvested and subjected to immunoprecipitation with the DP3 antibody, 7.2. DP3 was detected using 7.2 whilst the monoclonal anti-HA antibody was used to detect E2F5. The * and ** indicate the upper and lower bands of DP3 respectively and the position of the IgG is indicated.

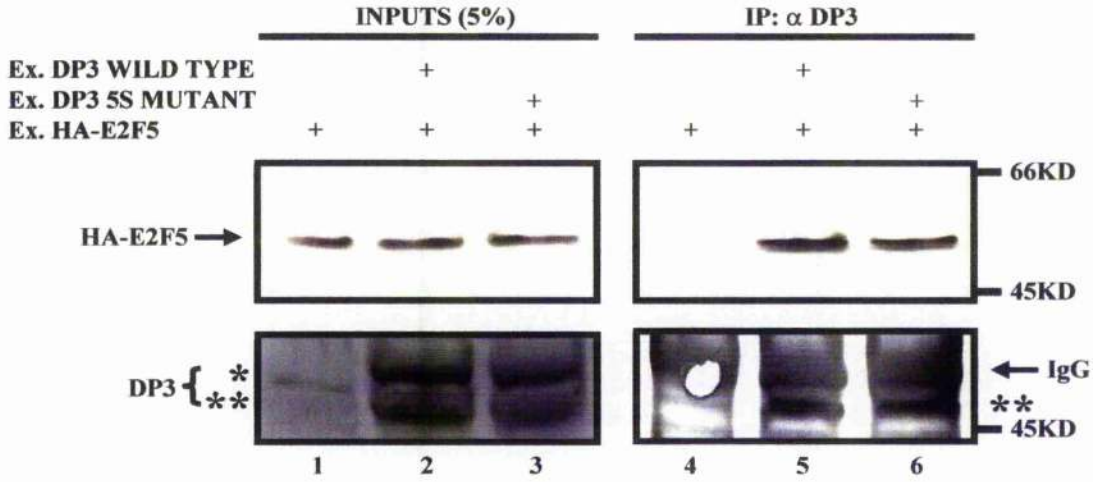

INPUTS (5%)
IP: α DP3
Ex. DP3 WILD TYPE
Ex. DP3 5S MUTANT
Ex. HA-E2F5
+
HA-E2F5
DP3
*
**
66KD
45KD
IgG
1
2
3
4
5
6

Figure 4.2C: The 5S mutant localises E2F5 to the nucleus

COS-7 cells were transiently transfected with the following amounts of the indicated plasmids: 2μg HA-tagged E2F5, 2μg DP3 wild type and 2μg DP3 5S mutant. Around 65 hours post-transfection the cells were harvested and subjected to immunofluorescence. Cellular DNA was stained with DAPI (parts A-C) whilst detection of the exogenously (Ex.) expressed E2F5 and DP3 was carried out using a monoclonal anti-HA antibody and the 7.2 polyclonal anti-DP3 antibody respectively. The assay was carried out blind and the data shown is representative of three independent experiments. E2F5 alone (part G); DP3 wt and E2F5 (parts E and H); DP3 5S and E2F5 (parts F and I).

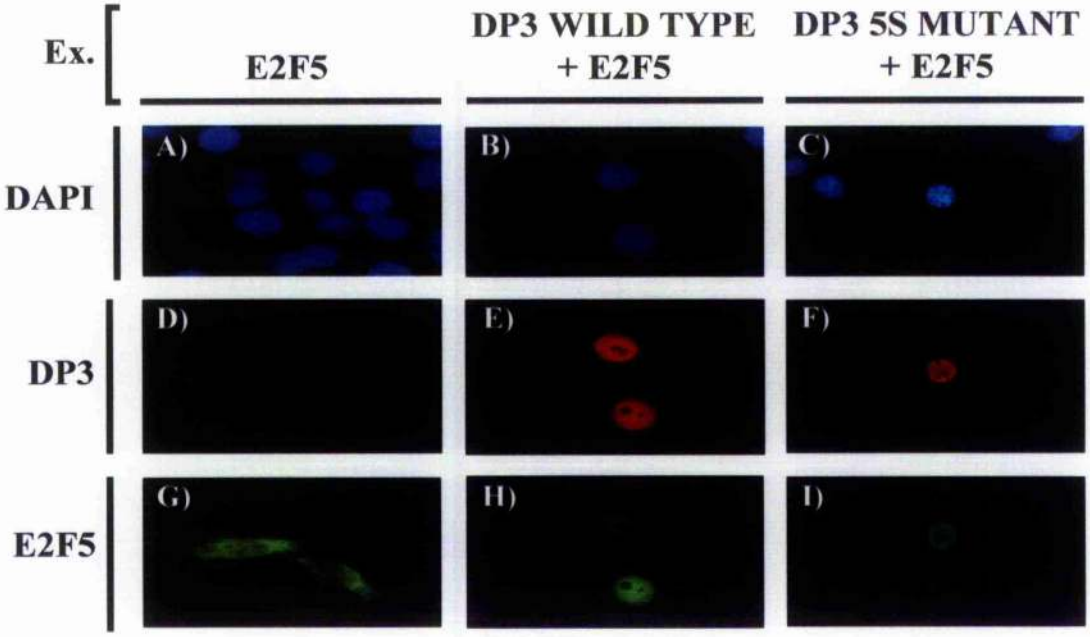
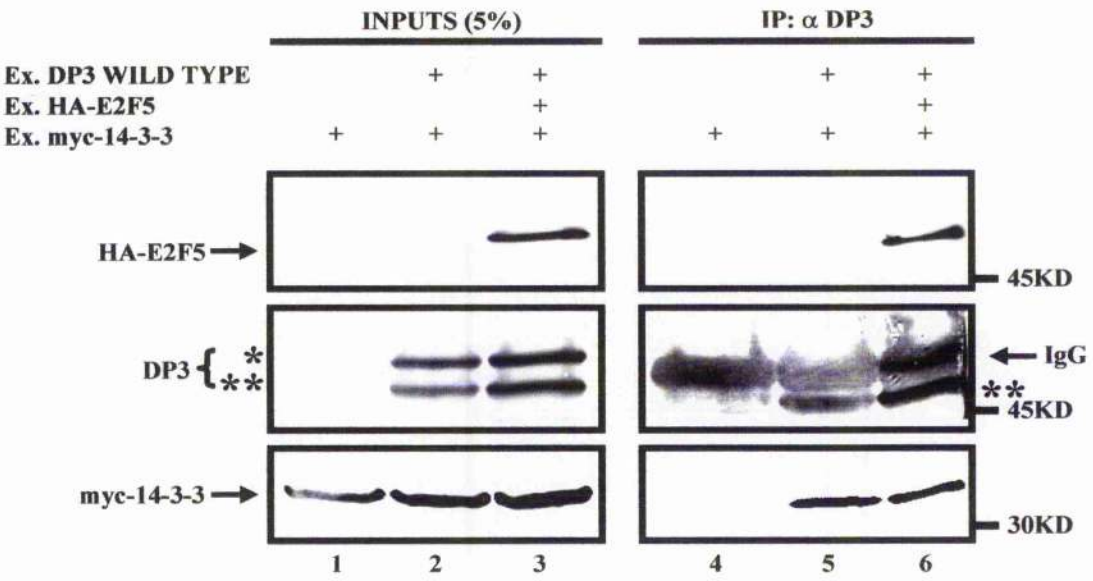

Ex.
E2F5
DP3 WILD TYPE + E2F5
DP3 5S MUTANT + E2F5
DAPI
DP3
E2F5
A)
B)
C)
D)
E)
F)
G)
H)
I)

Figure 4.2D: E2F5 has no effect on the ability of DP3 to bind to 14-3-3

COS-7 cells were transiently transfected with the following amounts of the indicated plasmids: 20μg HA-tagged E2F5, 20μg DP3 wild type, 40μg myc-tagged 14-3-3 and 5μg β-Gal as an internal transfection control (Ex.=Exogenous). Around 65 hours post-transfection the samples were harvested and subjected to immunoprecipitation with the DP3 antibody, 7.2. Detection of DP3 was carried out using 7.2, E2F5 was detected by an anti-HA antibody whilst 14-3-3 was detected by the anti-myc antibody. The * and ** indicate the upper and lower bands of DP3 respectively and the position of the IgG is indicated.


INPUTS (5%)
IP: α DP3
Ex. DP3 WILD TYPE
Ex. HA-E2F5
Ex. myc-14-3-3
HA-E2F5
DP3
*
**
myc-14-3-3
45KD
IgG
**
45KD
30KD
1
2
3
4
5
6

Chapter 5: 14-3-3 does not alter the sub-cellular localisation of E2F/DP3

5.1: Introduction

As previously described in chapter 1, 14-3-3 proteins are able to regulate the activity of a wide range of their ligands by altering the sub-cellular localisation of the target protein. Examples include the cytoplasmic tethering of cdc25C (Kumagai and Dunphy, 1999), cdk2 (Laronga *et al.*, 2000) and HDAC4 and HDAC5 (Grozinger and Schreiber, 2000). In this way 14-3-3 proteins can indirectly influence the activity of target ligands by altering their sub-cellular localisation.

Given this it was of interest to examine if the sub-cellular localisation of DP3 was altered when co-expressed with 14-3-3 and also whether a lack of binding to the DP3 5S mutant influenced the cellular location of 14-3-3. This would allow the further understanding of whether 14-3-3 could influence the activity of the E2F/DP3 heterodimer by altering the sub-cellular localisation of the complex.

5.2: RESULTS

5.2A: 14-3-3 unable to bind DP3 localises in the cytoplasm

14-3-3 proteins can regulate the cell cycle by tethering the target ligand in the cytoplasm, thereby acting as 'anchor' molecules. It is thought that 14-3-3s are able to do this by binding near, and thereby interrupting, nuclear localisation signals in the target molecule, which prevents the NLS from complexing with the nuclear import machinery.

To address the question of whether 14-3-3 could influence the sub-cellular localisation of E2F/DP3, COS-7 cells were transiently transfected with plasmids expressing myc-tagged 14-3-3 alone or together with both E2F5 and wt DP3 or E2F5 and the 5S mutant. The cells were harvested and subjected to immunofluorescence. Figure 5.2A(i) shows that, in accordance with Figure 3.2E, wt DP3 localises to the nucleus (parts E and F). When expressed alone, 14-3-3 displayed a mostly uniform staining pattern throughout the cell (part G). Upon the co-expression with wt DP3, 14-3-3 localised mostly in the nucleus (parts H and I) and the cellular location of DP3 was not seen to change.

Again, Figure 5.2A(ii) shows that the DP3 5S mutant localised exclusively to the nucleus (parts E and F). Interestingly, when 14-3-3 was co-expressed with the DP3 5S mutant it localised mostly to the cytoplasm (parts H and I). Taken together these results show that 14-3-3 does not act to alter the sub-cellular localisation of E2F/DP3. Indeed Figure 5.2(ii) suggests that DP3 may act to drive

14-3-3 into the nucleus and then act as a nuclear anchor for 14-3-3, since a lack of binding to DP3 causes a significant increase in the cytoplasmic localisation of 14-3-3. Figure 5.2A(iii) shows the typical example in the nuclear and cytoplasmic distribution of 14-3-3 when co-expressed with either wt DP3 or the DP3 5S mutant.

5.2B: 14-3-3 unable to bind DP3 can localise to the nucleus

Figure 5.2A(ii) showed that 14-3-3 that was unable to bind the DP3 5S mutant predominantly localised in the cytoplasm. This suggested that DP3 was acting as a nuclear anchor for 14-3-3. However this did not rule out the possibility that 14-3-3 could actively shuttle between the nucleus and the cytoplasm and that it was not dependent on the binding to DP3 to localise it to the nucleus. To address this point it was decided to repeat the experiment carried out in Figure 5.2A with the use of leptomycin B, which can block the nuclear export of certain proteins.

COS-7 cells were transiently transfected with plasmids expressing myc-tagged 14-3-3 alone or together with both E2F5 and wt DP3 or E2F5 and the 5S mutant. The cells were treated with leptomycin B and subjected to immunofluorescence. Figure 5.2B(i) shows that, when expressed alone, 14-3-3 again displayed a mostly uniform staining pattern (part C). Upon the addition of leptomycin B 14-3-3 was seen to accumulate in the nucleus (part D). Upon the co-expression of wt DP3, 14-3-3 localised mostly in the nucleus [Figure 5.2B(ii) part E], results that were observed previously [Figure 5.2A(i)]. In the presence of leptomycin B 14-3-3 was

seen to accumulate in the nucleus (part F). In accordance with previous results, when 14-3-3 was co-expressed with the DP3 5S mutant it localised mostly to the cytoplasm [Figure 5.2B(iii) part E]. Upon the addition of leptomycin B 14-3-3 localised exclusively in the nucleus (part F). These results indicate that the nuclear localisation of 14-3-3 is not dependent on the binding to DP3 and indeed other mechanisms may be involved which allow 14-3-3 to shuttle between the nucleus and the cytoplasm, independently of binding to DP3.

5.3 CONCLUSIONS

The 14-3-3 proteins have been shown to bind to a variety of proteins critical in controlling the mammalian cell cycle and to alter their sub-cellular localisation (Fu *et al.*, 2000). However the results shown in this chapter indicate that the binding to 14-3-3 does not alter the cellular location of E2F/DP3.

In the presence of wt DP3, 14-3-3 mostly localises to the nucleus [Figure 5.2A(i) parts H and I]. Whilst this was true for the wt DP3, in the presence of the DP3 5S mutant, there was an increase in the cytoplasmic 14-3-3 seen in the cell [Figure 5.2A(ii) parts H and I]. This was an interesting result since it suggested that, rather than 14-3-3 influencing the sub-cellular localisation of DP3, that DP3 could have been actively blocking the nuclear export of 14-3-3.

Recent work by Brunet *et al.*, 2002 has now shown that 14-3-3 proteins do not contain a C-terminal NES, as was previously thought. Indeed, this region of 14-3-3 has been shown to be involved in ligand binding (Brunet *et al.*, 2002). The fact that the 14-3-3 that was unable to bind the DP3 5S mutant was localised to the cytoplasm suggested two possibilities. Either it was mostly localised to the cytoplasm in the first instance and was therefore unable to localise to the nucleus since it could not complex with DP3 5S or it was indeed able to enter the nucleus and was exported by some other mechanism. This result raised the question of whether 14-3-3 was indeed dependent on DP3 for its nuclear localisation or whether 14-3-3 could still enter the nucleus itself and that subsequent complex formation occurred in the nucleus.

Indeed, Figure 5.2B showed that, whilst 14-3-3 localised in the cytoplasm when unable to bind DP3 5S, it could still enter the nucleus itself since leptomycin B treatment, which blocks nuclear export, caused an accumulation of 14-3-3 in the nucleus in the presence of the DP3 5S mutant. This result showed that the nuclear localisation of 14-3-3 was not dependent on binding to DP3, rather 14-3-3 seems able to independently shuttle in and out of the nucleus.

This allows the conclusion that the nature of the relationship between DP3 and 14-3-3 may be of a 'fluid' nature. If 14-3-3 was dependent on DP3 for its nuclear import then this would mean that E2F/DP3 would be targeted to specific promoter regions with 14-3-3 already in complex. This could presumably allow 14-3-3 to influence the activity of E2F/DP3 without any prior signalling pathway being required to induce 14-3-3 to enter the nucleus and to bind to DP3. However, if 14-3-3 can enter and exit the nucleus by itself it suggests that the binding between DP3 and 14-3-3 could occur once the E2F5/DP3 heterodimer is in the nucleus and that some signalling event such as phosphorylation is required to initiate binding.

Figure 5.2B raises the interesting question of how 14-3-3 that is unable to bind to DP3 is still able to actively shuttle between the nucleus and the cytoplasm. Although the work in chapter 3 showed that the DP3 5S mutant and 14-3-3 are unable to form a complex, this does not prove that the interaction is direct. The result in Figure 5.2B suggests that there may be another unidentified protein within the complex that is still able to bind to 14-3-3 and which possesses both an NLS and an NES. This would explain how 14-3-3 that is unable to bind to DP3 is

able to shuttle between the nucleus and the cytoplasm. It also cannot be ruled out that the 14-3-3 that is unable to bind to DP3 is then targeted to other endogenous partners that localise it predominantly to the cytoplasm. If this was the case however it could be postulated that the same pattern of distribution of 14-3-3 would be observed when 14-3-3 is expressed alone. However it may be that 14-3-3 that is co-expressed with the DP3 5S mutant behaves in a different manner from 14-3-3 that is expressed alone.

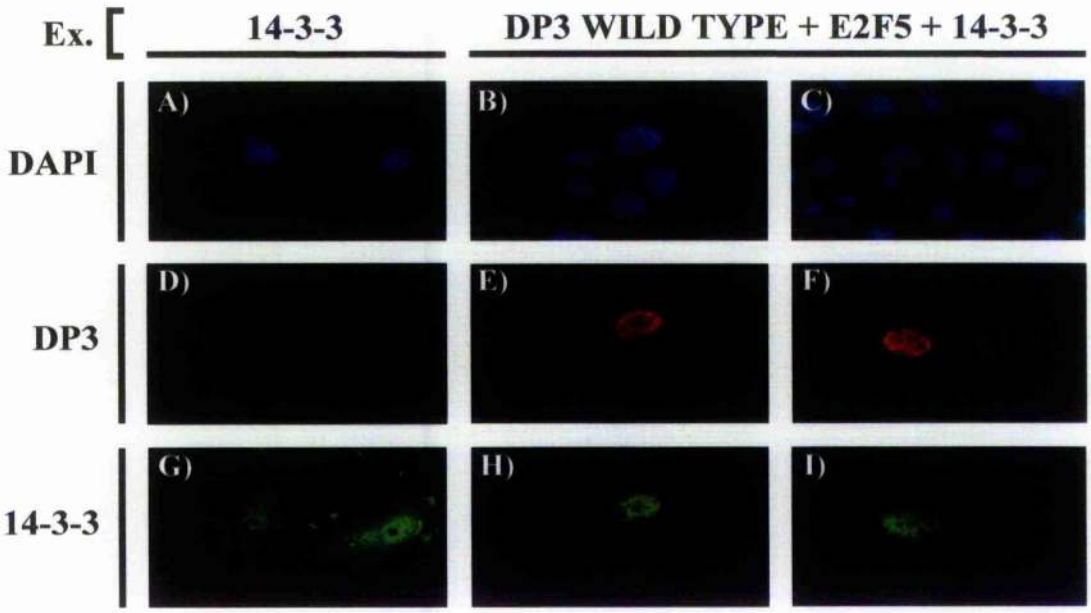
Also, despite the fact that the sub-cellular localisation of DP3 is not influenced by co-expression with 14-3-3, it cannot be ruled out that this may not happen if some sort of intra- or extra-cellular signalling event was to occur.

Figure 5.2A: <u>14-3-3 unable to bind DP3 localises in the cytoplasm</u>

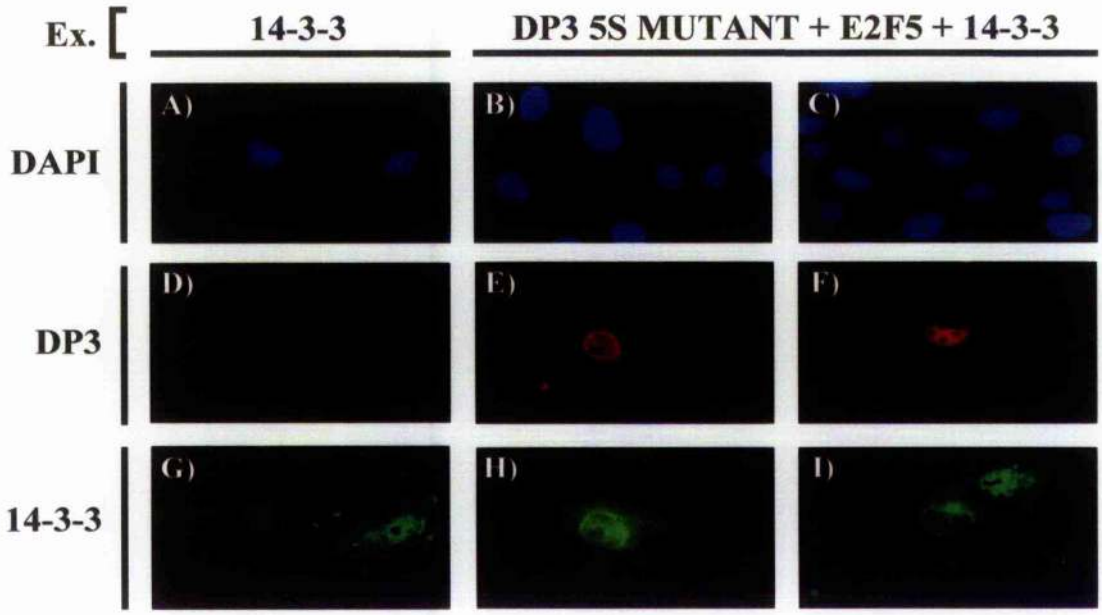
i) and ii) COS-7 cells were transiently transfected with the following amounts of the indicated plasmids: 2μg HA-tagged E2F5, 2μg DP3 wt, 2μg DP3 5S mutant, 4μg myc-tagged 14-3-3. Around 65 hours post-transfection the cells were fixed and subjected to immunofluorescence. Cellular DNA was stained with DAPI (i and ii, parts A-C) whilst detection of the exogenously (Ex.) expressed 14-3-3 and DP3 was carried out using a monoclonal anti-myc antibody and the polyclonal anti-DP3 antibody, 7.2, respectively. The assay was carried out blind and 200 cells were counted. Data shown is representative of three independent experiments. 14-3-3 alone (part G); DP3 wt and 14-3-3 (parts E and H); DP3 5S and 14-3-3 (parts F and I).

iii) Graphical representation of the cellular distribution of 14-3-3 in the presence of either wt DP3 or the 5S mutant.

i)



ii)



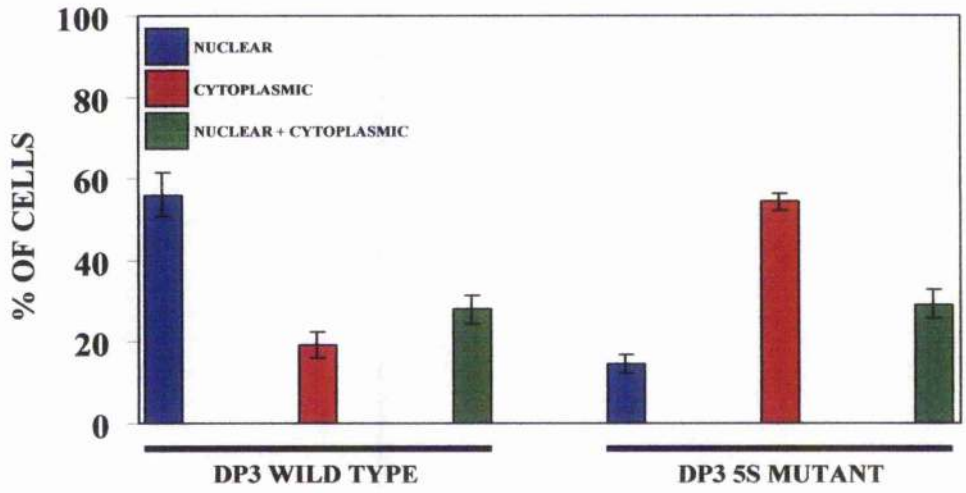
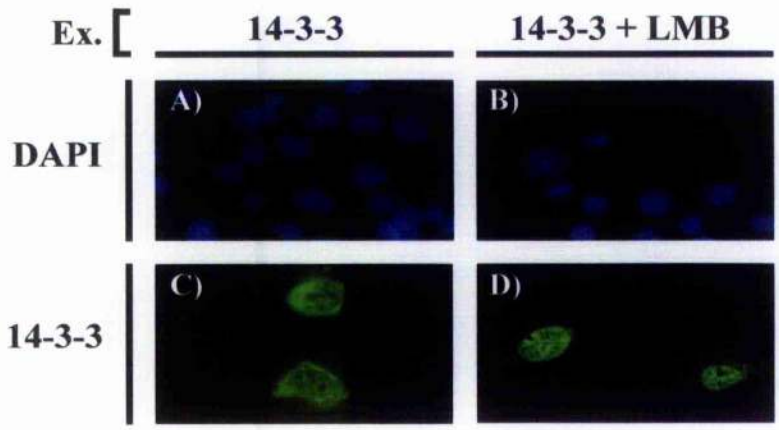

100
80
60
40
20
0
% OF CELLS
NUCLEAR
CYTOPLASMIC
NUCLEAR + CYTOPLASMIC
DP3 WILD TYPE
DP3 5S MUTANT

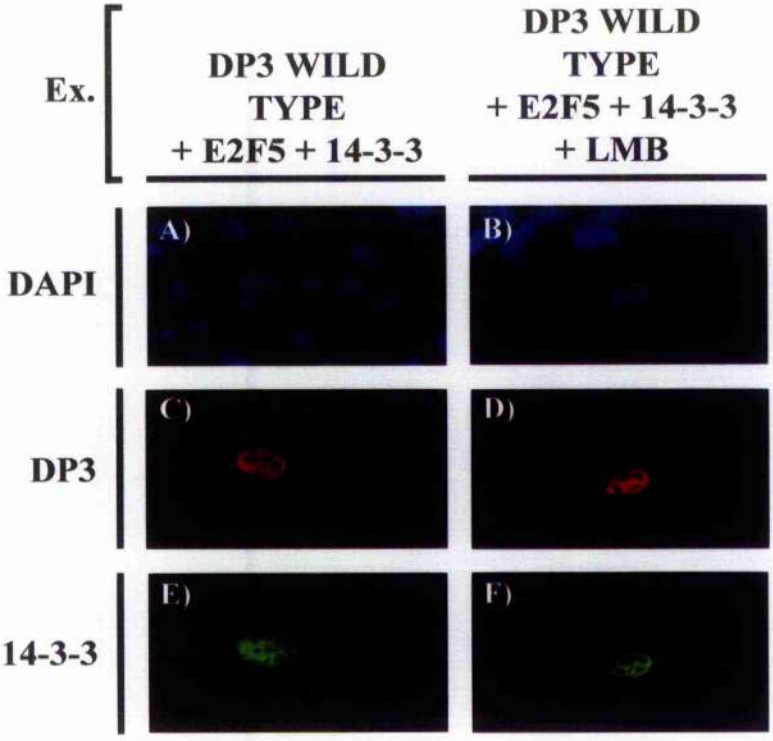
Figure 5.2B: <u>14-3-3 unable to bind DP3 can localise to the nucleus</u>

i), ii) and iii) COS-7 cells were transiently transfected with the following amounts of the indicated plasmids: 2μg HA-tagged E2F5, 2μg DP3 wt, 2μg DP3 5S mutant, 4μg myc-tagged 14-3-3. Around 48 hours post-transfection the cells were treated with 1μM leptomycin B (LMB). Around 17 hours later the cells were fixed and subjected to immunofluorescence. Cellular DNA was stained with DAPI (i-iii, parts A and B) whilst detection of the exogenously (Ex.) expressed 14-3-3 and DP3 was carried out using a monoclonal anti-myc antibody and the polyclonal anti-DP3 antibody, 7.2, respectively. The assay was carried out blind and the data shown is representative of three independent experiments. 14-3-3 alone (i, part C); 14-3-3 and LMB (i, part D); DP3 wt and 14-3-3 (ii, parts C and E); DP3 wt, 14-3-3 and LMB (ii, parts D and F); DP3 5S and 14-3-3 (iii, parts C and E); DP3 5S, 14-3-3 and LMB (iii, parts D and F).

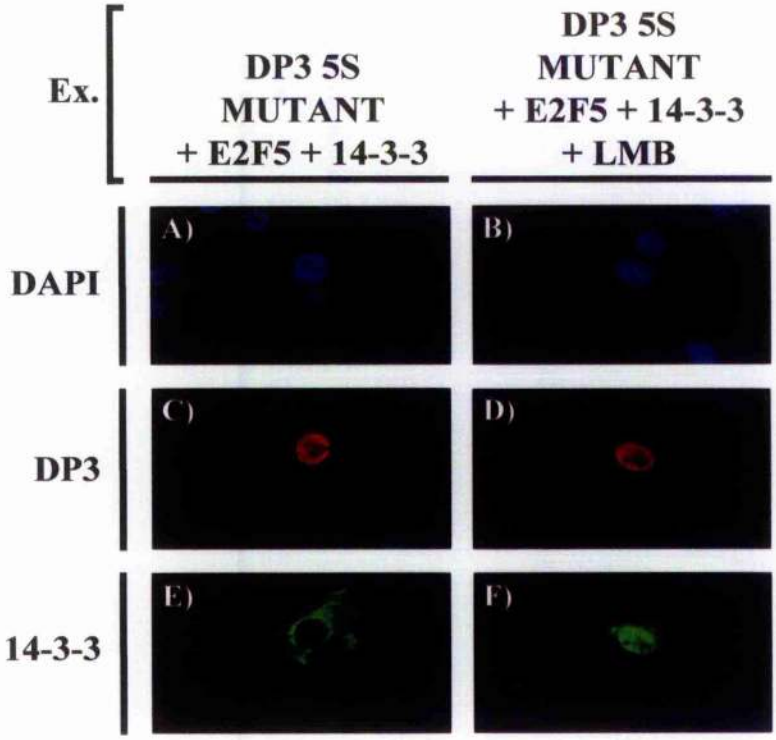
i)



ii)


Ex.
DP3 WILD TYPE + E2F5 + 14-3-3
DP3 WILD TYPE + E2F5 + 14-3-3 + LMB
DAPI
DP3
14-3-3
A)
B)
C)
D)
E)
F)

iii)


Ex.
DP3 5S MUTANT + E2F5 + 14-3-3
DP3 5S MUTANT + E2F5 + 14-3-3 + LMB
DAPI
DP3
14-3-3
A)
B)
C)
D)
E)
F)

Chapter 6: 14-3-3 has an effect on the cell cycle through the E2F pathway

6.1: Introduction

14-3-3 proteins have an integral role to play in controlling the mammalian cell cycle (Fu *et al.*, 2000). Indeed, 14-3-3 proteins are able to bind to, and regulate, a wide variety of proteins involved in control of the cell cycle including cdc25C (Peng *et al.*, 1997), Wee1 (Rothblum-Oviatt *et al.*, 2001), p53 (Waterman *et al.*, 1998) and both HDAC4 and HDAC5 (Grozinger *et al.*, 2000).

E2F4 and E2F5, which both lack an NLS, are known to negatively regulate the cell cycle (Stevens and La Thangue, 2003). In particular, they acquire nuclear localisation via one of the pocket proteins and therefore they exert a repressive role on the cell cycle (Stevens and La Thangue, 2003). If however, either of these E2Fs is in complex with an NLS containing splice variant of the DP3 family, activation of cell cycle genes is observed since nuclear localisation is attained through the DP3 component. (de la Luna *et al.*, 1996) [Figure 1.2B(i)].

It was therefore of interest to examine whether 14-3-3 could exert an influence on the cell cycle, via the interaction with the DP3/E2F heterodimer.

6.2: RESULTS

6.2A: 14-3-3 has an effect on DP3/E2F5 mediated transcriptional activity *in vitro*

It was firstly of interest to analyse whether the cytoplasmic localisation of 14-3-3 in the presence of the DP3 5S mutant had any effect on the cell cycle. To address this question the co-activation function of either the wt DP3 or the 5S mutant in the presence or absence of 14-3-3 was assayed using a luciferase reporter assay on a synthetic E2F5 responsive promoter. Figure 6.2A shows that the luciferase activity was low in the samples expressing E2F5 (track 3), DP3 wt (track 4) or 5S mutant alone (track 5). The luciferase activity was also low in the samples where E2F5 was co-expressed with either DP3 wt (track 12) or the 5S mutant (track 13).

When 14-3-3 was co-expressed with E2F5 and wt DP3 (tracks 14-16), there was a significant and titratable increase in the expression of the cyclin-E gene. Indeed, when the ratio of transfected 14-3-3 to wt DP3 and E2F5 was 2:1, the amount of luciferase activity was around six-fold higher than wt DP3 and E2F5 alone (track 16). However, when 14-3-3 was titrated on top of E2F5 and the DP3 5S mutant (tracks 17-19) only a marginal increase in the expression of the cyclin E reporter was observed and no titratable effect was seen. These data suggested that 14-3-3 has a significant effect on the transcriptional activity of the wt DP3/E2F5 heterodimer but that it is unable to exert the same effect over the DP3 5S/E2F5 complex.

6.2B: 14-3-3 has an effect on DP3/E2F5 mediated cell cycle progression

Given the effect of 14-3-3 on the transcriptional activity of the DP3/E2F5 heterodimer *in vitro*, it was of interest to see whether this regulation had any consequences on the cell cycle *in vivo*. For this purpose, Fluorescence Activated Cell Sorting (FACS) analysis was carried out. COS-7 cells were transiently transfected with the indicated plasmids shown in Figure 6.2B(i), along with the cell surface marker CD20.

Figure 6.2B(i) shows that 14-3-3 alone (part B) is able to cause a partial induction of cell cycle progression over the mock sample (part A). When E2F5 and wt DP3 are co-expressed together (part C), a decrease in the G1 population is observed compared to mock with a slight rise in the G2 population. However, when 14-3-3 is co-expressed with both E2F5 and wt DP3 (part D) a significant amount of cell cycle progression is observed compared to both E2F5 and DP3 alone and the mock sample (compare parts A, C and D).

In contrast, when the DP3 5S mutant and E2F5 are co-expressed together only a marginal amount of cell cycle progression is observed over the mock (compare parts A and E). Strikingly, the co-expression of 14-3-3 with the DP3 5S/E2F5 heterodimer had only a marginal effect on cell cycle progression compared to DP3 5S and E2F5 alone or the mock (compare A, E and F). This data allowed the conclusion that 14-3-3 has a significant effect on cell cycle progression, mediated through the regulation of E2F. Importantly, this effect can be seen to be dependent

on the ability of 14-3-3 to bind to DP3 since the ability of 14-3-3 to cause cell cycle progression was impaired when it was co-expressed with E2F5 and the DP3 5S mutant. These *in vivo* data therefore substantiate the *in vitro* results, suggesting that 14-3-3 functions as a positive regulator of the transcriptional activity of the wt DP3/E2F5 heterodimer and that this effect is dependent on binding to DP3. Figure 6.2B(ii) represents the percentage increase in G1, S-phase, G2/M and Sub-G1 of each of the samples, relative to the mock.

6.2C: 14-3-3 regulates S-phase entry

Figure 6.2B had provided *in vivo* evidence that showed that 14-3-3 could positively influence cell cycle progression through its effects on E2F. However the FACS data could not provide an accurate observation of the induction of S-phase entry, a part of the cell cycle where E2F plays an essential role. It was therefore of interest to see if 14-3-3 was regulating the ability of E2F to induce the exit from G1 and the entry into S-phase.

In order to analyse the possible effect of 14-3-3 on the entry into S-phase the Brdu incorporation method was employed. COS-7 cells were transiently transfected with the plasmids indicated in Figure 6.2C as well as red fluorescent protein (RFP), to enable transfection efficiency to be measured. The cells were fixed onto coverslips, subjected to Brdu incorporation and then an anti-Brdu fluorescent antibody was added to allow detection of those cells that had entered S-phase.

Figure 6.2C (i) shows that around 22% of the cells in the mock sample were in S-phase (part 1). No significant induction of S-phase was observed when 14-3-3 was expressed alone (part 2). When E2F5 and wt DP3 were co-expressed together only a marginal amount of S-phase entry was observed (part 3). However, when 14-3-3 was co-expressed with E2F5 and wt DP3 (part 4) there was a dramatic increase in the number of cells in S-phase compared to E2F5 and wt DP3 together and the mock alone. Indeed there was almost a two-fold increase in the number of S-phase cells in this sample compared to the mock.

In contrast, only a marginal amount of S-phase induction was observed with both E2F5 and DP3 5S mutant alone (part 5) and 14-3-3 co-expressed together with E2F5 and the DP3 5S mutant (part 6). Taken together these results suggest that the role that 14-3-3 plays in the positive regulation of the cell cycle through E2F is to control the entry into S-phase. Again, the influence that 14-3-3 exerts over E2F is dependent on its ability to bind to the DP3 component of the heterodimer. Figure 6.2C(ii) represents the percentage increase in S-phase of each of the samples, relative to the mock.

6.3 CONCLUSIONS

A large body of evidence has been published recently regarding the involvement of 14-3-3 in the p53 pathway. In particular, 14-3-3 enhances p53 transcriptional activity by increasing the affinity of this transcription factor for the DNA (Waterman *et al.*, 1998). It was therefore interesting to test whether 14-3-3 had any effect on E2F5 transcriptional activity in a similar manner. Indeed this was proved to be the case since a titration of 14-3-3 over the wt DP3/E2F5 heterodimer caused a considerable increase in the transcriptional activity of E2F5 measured on a known E2F responsive gene. This was not the case when 14-3-3 was titrated on top of the DP3 5S mutant/E2F5 complex, since only a marginal effect was observed under these conditions (Figure 6.2A). This result suggested that, i*n vitro* at least, that 14-3-3 can exert a positive influence on the transcriptional activity of E2F, via DP3.

The data presented in Figure 6.2B also suggested that 14-3-3 could exert a positive influence over E2F-mediated transcriptional activity *in vivo*. 14-3-3 was seen to induce a significant level of cell cycle progression over the mock when co-expressed with E2F5 and wt DP3 however this effect was not repeated when 14-3-3 was co-expressed with E2F5 and the DP3 5S mutant. This result suggested that 14-3-3 can cause efficient cell cycle progression only when it is able to efficiently complex with DP3. Interestingly, previous evidence indicates that the 14-3-3 family largely exerts a negative influence on the mammalian cell cycle. This result highlighted the possibility that the influences exerted by the 14-3-3 family over the cell cycle may be isoform specific since the 14-3-3σ isoform plays

a large part in the negative regulation of cell cycle progression through its regulation of both cdc2 and p53.

Given that E2F is known to play a crucial role in the transition from G1 into S-phase it was thought that perhaps 14-3-3 was influencing the induction of S-phase, mediated by DP3/E2F5. Indeed Figure 6.2C shows that when 14-3-3 is able to form a complex with the DP3/E2F5 heterodimer, there is around a two-fold increase in the number of cells in S-phase. This phenotype was not reproduced when 14-3-3 was co-expressed with E2F5 and the DP3 5S mutant. Also, no significant increase in S-phase entry was seen with either the wild type or the mutant complex alone suggesting that the presence of 14-3-3 is essential in allowing efficient S-phase progression. These results were slightly in contrast to those observed with the FACS data attained in Figure 6.2B, where no significant increase in S-phase was observed. However, the Brdu assay provides a far more accurate measurement of S-phase than FACS analysis.

Taken together these data suggest an integral role for 14-3-3 in the control of the mammalian cell cycle and specifically, control of the G1/S transition, through the E2F pathway. Whether the positive effects seen on the cell cycle are due to an enhanced level of DNA binding by the wild type DP3/E2F5 heterodimer, mediated by 14-3-3, is unclear. However, given the effects that 14-3-3 can exert on p53 DNA binding, this is a possibility. It can however be seen from analysis of the effects of 14-3-3 over E2F5 and wt DP3 that 14-3-3 can exert a positive

influence on the mammalian cell cycle, through E2F, and that this effect is dependent on the binding to the DP3 sub-unit.

Figure 6.2A: 14-3-3 has an effect on DP3/E2F5 mediated transcriptional activity *in vitro*

COS-7 cells were transiently transfected with the following amounts of the indicated plasmids as follows: 0.5μg cyclin-E reporter, 1μg HA-tagged E2F5, 1μg DP3 wt, 1μg DP3 5S mutant, 0.5,1 and 2μg of myc-tagged 14-3-3 as a titration and 1μg β-Gal as an internal transfection control. Where no 14-3-3 titration is indicated, 1μg of 14-3-3 was transfected. Around 65 hours post-transfection the cells were harvested and subjected to the luciferase reporter system. The data shown is representative of three independent experiments and the values depict the relative level of luciferase to β-Gal expression. Each point was done in duplicate plates.

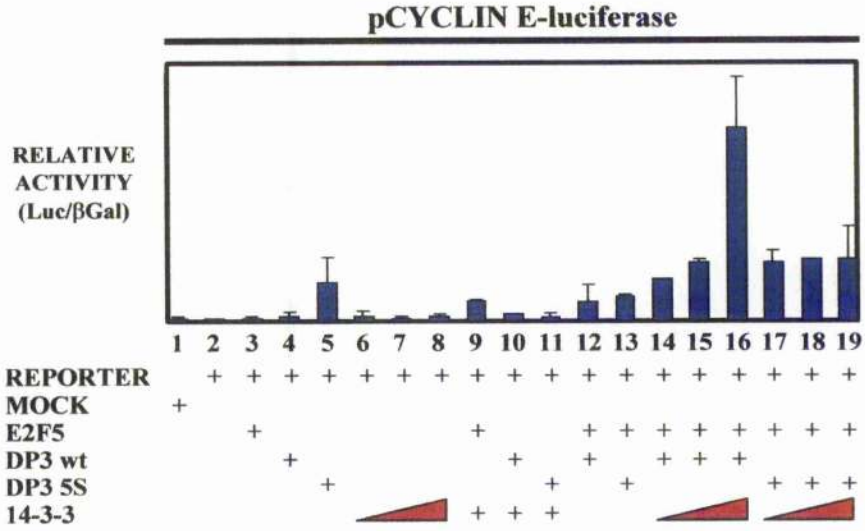
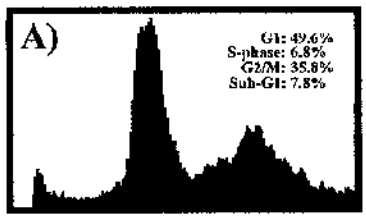

pCYCLIN E-luciferase
RELATIVE ACTIVITY (Luc/βGal)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
REPORTER + + + + + + + + + + + + + + + + + +
MOCK +
E2F5 + + + + + + + + + + + + + + + +
DP3 wt + + + + + + + + + + + +
DP3 5S + + + + + + + + + +
14-3-3

Figure 6.2B: 14-3-3 has an effect on DP3/E2F5 mediated cell cycle progression

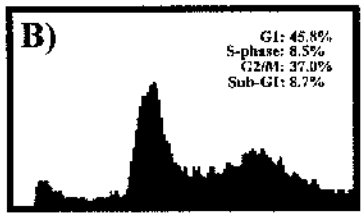
i) COS-7 cells were transiently transfected with the following amounts of the indicated plasmids: 5μg CD20, 5μg HA-tagged E2F5, 5μg DP3 wt, 5μg DP3 5S mutant and 10μg of myc-tagged 14-3-3. Around 65 hours post-transfection the cells were harvested and subjected to FACS analysis. Cellular sorting and analysis was carried out using a Fluorescence Activated Cell Sorter. The data shown is representative of three independent experiments. Sub-G1, G1, S-phase and G2/M percentages are shown inset.

ii) Graphical representation, from one of the three independent experiments, of the percentage increase in G1, S-phase, G2/M and Sub-G1 of each sample, relative to mock.

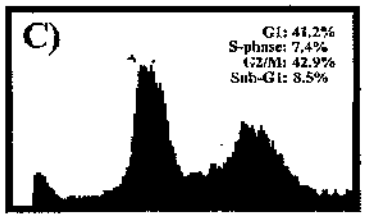
i)



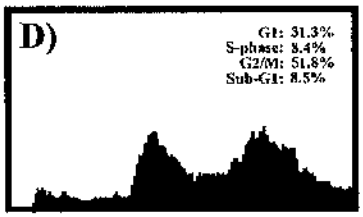
MOCK



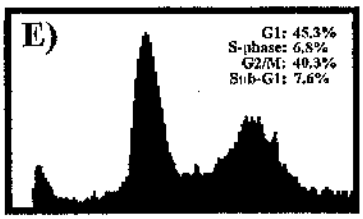
14-3-3



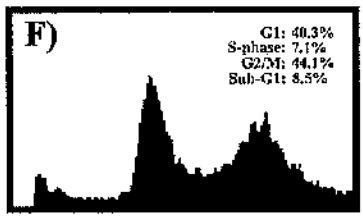
DP3 WILD TYPE + E2F5



DP3 WILD TYPE + E2F5 + 14-3-3



DP3 5S MUTANT + E2F5



DP3 5S MUTANT + E2F5 + 14-3-3

ii)

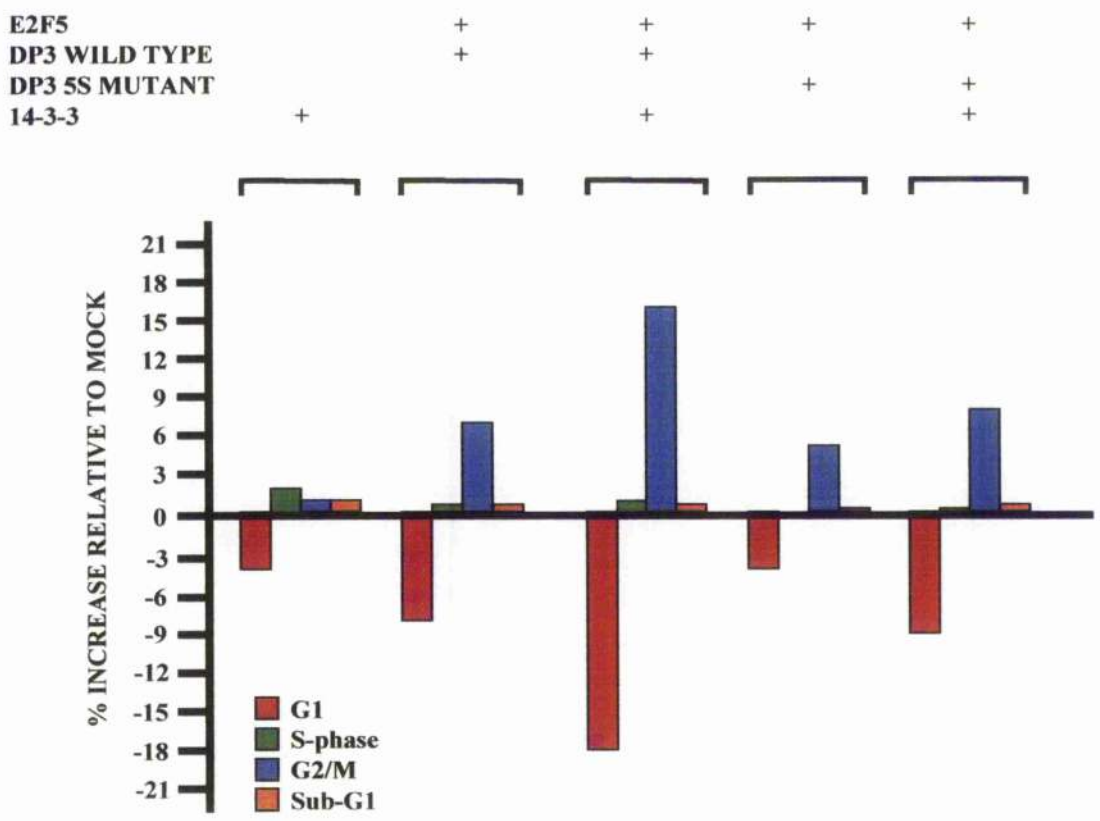
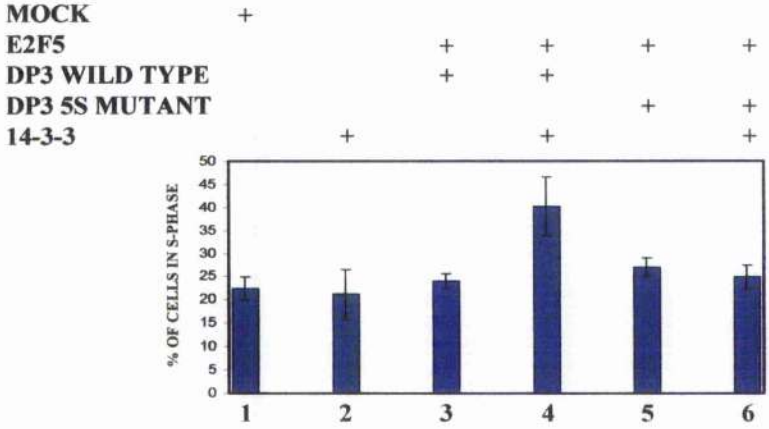

E2F5
DP3 WILD TYPE
DP3 5S MUTANT
14-3-3
+
+
+
+
+
+
+
+
+
+
% INCREASE RELATIVE TO MOCK
21
18
15
12
9
6
3
0
-3
-6
-9
-12
-15
-18
-21
G1
S-phase
G2/M
Sub-G1

Figure 6.2C: 14-3-3 regulates S-phase entry

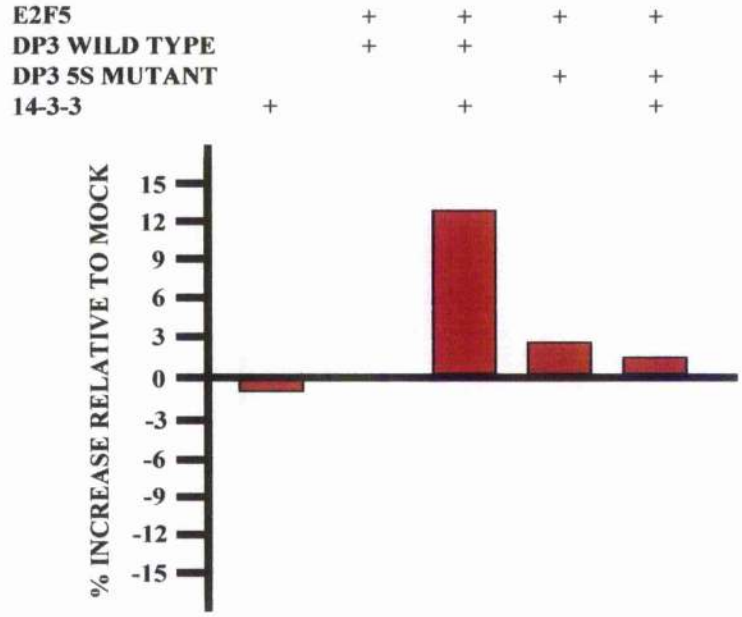
i) COS-7 cells were transiently transfected with the following amounts of the indicated plasmids: 2μg HA-tagged E2F5, 2μg DP3 wt, 2μg DP3 5S mutant, 4μg myc-tagged 14-3-3 and 1μg RFP. Around 65 hours post-transfection the cells were harvested and subjected to Brdu treatment. Detection of the total cell population was achieved by staining the cellular DNA with DAPI. The assay was carried out blind and 200 cells were counted. The values shown represent the average of three separate experiments.

ii) Graphical representation, from one of the three independent experiments, of the percentage increase in S-phase of each sample, relative to mock.

i)


MOCK +
E2F5 + + + +
DP3 WILD TYPE + +
DP3 5S MUTANT + +
14-3-3 + + +
% OF CELLS IN S-PHASE
50
45
40
35
30
25
20
15
10
5
0
1 2 3 4 5 6

ii)


E2F5 + + + +
DP3 WILD TYPE + +
DP3 5S MUTANT + +
14-3-3 + + +
% INCREASE RELATIVE TO MOCK
15
12
9
6
3
0
-3
-6
-9
-12
-15

Chapter 7: 14-3-3 regulates DP3/E2F5 mediated apoptosis

7.1: Introduction

As in cell cycle control and signal transduction, the 14-3-3 proteins have a major function in the control of apoptosis (van Hemert *et al.*, 2001). 14-3-3 proteins are seen to negatively regulate pro-apoptotic proteins such as Bad (Zha *et al.*, 1996), FKHRL1 (Brunet *et al.*, 1999) and ASK1 (Zhang *et al.*, 1999). The regulation of these proteins results in the inhibition of apoptosis and 14-3-3 proteins are therefore seen as molecules that are intricately involved in the anti-apoptotic effect.

In addition to its role in proliferation, E2F1 can also trigger apoptosis (Stevens and La Thangue, 2003). E2F1 has now been shown to induce the expression of various pro-apoptotic proteins such as Apaf-1 (Moroni *et al.*, 2001), the p53 homologue, p73 (Irwin *et al.*, 2000) and $p14^{ARF}$ (Bates *et al.*, 1998). It is not known whether other E2F family members are involved in the regulation of apoptosis. However, recent work has shown that E2F5 can induce apoptosis dependent on the nuclear localisation of the protein and the presence of a functional transcriptional activation domain (Loughran and La Thangue, 2000). Interestingly an intact DNA binding domain within the DP3 partner used in the study augmented this apoptotic effect.

Based on these findings, and the fact that many studies have now documented the role of 14-3-3 in the regulation of apoptosis, it was of interest to examine if 14-3-3 could influence the apoptotic effects of E2F5 through DP3. The experimental approach implemented to understand this 14-3-3 function is described in this chapter.

7.2: RESULTS

7.2A: 14-3-3 inhibits DP3/E2F5 mediated apoptosis

In order to examine the role of 14-3-3 in the regulation of apoptosis via the DP3/E2F heterodimer the TUNEL (**T**dT-mediated d**U**TP-X **n**ick **e**nd **l**abelling) assay was used. This method is used to detect apoptotic cells and can distinguish between apoptosis and other types of cell death such as necrosis.

Figure 7.2A(i) shows that, when transfected alone, 14-3-3 could induce around a 3-fold increase in apoptosis over mock (part 1). The amount of apoptosis induced by the wt DP3/E2F5 heterodimer was twenty times higher than the mock (part 2), a result consistent with data published recently (Loughran and La Thangue, 2000). Co-expression of E2F5 and the DP3 5S mutant exerted the same apoptotic effect (part 4).

However the amount of apoptosis observed differed quite remarkably between these samples when 14-3-3 was co-transfected. Whereas 14-3-3 was seen to considerably inhibit apoptosis mediated by the wt DP3/E2F5 heterodimer (part 3) it did not have any significant effect on the apoptosis mediated by the DP3 5S/E2F5 heterodimer (part 5). Rather, a slight increase in apoptosis over DP3 5S/E2F5 alone was observed. These data indicate that the DP3/E2F5 heterodimer has a potent pro-apoptotic function, which can be negatively regulated by 14-3-3. The data suggests that 14-3-3 inhibits the DP3/E2F5 dependent apoptosis, presumably through its DP3 binding

activity since this inhibitory effect is not observed with the DP3 5S mutant/E2F5 heterodimer, which is unable to bind 14-3-3.

7.2B: 14-3-3 anti-apoptotic effect is transcription independent

It has recently been shown that the apoptotic effect of E2F5 is linked to the presence of the transcriptional domain (Loughran and La Thangue, 2000). With this in mind it was therefore of interest to analyse the ability of 14-3-3 to inhibit apoptosis induced by a TrAnscriptionally Dead E2F5 (TAD E2F5) mutant. If similar patterns were observed as in TUNEL carried out with the wt E2F5 then it could be concluded that 14-3-3 reduces DP3/E2F5 mediated apoptosis independently of the transcriptional domain of E2F5.

Figure 7.2B shows that, in accordance with the findings reported by Loughran and La Thangue, (2000), the levels of apoptosis in cells expressing TAD E2F5 was reduced in comparison with cells transfected with the transcriptionally active wt E2F5 (compare Figure 7.2A parts 2-5 with Figure 7.2B parts 2-5). Again, 14-3-3 alone caused around a three-fold increase in apoptosis compared to the mock sample (part 1). The apoptotic pattern remained the same in the samples expressing TAD E2F5 and either wt DP3 (part 2) or the 5S mutant (part 4) or when 14-3-3 was co-transfected (parts 3 and 5 respectively). In particular, addition of 14-3-3 significantly reduced the wt DP3/E2F5 dependent apoptosis (part 3) but it had no effect on apoptosis mediated by the DP3 5S mutant/E2F5 complex (part 5), which again

showed a slight increase. These data suggest that the observed inhibitory effect of 14-3-3 on the DP3/E2F5 mediated apoptosis is exerted in a manner which is independent of the transcriptional activity of the complex.

7.2C: 14-3-3 affects the stability of the DP3/E2F5 heterodimer

The data presented so far in Figures 7.2A and 7.2B showed that 14-3-3 could negatively regulate the pro-apoptotic effect of the wt DP3/E2F5 heterodimer and that this effect was independent of the transcriptional activity of E2F5. One possibility for the regulation of DP3/E2F5 mediated apoptosis by 14-3-3 could be the stability of the proteins involved. To examine this, COS-7 cells were transiently transfected with the plasmids indicated in Figure 7.2C parts i, iii, v and vii. The cells were treated with cycloheximide, which inhibits protein synthesis, thereby allowing measurement of the degradation profile (persistence in the cell) of the transfected proteins.

Figure 7.2C(i) shows that E2F5 had a persisted in the cell past 150 minutes (part A). The upper form of DP3 also persisted past 150 minutes (part B)] whilst the lower form persisted past 90 minutes (part B). Co-transfection of 14-3-3 had a considerable effect on the stability of both DP3 and E2F5. The stability of the lower form of wt DP3 and the upper form was reduced compared to expression in the absence of 14-3-3 [Figure 7.2C(iii)(B)]. Similarly, the stability of E2F5 was also reduced compared to expression in the absence of 14-3-3 (part A).

Figure 7.2C(v)(A) shows that E2F5, in the presence of the DP3 5S mutant still persisted in the cell over 150 minutes. However both forms of the 5S mutant had extended stability's compared to the wt protein [compare Figure 7.2C(i)(B) and Figure 7.2C(v)(B)]. Furthermore, the influence that 14-3-3 exerted on the stability of both E2F5 and wt DP3 was not observed when 14-3-3 was co-expressed with E2F5 and the DP3 5S mutant [compare Figure 7.2C (iii)(parts A and B) with Figure 7.2C(vii)(parts A and B)]. Interestingly, the persistence of 14-3-3 was extended when it was co-expressed with E2F5 and the DP3 5S mutant [compare Figure 7.2C(iii)(part C) with Figure 7.2C(vii)(part C)]. Taken together these data suggest that 14-3-3 plays an essential role in the regulation of the stability of the DP3/E2F5 complex and that this regulation is dependent on the ability of 14-3-3 to bind the DP3 component of the DP3/E2F5 heterodimer.

7.3 CONCLUSIONS

Given the intricate roles that both E2F and 14-3-3 play in the regulation of apoptosis, it was interesting to see if 14-3-3 could affect the DP3/E2F5-mediated apoptotic response.

TUNEL analysis showed that 14-3-3 negatively regulates the levels of apoptosis induced by the wild type DP3/E2F5 but not the DP3 5S mutant/E2F5 heterodimer. Indeed, the levels of apoptosis observed when 14-3-3 was co-transfected with DP3 5S and E2F5 were around four-fold higher than when 14-3-3 was co-expressed with wt DP3 and E2F5. Interestingly co-transfection of 14-3-3 with the DP3 5S mutant and E2F5 resulted in a slight increase of the apoptotic population compared to the DP3 5S mutant and E2F5 alone. This could be attributed to the 14-3-3 that is unable to bind the DP3 5S mutant acting in the same manner as when 14-3-3 is expressed alone. Indeed, the extra apoptosis observed is approximately equal to that seen when 14-3-3 is expressed alone. It may also be that this extra apoptotic effect is perhaps due to an as of yet undefined function of the 14-3-3 that cannot bind to the DP3/E2F5 heterodimer through inactivation (by re-localisation to the cytoplasm) of an anti-apoptotic protein. Indeed, Figure 5.2A(ii) highlighted the fact that most 14-3-3 in the presence of the DP3 5S mutant/E2F5 complex re-localised to the cytoplasm.

Recent work has shown that E2F5 can induce apoptosis in a manner dependent on its nuclear localisation and the presence of an intact transcriptional domain (Loughran

and La Thangue, 2000). In order to investigate whether 14-3-3 regulates DP3/E2F5 mediated apoptosis in a manner dependent on the transcriptional domain, the TUNEL assay was repeated with a transcriptionally dead E2F5 (TAD E2F5). Although the levels of apoptosis were reduced under these conditions (as expected), the pattern of apoptosis remained the same. 14-3-3 inhibited the apoptosis induced by E2F5 and wt DP3 but it did not have any effect when co-expressed with E2F5 and the DP3 5S mutant. Again, the sample expressing 14-3-3, E2F5 and the DP3 5S mutant showed around a four-fold increase in apoptosis compared to when 14-3-3 was co-expressed with wt DP3 and E2F5. Under these conditions the same slight increase in the apoptotic population was observed suggesting that the 14-3-3 that is unable to complex with the DP3 5S mutant causes an increase in apoptosis through an unknown mechanism. This result allowed the conclusion that 14-3-3 is able to regulate the levels of apoptosis induced by wt DP3 and E2F5 in a manner that is independent of the presence of the transcriptional domain of E2F.

In order to investigate further the mechanism by which 14-3-3 regulates the DP3/E2F5 dependent apoptosis the possibility that 14-3-3 inhibits this apoptotic pathway by destabilising the DP3/E2F5 complex was analysed. Analysis of the persistence in the cell of both the wt DP3/E2F5 and the 5S mutant/E2F5 complexes in the absence and the presence of 14-3-3 showed that 14-3-3 was able to reduce the stability of the wt DP3/E2F5 heterodimer. However 14-3-3 was unable to affect the levels of the DP3 5S mutant/E2F5 complex. This is presumably because 14-3-3 is unable to effectively complex with DP3. The results obtained in Figure 7.2C suggest

that 14-3-3 is essential in regulating the stability of both E2F5 and DP3 and that this regulation is dependent on the ability of 14-3-3 to bind DP3.

Recent reports suggest that E2F1 associates with cyclin A during S-Phase. This results in DP1 phosphorylation and the subsequent loss of the DNA binding activity of E2F1 due to phosphorylation in the DNA binding domain (Krek *et al.*, 1995). This process thereby allows a tight control over the transcriptional activity of proteins that are involved in cell cycle progression. Improper regulation of such proteins leads to a mis-regulated cell cycle and, ultimately, apoptosis.

It is possible that apoptosis induced by the DP3 5S mutant/E2F5 heterodimer is a result of an enhanced stability of the complex on the DNA. Cells unable to regulate the protein levels of transcription factor complexes bound to DNA activate programmed cell death. Presumably one of the main roles of 14-3-3 is therefore to regulate the levels of such complexes by linking them to the ubiquination and subsequent degradation process.

14-3-3 protein levels also seem to be under similar regulation since 14-3-3 protein levels were reduced in the presence of the wild type but not the mutant DP3/E2F5 complex. This is another indication that 14-3-3 acts as a linker molecule between the protein degradation process and the DP3/E2F5 heterodimer. Indeed, 14-3-3s often act as adaptor proteins, bringing and holding together different protein complexes (Fu *et al.*, 2000).

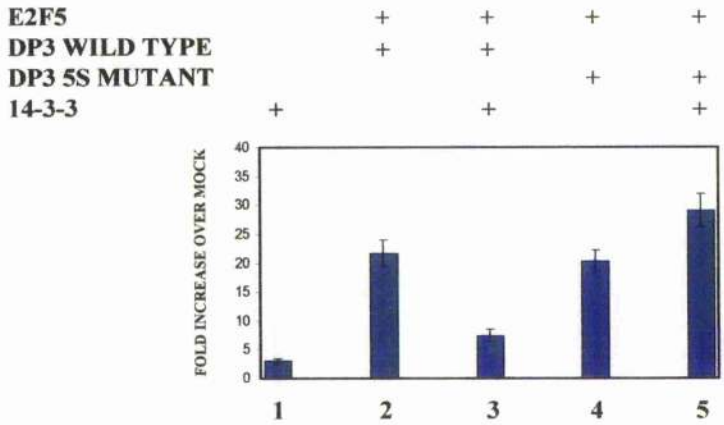
Another interesting observation was that the upper form of DP3 had enhanced stability compared to the lower form. Although 14-3-3 could regulate the stability of all the forms of DP3, the levels of the upper form were not affected to the same extent compared to the lower form of the protein. As described in chapter 3 it is not known exactly what both forms of DP3 represent. Indeed it may be that if the lower band is a smaller form of DP3 due to tryptic digestion then this may explain why this form may be less stable as is shown in the cycloheximide treatment. This therefore points to an additional level of control that 14-3-3 can exert over the DP3/E2F complex, based, not only on the choice of DP partner, but also on the distinction of DP3 protein forms that are in complex with E2F.

Figure 7.2A: 14-3-3 inhibits DP3/E2F5 mediated apoptosis

i) COS-7 cells were transiently transfected with the following amounts of the indicated plasmids: 2μg HA-tagged E2F5, 2μg DP3 wt, 2μg DP3 5S mutant, 4μg myc-tagged 14-3-3 and 1μg RFP. The cells were harvested, fixed onto coverslips and subjected to the TUNEL assay. The assay was carried out blind and 200 cells were counted. The amount of apoptosis is presented as fold increase over mock and the data shown is the average of three independent experiments.

ii) Tabular data representing the values of one of the three experiments carried out.

i)



ii)

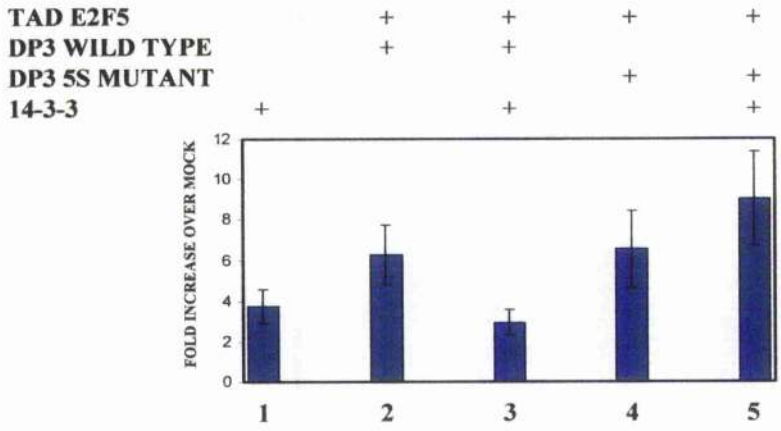
	SAMPLE	TRANSFECTION EFFICIENCY (%)	TUNEL POSITIVE CELLS (%)	TUNEL POSITIVE CELLS RELATIVE TO TRANSFECTION EFFICIENCY (%)
	MOCK	22	0.5	2.3
1	14.3.3	29	2	6.9
2	DP3 WILD TYPE + E2F5	26	12	46.2
3	DP3 WILD TYPE + E2F5 + 14.3.3	22	4	18.2
4	DP3 MUTANT + E2F5	31	13	42
5	DP3 MUTANT + E2F5 + 14.3.3	21	14	66.7

Figure 7.2B: 14-3-3 anti-apoptotic effect is transcription independent

i) COS-7 cells were transiently transfected with the following amounts of the indicated plasmids: 2μg TAD HA-tagged E2F5, 2μg DP3 wt, 2μg DP3 5S mutant, 4μg myc-tagged 14-3-3 and 1μg RFP. The cells were harvested, fixed onto coverslips and subjected to the TUNEL assay. The assay was carried out blind and 200 cells were counted. The amount of apoptosis is presented as fold increase over mock and the data shown is the average of three independent experiments.

ii) Tabular data representing the values of one of the three experiments carried out.

i)



ii)

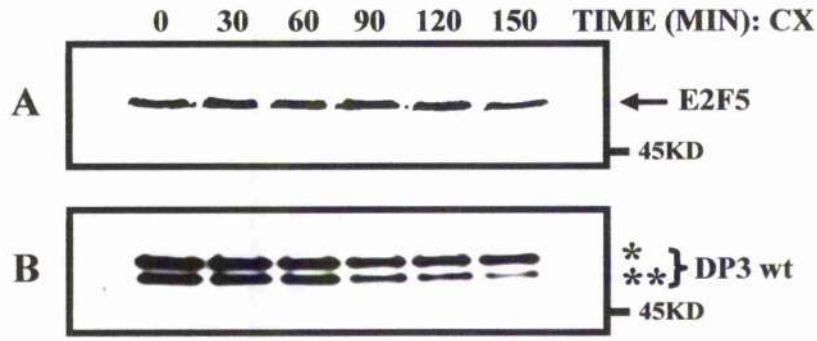
	SAMPLE	TRANSFECTION EFFICIENCY (%)	TUNEL POSITIVE CELLS (%)	TUNEL POSITIVE CELLS RELATIVE TO TRANSFECTION EFFICIENCY (%)
	MOCK	20	1	5
1	14.3.3	18	3	16.7
2	DP3 WILD TYPE + TAD E2F5	20	5	25
3	DP3 WILD TYPE + TAD E2F5 + 14.3.3	18	2	11.1
4	DP3 MUTANT + TAD E2F5	18	5	27.8
5	DP3 MUTANT + TAD E2F5 + 14.3.3	13	5	38.5

Figure 7.2C: 14-3-3 affects the stability of the DP3/E2F5 heterodimer

i) COS-7 cells were transiently transfected with the following amounts of the indicated plasmids: 5μg HA-tagged E2F5 and 5μg DP3 wt. The cells were treated with cycloheximide (CX) and then harvested at the indicated time points. DP3 was detected using the polyclonal antibody, 7.2 and E2F5 with monoclonal anti-HA. The * and ** indicate the upper and lower bands of DP3 respectively.

ii) Graphical representation of the stability of the indicated proteins.

i)


0 30 60 90 120 150 TIME (MIN): CX
A
E2F5
45KD
B
*
**
DP3 wt
45KD

ii)

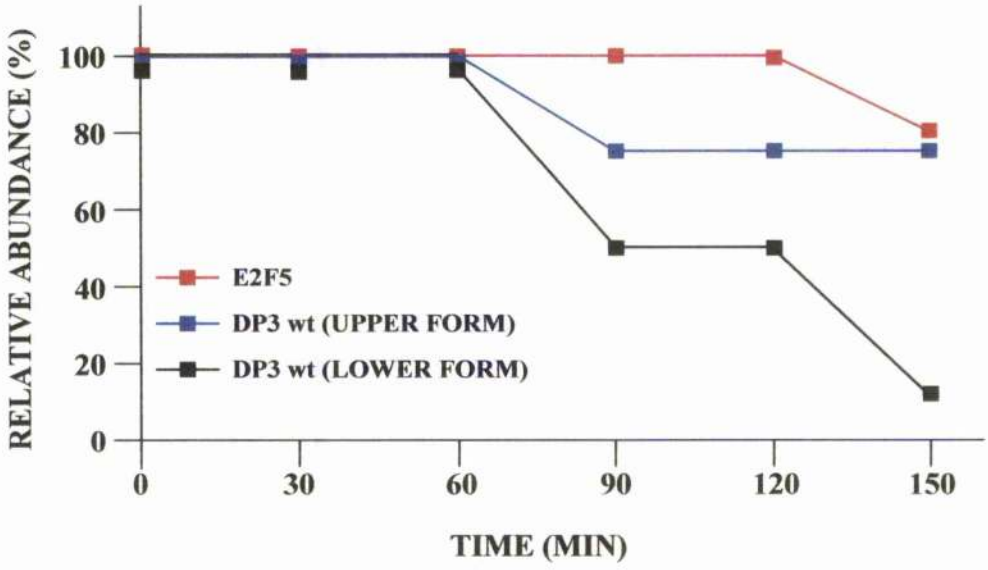
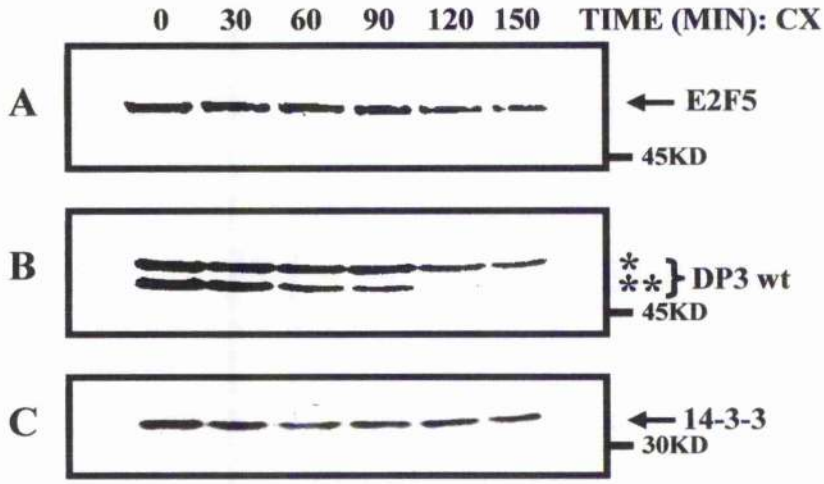

RELATIVE ABUNDANCE (%)
100
80
60
40
20
0
E2F5
DP3 wt (UPPER FORM)
DP3 wt (LOWER FORM)
0
30
60
90
120
150
TIME (MIN)

Figure 7.2C: 14-3-3 affects the stability of the DP3/E2F5 heterodimer

iii) COS-7 cells were transiently transfected with the following amounts of the indicated plasmids: 5μg HA-tagged E2F5, 5μg DP3 wt and 10μg myc-tagged 14-3-3. The cells were treated with cycloheximide (CX) and then harvested at the indicated time points. DP3 was detected using the polyclonal antibody, 7.2, E2F5 with monoclonal anti-HA and 14-3-3 with monoclonal anti-myc. The * and ** indicate the upper and lower bands of DP3 respectively.

iv) Graphical representation of the stability of the indicated proteins.

iii)


0 30 60 90 120 150 TIME (MIN): CX
A
E2F5
45KD
B
DP3 wt
45KD
C
14-3-3
30KD

iv)

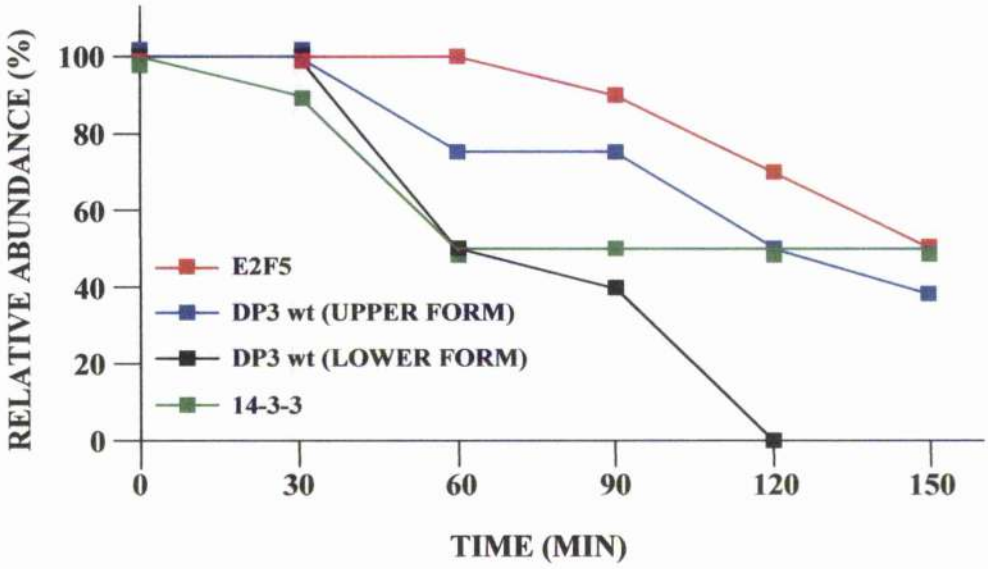
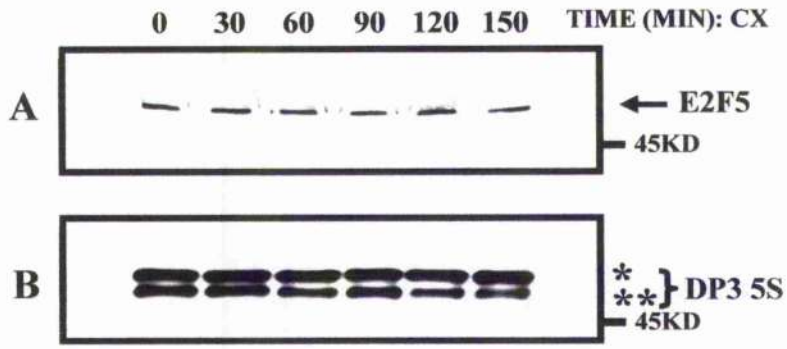

RELATIVE ABUNDANCE (%)
100
80
60
40
20
0
E2F5
DP3 wt (UPPER FORM)
DP3 wt (LOWER FORM)
14-3-3
0
30
60
90
120
150
TIME (MIN)

Figure 7.2C: 14-3-3 affects the stability of the DP3/E2F5 heterodimer

v) COS-7 cells were transiently transfected with the following amounts of the indicated plasmids: 5μg HA-tagged E2F5 and 5μg DP3 5S mutant. The cells were treated with cycloheximide (CX) and then harvested at the indicated time points. DP3 was detected using the polyclonal antibody, 7.2 and E2F5 with monoclonal anti-HA. The * and ** indicate the upper and lower bands of DP3 respectively.

vi) Graphical representation of the stability of the indicated proteins.

v)


0 30 60 90 120 150 TIME (MIN): CX
A
E2F5
45KD
B
*
**
DP3 5S
45KD

vi)

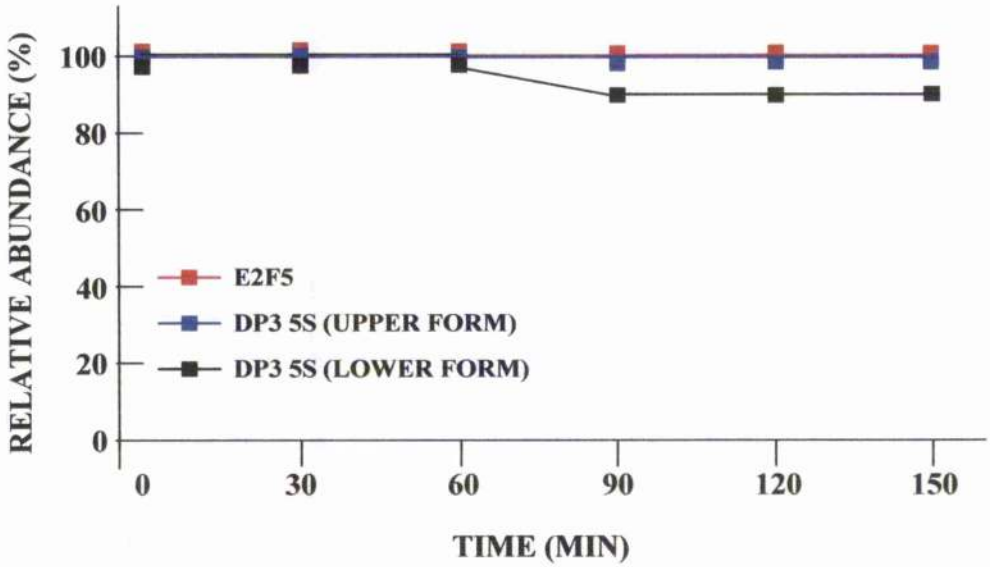
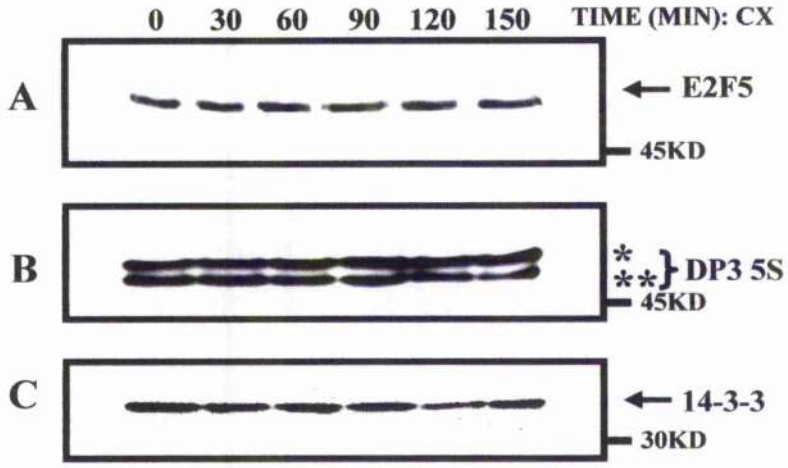

RELATIVE ABUNDANCE (%)
100
80
60
40
20
0
E2F5
DP3 5S (UPPER FORM)
DP3 5S (LOWER FORM)
0
30
60
90
120
150
TIME (MIN)

Figure 7.2C: 14-3-3 affects the stability of the DP3/E2F5 heterodimer

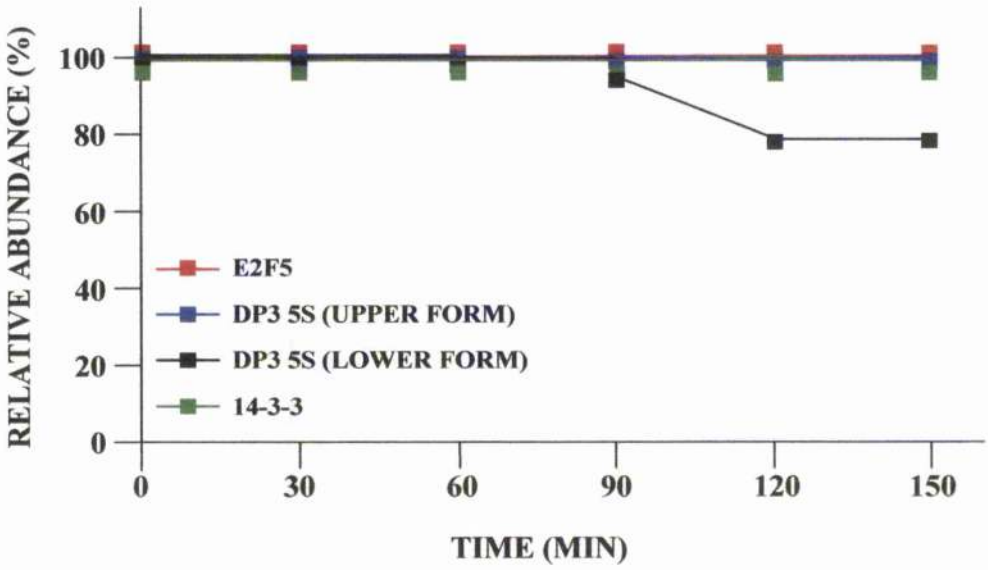
vii) COS-7 cells were transiently transfected with the following amounts of the indicated plasmids: 5μg HA-tagged E2F5, 5μg DP3 5S mutant and 10μg myc-tagged 14-3-3. The cells were treated with cycloheximide (CX) and then harvested at the indicated time points. DP3 was detected using the polyclonal antibody, 7.2, E2F5 with monoclonal anti-HA and 14-3-3 with monoclonal anti-myc. The * and ** indicate the upper and lower bands of DP3 respectively.

viii) Graphical representation of the stability of the indicated proteins.

vii)


0 30 60 90 120 150 TIME (MIN): CX
A
E2F5
45KD
B
*
**
DP3 5S
45KD
C
14-3-3
30KD

viii)


RELATIVE ABUNDANCE (%)
100
80
60
40
20
0
E2F5
DP3 5S (UPPER FORM)
DP3 5S (LOWER FORM)
14-3-3
0
30
60
90
120
150
TIME (MIN)

Chapter 8: The DP3/14-3-3 interaction is DNA damage responsive

8.1: Introduction

14-3-3 proteins are intricately involved in the DNA damage response. 14-3-3σ is able to re-localise cdc2 to the cytoplasm after DNA damage and thereby prevent mitotic onset (Chan *et al.*, 1999). Qi and Martinez, (2003) also showed that following irradiation, 14-3-3ζ associates with cdc25C, resulting in G2 checkpoint activation. These results show that various isoforms of 14-3-3 are essential in preventing improper cell cycle progression following DNA damage.

Given the essential role played by E2F in the response to cellular insults, it was of great interest to investigate whether the relationship between 14-3-3 and DP3/E2F was DNA damage responsive. The purpose of this chapter was to examine if this was the case.

8.2: RESULTS

8.2A: The DP3/14-3-3 interaction is DNA damage responsive

We analysed whether the binding between DP3 and 14-3-3 was responsive to DNA damage. To address this, COS-7 cells were transiently transfected with the plasmids indicated in Figure 8.2A. The cells were treated with etoposide, which induces double stranded breaks in the DNA, and then harvested and subjected to immunoprecipitation with the anti-DP3 antibody, 7.2.

Figure 8.2A clearly shows that less 14-3-3 was seen to co-immunoprecipitate with DP3 after the cells had been treated with etoposide (compare lower track, lanes 7 and 8). Specifically, there was a reduction in the amount of 14-3-3 in complex with DP3 after DNA damage. Comparing the upper track, lanes 7 and 8, it can clearly be seen that equal amounts of DP3 were immunoprecipitated with the anti-DP3 antibody. This implied that the reduction observed in the levels of 14-3-3 in lane 8 was not due to a reduction in the amount of DP3 being immunoprecipitated in that sample.

Incubation of the anti-DP3 antibody with 14-3-3 before and after damage (lower track, lanes 5 and 6) did not result in any 14-3-3 forming a complex with the antibody alone, suggesting that the complex formation between DP3 and 14-3-3 was specific. This result therefore suggested that the interaction between DP3 and 14-3-3 was responsive to DNA damage and, specifically, that the interaction

between these two proteins was reduced following treatment with the DNA damaging agent, etoposide.

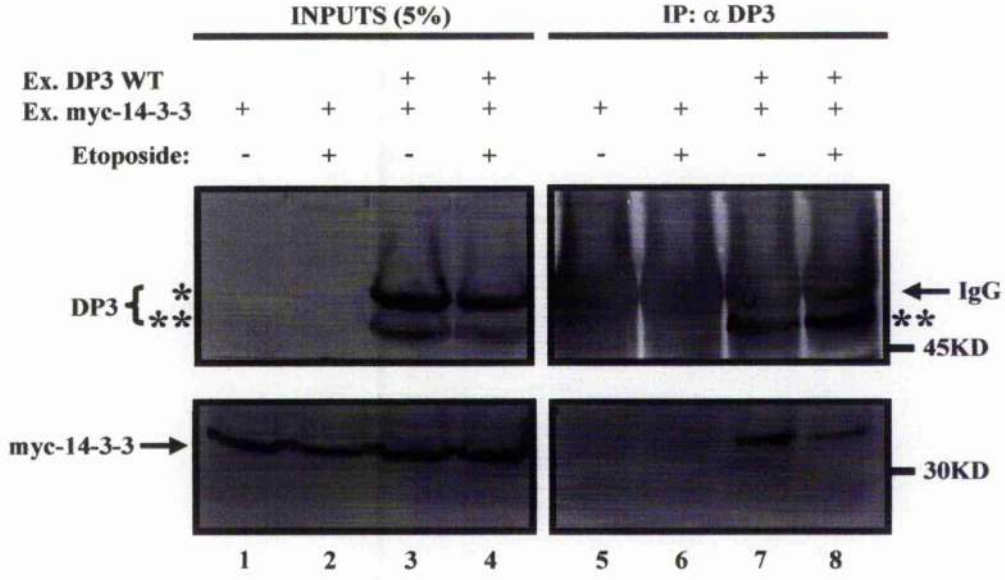
8.3: CONCLUSIONS

Although only a preliminary result, Figure 8.2A provides some indication that the regulation that 14-3-3 exerts over E2F, via DP3, is regulated by the response to DNA damage. Specifically, the level of binding between these two proteins is reduced following double strand breaks in the DNA. Interestingly, this would imply that, following cellular insults, 14-3-3 dissociates from DP3. This would presumably produce the same phenotypes observed with the DP3 5S mutant, which caused a negative regulation on the cell cycle and an increase in apoptosis.

This would certainly seem to be an advantageous situation for the cell, which would need to be able to tightly regulate cell cycle progression following DNA damage. Also, if unable to efficiently repair the damage, the cell would need to initiate the apoptotic response. The result presented in Figure 8.2A would therefore certainly seem to support this idea. Despite this, more work is required to analyse if indeed the interaction between DP3 and 14-3-3 forms part of a checkpoint control mechanism.

Figure 8.2A: The DP3/14-3-3 interaction is DNA damage responsive

COS-7 cells were transiently transfected with the following amounts of the indicated plasmids: 20μg DP3 wt, 40μg myc-tagged 14-3-3 as well as 5μg β-Gal as an internal transfection control (Ex.=Exogenous). Around 50 hours post-transfection the indicated samples were treated with etoposide. Around 15 hours later the samples were harvested and subjected to immunoprecipitation with the specific anti-DP3 antibody, 7.2. DP3 was detected using 7.2 whilst detection of 14-3-3 was carried out using a monoclonal anti-myc antibody. The * and ** indicate the upper and lower bands of DP3 respectively and the position of the IgG is indicated. The assay was carried out twice.


INPUTS (5%)
IP: α DP3
Ex. DP3 WT
Ex. myc-14-3-3
Etoposide:
DP3
*
**
IgG
**
45KD
myc-14-3-3
30KD
1
2
3
4
5
6
7
8

Chapter 9: Discussion

9.1: The association of E2F and 14-3-3 mediated by DP3

The activity of the E2F transcription factor complex is regulated in a number of different ways. These include regulation by the RB family (Helin *et al.*, 1993), phosphorylation by ATM/ATR (Lin *et al.*, 2001) and the cyclin/cdk complexes (Krek *et al.*, 1995; Xu *et al.*, 1994; Guida and Zhu, 1999) and acetylation (Morris *et al.*, 2000; Martinez-Balbas *et al.*, 2000). This study has now shown that the activity of E2F can be regulated by the 14-3-3 family of signalling molecules, through the DP3 partner. Specifically I have shown that 14-3-3 can regulate E2F-dependent cell cycle progression and apoptosis through the DP3 component.

The activity of the E2F heterodimer is dependent on its ability to accumulate in the nucleus. Since E2F4 and E2F5 lack an NLS and require to be in complex with a pocket protein to enter the nucleus (Allen *et al.*, 1997; Verona *et al.*, 1997), this creates a repressive E2F complex. However, work by de la Luna *et al.* (1996) showed that E2F4 or E2F5 could enter the nucleus in a transcriptionally active state by the choice of DP partner. If in complex with either DP3 α or δ, an E2F lacking an intrinsic NLS was driven into the nucleus. This was dependent on the N-terminal bi-partite NLS present in both DP3α and DP3δ. This NLS contained a basic region present in all four DP3 splice variants and an extra (E) region directly N-terminal of the basic region [Figure 1.2B(i)].

Subsequent work in the laboratory showed that removal of the basic region resulted in the cytoplasmic accumulation of DP3 but that this mutant (termed DP3ΔB) could not be re-localised to the nucleus with an NLS-containing E2F. The re-localisation of DP3ΔB was possible with a non-NLS containing E2F, complexed with a pocket protein suggesting that downstream E2F functions are regulated differently after pocket protein-mediated nuclear transport compared with free E2F nuclear transport (Cruickshank, 2000).

With a view towards understanding the role of the DP sub-unit in the E2F/DP heterodimer it was reasoned that the basic region of DP3 was involved in regulating the interaction of the DP sub-unit with another protein. By employing a yeast two-hybrid screen it was found that 14-3-3ε could bind to DP3, via the basic region. Subsequent biochemical analysis confirmed the interaction and, interestingly, it was shown that 14-3-3 was unable to complex with E2F5, suggesting that 14-3-3 influences E2F activity indirectly, through the DP3 partner. It was subsequently shown that the wild type DP3 protein and 14-3-3 could co-localise in the nucleus (Cruickshank, 2000).

Given that the interaction was mediated through the basic region, this posed the question of whether 14-3-3 was influencing the nuclear localisation of E2F, via the interaction with the basic region of DP3 or whether the bi-partite NLS was solely responsible for the nuclear accumulation of the E2F/DP3 heterodimer. 14-3-3s are known to alter the sub-cellular localisation of a variety of target ligands including

cdc25C (Peng *et al.*, 1997), HDAC4 and HDAC5 (Grozinger and Schreiber, 2000) and cdc2 (Chan *et al.*, 1999).

The 14-3-3 binding site in cdc25C is located near a bi-partite NLS (Peng *et al.*, 1997) which is prevented from associating with the importin α/β machinery by 14-3-3 (Zeng and Piwnica-Worms, 1999; Kumagai and Dunphy, 1999). In this way, 14-3-3 can affect the sub-cellular localisation of some of its ligands by altering such signals. Given this, it was of interest to firstly ask whether 14-3-3 was affecting the nuclear accumulation of DP3, via the interaction with the basic region within the bi-partite NLS of DP3.

Given that the vast majority of 14-3-3 ligands are bound via phosphorylated serines (Fu *et al.*, 2000) it was queried whether this was the case with DP3. The bi-partite NLS of DP3 contains five serine residues (Figure 3.1). Subsequent *in vitro* biochemical analysis of the ability of 14-3-3 to bind a range of DP3 mutants bearing a combination of serine to alanine changes resulted in the identification of a mutant that was unable to bind to 14-3-3 (Figure 3.2A). This mutant had changes to all five serine residues and was termed the 5S mutant. The 5S mutant was then shown to be defective in its ability to bind to 14-3-3 in both a pull-down system (Figure 3.2B) and in an *in vivo* IP (Figure 3.2C). Importantly, the 5S mutant was able to localise to the nucleus (Figure 3.2E). This result showed that 14-3-3 does not influence the nuclear localisation of DP3. Interestingly, further biochemical analysis (Figure 3.2D) showed that 14-3-3 and DP2, the human form of DP3,

formed a complex at an endogenous level. This pointed to the conclusion that the interaction between DP2 and 14-3-3 was physiologically relevant.

Further characterisation of the DP3 5S mutant revealed that it was only defective in its ability to bind 14-3-3 and yet behaved like the wild type protein in every other respect (Figures 4.2A-D). Interestingly, it was shown that 14-3-3 and DP3 could still bind whether E2F5 was present in the complex or not (Figure 4.2E). This was an important point to address since it showed that the DP3/14-3-3 interaction is not dependent on the presence of other factors in the complex.

The 5S mutant now provided a powerful tool in analysing the role of the 14-3-3/E2F association, via the DP3 sub-unit. The DP3ΔB mutant had limitations since, due to the removal of the basic region, it was largely cytoplasmic. This was undesirable in elucidating the role of 14-3-3 with a nuclear transcription factor complex. Identification of a mutant of DP3 that was unable to bind 14-3-3 and yet retained its nuclear localisation therefore allowed the detailed examination of the nature of the DP3/E2F/14-3-3 association.

9.2: 14-3-3 does not influence the sub-cellular location of DP3

14-3-3 proteins can indirectly influence the activity of target ligands by altering their sub-cellular localisation. It was therefore of interest to examine whether the binding to 14-3-3 could influence the sub-cellular localisation of DP3. Furthermore, did a lack of binding to the DP3 5S mutant influence the cellular location of 14-3-3. Immunostaining studies showed that 14-3-3 predominantly localised to the nucleus when co-expressed with wt DP3 [Figure 5.2A(i)]. However, analysis of the DP3 5S mutant and 14-3-3 showed a significant amount of cytoplasmic 14-3-3, indicating that the inability to complex with DP3 resulted in the re-localisation of 14-3-3 from the nucleus to the cytoplasm [Figure 5.2A(ii)]. This suggested that DP3 acts as some sort of nuclear anchor for 14-3-3, preventing its export to the cytoplasm.

This posed the interesting question of whether DP3 was influencing the nuclear accumulation of 14-3-3 or whether 14-3-3 that was unable to bind DP3 was simply exported back into the cytoplasm via some undefined mechanism. To address this question leptomycin B was used to block nuclear export and the immunostaining study performed in Figure 5.2A was repeated. Unsurprisingly, 14-3-3 accumulated almost exclusively in the nucleus in the presence of the wild type DP3 protein [Figure 5.2B(ii)]. However, when 14-3-3 was co-expressed with the 5S mutant it again localised predominantly to the nucleus [Figure 5.2B(iii)].

This result indicates that 14-3-3 is not dependent on the ability to bind DP3 to enter the nucleus and that it can actively shuttle between the nucleus and the cytoplasm. Given that recent work by Brunet *et al.*, (2002) has now shown that 14-3-3 proteins do not contain a nuclear export signal this poses the interesting question of how exactly 14-3-3 that is unable to bind to DP3 can enter and exit the nucleus. Although the work in chapter 3 has shown that the DP3 5S mutant and 14-3-3 are unable to form a complex, this does not prove that the interaction is direct. Figure 5.2B suggests that there may be another protein within the complex that is still able to bind to 14-3-3 and which is able to localises it between the nucleus and the cytoplasm. This would explain how 14-3-3 that is unable to bind to DP3 is able to shuttle between the nucleus and the cytoplasm.

As previously stated in the conclusions in chapter 5 it also cannot be ruled out that 14-3-3 that is unable to bind to DP3 is then targeted to other endogenous partners that localise it predominantly to the cytoplasm. However, if this was the case it could be expected that the same pattern of distribution of 14-3-3 in the presence of the DP3 5S mutant would be observed when 14-3-3 is expressed alone. However it may be that 14-3-3 that is co-expressed with the DP3 5S mutant behaves in a different manner from 14-3-3 that is expressed alone.

If 14-3-3 is not dependent on DP3 to enter the nucleus then this would allow the interaction to be more dynamic, with 14-3-3 responding to the signals to bind DP3 at any point. Despite this, it may be that the interaction is of a more rigid nature since loss of 14-3-3 regulation results in a block in cell cycle progression and an apoptotic response.

9.3: 14-3-3 can positively influence E2F-dependent cell cycle progression

Both E2F and 14-3-3 proteins play an integral role in the control of the mammalian cell cycle. E2F co-ordinates gene expression with S-phase induction whilst 14-3-3 proteins play an essential role in controlling the activity of proteins involved in cell cycle progression. Given this, it was of interest to examine if 14-3-3 had an effect on E2F dependent cell cycle progression. The *in vitro* effect of 14-3-3 on the transcriptional activity of E2F via either DP3 wild type or the 5S mutant was examined (Figure 6.2A).

Both E2F5/DP3 wild type and E2F5/DP3 5S mutant showed similar levels of transcriptional activity on the cyclinE promoter. However, when 14-3-3 was titrated in along with the heterodimers the results differed remarkably. Whereas the levels of transcriptional activity of the wild type heterodimer increased as more 14-3-3 was titrated into the system, the transcriptional activity of the mutant heterodimer rose slightly but did not increase in the same manner as E2F5/DP3 wild type. This

result suggested that 14-3-3 can increase the transcriptional activity of E2F5, via the DP3 sub-unit. Presumably the lack of binding to the E2F5/DP3 5S complex prevents 14-3-3 from enhancing the transcriptional activation of the heterodimer. Indeed, work by Waterman *et al.* (1998) showed that dephosphorylation of p53 at serine-376 creates a 14-3-3 binding site at serine-378. This leads to an enhancement of p53 DNA binding activity and more effective transcriptional activity.

Given this result, it was of interest to examine what effect this would have *in vivo*. To address this question FACS and Brdu analysis were carried out. The FACS analysis (Figure 6.2B) revealed that the E2F5/DP3 wild type heterodimer could induce cell cycle progression marginally more than the E2F5/DP3 5S mutant construct. However, when 14-3-3 was co-transfected, the amount of cell cycle progression observed in the E2F5/DP3 wild type increased significantly whereas the E2F5/DP3 5S mutant heterodimer showed only a marginal increase.

Similarly, Brdu analysis, which examines S-phase induction, showed significant differences between the wild type and mutant heterodimers (Figure 6.2C). Neither the wild type nor mutant heterodimers showed a significant increase in S-phase entry over mock when expressed alone. When 14-3-3 was co-expressed, the amount of cells in S-phase increased two-fold with the E2F5/DP3 wild type construct however no effect was observed with the E2F5/DP3 5S heterodimer. Taken together these *in vivo* results suggest that 14-3-3 tightly controls E2F-mediated S-phase entry.

These observations are slightly surprising given the inhibitory role traditionally played by the 14-3-3 proteins in the control of the cell cycle. 14-3-3s are seen to block S-phase entry by negatively regulating cdk2 and cdc2 (Laronga *et al.*, 2000) and to prevent mitotic onset by sequestering cdc25C in the cytoplasm (Peng *et al.*, 1997). The sequestration of HDAC4 and HDAC5 by 14-3-3 proteins in the cytoplasm also prevents the gene silencing associated with these proteins (Grozinger and Schreiber, 2000).

The enhancement of cell cycle progression by 14-3-3, through E2F, is therefore unique within the roles that 14-3-3 plays in regulating ligands involved in the cell cycle. However, 14-3-3 can enhance the DNA binding activity of p53 (Waterman *et al.*, 1998), which may lead to an enhancement of transcription. Given that 14-3-3 may act in the same way to enhance the ability of the E2F5/DP3 heterodimer to bind the DNA, this could lead to an enhancement of transcriptional activity and more efficient cell cycle progression, as was observed in Figures 6.2A-C.

9.4: 14-3-3 negatively regulates E2F5/DP3-mediated apoptosis

14-3-3 proteins are known to regulate an array of proteins involved in the apoptotic response including Bad (Xing *et al.*, 2000), FKHRL1 (Brunet *et al.*, 1999) and ASK1 (Zhang *et al.*, 1999). Recent work by Loughran and La Thangue, (2000) showed that E2F4 and E2F5 could induce an apoptotic response in SAOS2 cells.

This response was dependent on the presence of the transcriptional activation domain of the proteins. Given this, it was of interest to investigate whether 14-3-3 had an effect on the ability of the E2F5/DP3 heterodimer to induce apoptosis.

Subsequent TUNEL analysis revealed similar results to the work by Loughran and La Thangue, (2000). Both the wild type and mutant heterodimers could induce apoptosis to a similar degree, and this was approximately twenty times higher than the mock sample (Figure 7.2A). Upon the co-expression of 14-3-3, the amount of apoptosis induced by E2F5/DP3 wild type was reduced significantly. This result is in accordance with published data, which support the fact that 14-3-3 proteins have a role in suppressing the apoptotic response. Interestingly, the amount of apoptosis induced by the DP3 5S mutant and E2F5, when co-expressed with 14-3-3, rose slightly. This could be attributed to the 14-3-3 that is unable to bind the DP3 5S mutant acting in a similar manner as when 14-3-3 is expressed alone since this itself caused around a three to four-fold increase in apoptosis over the mock sample. The extra apoptosis observed when 14-3-3 is co-expressed with E2F5 and the DP3 5S mutant could also be due to some other role that 14-3-3 carries out when unable to bind to the 5S mutant. This may involve the inactivation of an anti-apoptotic protein. This remains to be elucidated.

Interestingly, the suppression of E2F5/DP3 mediated apoptosis does not require the re-localisation of the complex to the cytoplasm, as is observed in many 14-3-3 associated apoptotic substrates. 14-3-3 must regulate E2F5/DP3-mediated apoptosis

in a different manner, possibly by influencing the choice of promoter occupied by the heterodimer. Perhaps when bound to 14-3-3 the E2F5/DP3 wild type complex is specifically targeted to either an anti-apoptotic gene or the E2F5/DP3/14-3-3 association could form an inhibitory complex on pro-apoptotic genes. However, one possibility was that 14-3-3 was influencing the apoptotic response through the regulation of the transactivation domain in E2F5. To test this, the TUNEL analysis was repeated with a transcriptionally dead E2F5 construct (Figure 7.2B). As expected, and in accordance with the work carried out by Loughran and La Thangue, (2000), the removal of the transactivation domain resulted in a significant decrease in the amount of apoptosis induced by E2F5. Despite this, the pattern of 14-3-3 repression of apoptosis remained the same with, yet again, an increase in apoptosis when 14-3-3 was co-expressed with the E2F5/DP3 5S heterodimer. This result suggested that 14-3-3 was able to regulate E2F5/DP3-mediated apoptosis in a manner that was independent of the presence of the transactivation domain.

During S-phase, the cyclinA/cdk2 complex regulates the activity of E2F1/DP1. Phosphorylation of DP1 and E2F1 results in the reduced binding affinity of the heterodimer and the subsequent degradation of both DP1 and E2F1 (Krek *et al.*, 1994, Xu *et al.*, 1994; Guida and Zhu, 1999; Marti *et al.*, 1999). This allows the regulation of the levels of E2F, something that is essential in preventing mis-regulation of E2F activity and apoptosis. Given this, it was of interest to examine if the regulation of E2F5/DP3-dependent apoptosis by 14-3-3 was through regulation of the stability of the heterodimer.

Subsequent cycloheximide treatment (Figure 7.2C) showed that the stability of the E2F5/DP3 wild type complex was reduced upon the co-expression with 14-3-3. The stability of the mutant heterodimer was not affected either in the presence or the absence of 14-3-3 and it can therefore be concluded that 14-3-3 regulates the protein levels of the E2F5/DP3 complex. Interestingly, the stability of 14-3-3 was affected when in association with E2F5/DP3 wild type. This suggests that 14-3-3 itself is regulated in a similar manner and that it may act as a linker molecule between E2F5/DP3 and the ubiquination process. In many cases 14-3-3 plays such a role, bringing together and linking up various protein-protein interactions.

Intriguingly, the upper form of DP3 had an extended persistence in the cell compared to the lower form. This suggests a further level of control that 14-3-3 can exert on E2F, based on the protein form of DP3 partner. Although it is unknown what both forms of DP3 represent it may be that the lower form has been tryptically digested and this would therefore explain why the lower form is less stable than the upper form. Mass spectroscopy should be able to determine exactly what both forms of DP3 are.

9.5: The DP3/14-3-3 interaction is DNA damage responsive

Given the role played by both E2F and 14-3-3 in the DNA damage response it was of interest to analyse if the regulation that 14-3-3 exerted over DP3/E2F was regulated by DNA damage. Indeed, Figure 8.2A showed that the interaction

between DP3 and 14-3-3 was abrogated following treatment with the DNA damaging agent, etoposide. This result suggested that following the damage response the phenotypes observed with the DP3 5S mutant would occur. This would therefore lead to a negative regulation on the cell cycle and an enhancement of the apoptotic response.

Despite this, much more work is required to prove this and Figure 8.2A only provides an initial, but interesting result, suggesting that the DP3/14-3-3 interaction can be regulated by the response to DNA damage and may be important in a checkpoint control mechanism. It may be that the level of binding is regulated depending on the time after the initiation of the DNA damage. Perhaps the interaction is completely abrogated after only a few hours and that, after this period, DP3 and 14-3-3 re-form a complex. This will be one of the many interesting experiments to carry out in the future.

9.6: Overall conclusions and future work

This study has now defined a unique level of control of E2F activity, mediated through the DP3 component, by the 14-3-3 family of proteins. The work presented here has shown that the abolition of the DP3/14-3-3 interaction results in an inhibition of cell cycle progression and an increase in apoptosis. Based on the results presented here I propose a 'dual role' model whereby the regulation of E2F activity by 14-3-3 proteins is dependent on the binding to the DP3 component and

that this interaction mediates the stability of the E2F5/DP3 heterodimer (Figure 9.1).

If a kinase induces the phosphorylation of DP3 on serine residues within the 14-3-3 binding site in DP3 this could lead to the association of 14-3-3 with DP3. 14-3-3 may be able to enhance the binding of the heterodimer to the DNA in a manner perhaps analogous to that of p53. This could perhaps lead to a more transcriptionally active E2F5/DP3 complex, which can then induce cell cycle progression. Given that Figure 6.2A shows that 14-3-3 can enhance the transcriptional activation of E2F5/DP3, and that this effect is titratable, this is an entirely plausible model.

I also propose that 14-3-3 can regulate the stability of the heterodimer by linking it to the protein degradation process. An inability to bind 14-3-3 leads to the accumulation of the E2F5/DP3 heterodimer on the DNA. Since the cell is unable to regulate the stability of the complex it initiates an apoptotic response. 14-3-3 can prevent this by regulating the levels of E2F5/DP3 and preventing the accumulation of the complex. In this way 14-3-3 acts to 'cap' the levels of E2F5/DP3 by allowing the continual turnover of the E2F5/DP3/14-3-3 complex.

This model poses some interesting questions. Whether the association between 14-3-3 and DP3 occurs in the cytoplasm or in the nucleus is unknown. Figure 5.2B suggests that 14-3-3 can actively shuttle between the nucleus and the cytoplasm,

suggesting that the association may take place when the heterodimer is in complex with the DNA. This is unlikely since this would lead to the phenotypes observed with the mutant complex. It is likely that the association takes place in the cytoplasm and the bi-partite NLS in DP3 causes the nuclear accumulation of both E2F5 and 14-3-3. As shown in Figure 7.2A, there is an increase in the levels of apoptosis when 14-3-3 is co-expressed with the E2F5/DP3 5S mutant heterodimer. Presumably this is caused by the 14-3-3 that is unable to bind to the E2F5/DP3 5S heterodimer. Perhaps this unbound 14-3-3 influences the sub-cellular localisation of an anti-apoptotic protein, leading to an increase in the apoptotic response.

The proposed model of the regulation of E2F by 14-3-3 raises a variety of questions that must be addressed in future work. Firstly, although the interaction is **possibly** mediated by the phosphorylation of the serine residues within the bi-partite NLS, this does not rule out that the mutations made merely disrupts the tertiary structure of DP3 sufficiently to abrogated 14-3-3 binding either within the NLS or at another site within DP3. 14-3-3 proteins have recently been shown to bind to non-phosphorylated motifs in ligands such as 5-phosphatase, exoenzyme S, A20 and the platelet glycoprotein IB-IX-V complex (Masters *et al.*, 1999; Vincenz and Dixit. 1996; Du *et al.*, 1996; Campbell *et al.*, 1997). It may therefore be the case that 14-3-3 can bind to non-phosphorylated motif(s) within DP3 since none of the serines that were mutated fit the consensus sequences for 14-3-3 binding ligands.

It will therefore be of interest to analyse if the binding between 14-3-3 and DP3 can be maintained in the presence of phosphatases. If so, and the binding can be shown to be phosphoserine-dependent, it will be of interest to investigate what kinases may be involved in mediating the binding. Given that all five serine residues were mutated, the role played by each one in the binding to 14-3-3 must be elucidated. Figure 3.2A shows that the 2S, 3S and 4S mutants have similar levels of reduced binding affinity for 14-3-3 compared to the wild type, whilst the 5S mutant is completely unable to bind 14-3-3. This suggests that serine-42 may be involved in binding and either, or both, of serines-58 or 61 [Figure 3.1(i)].

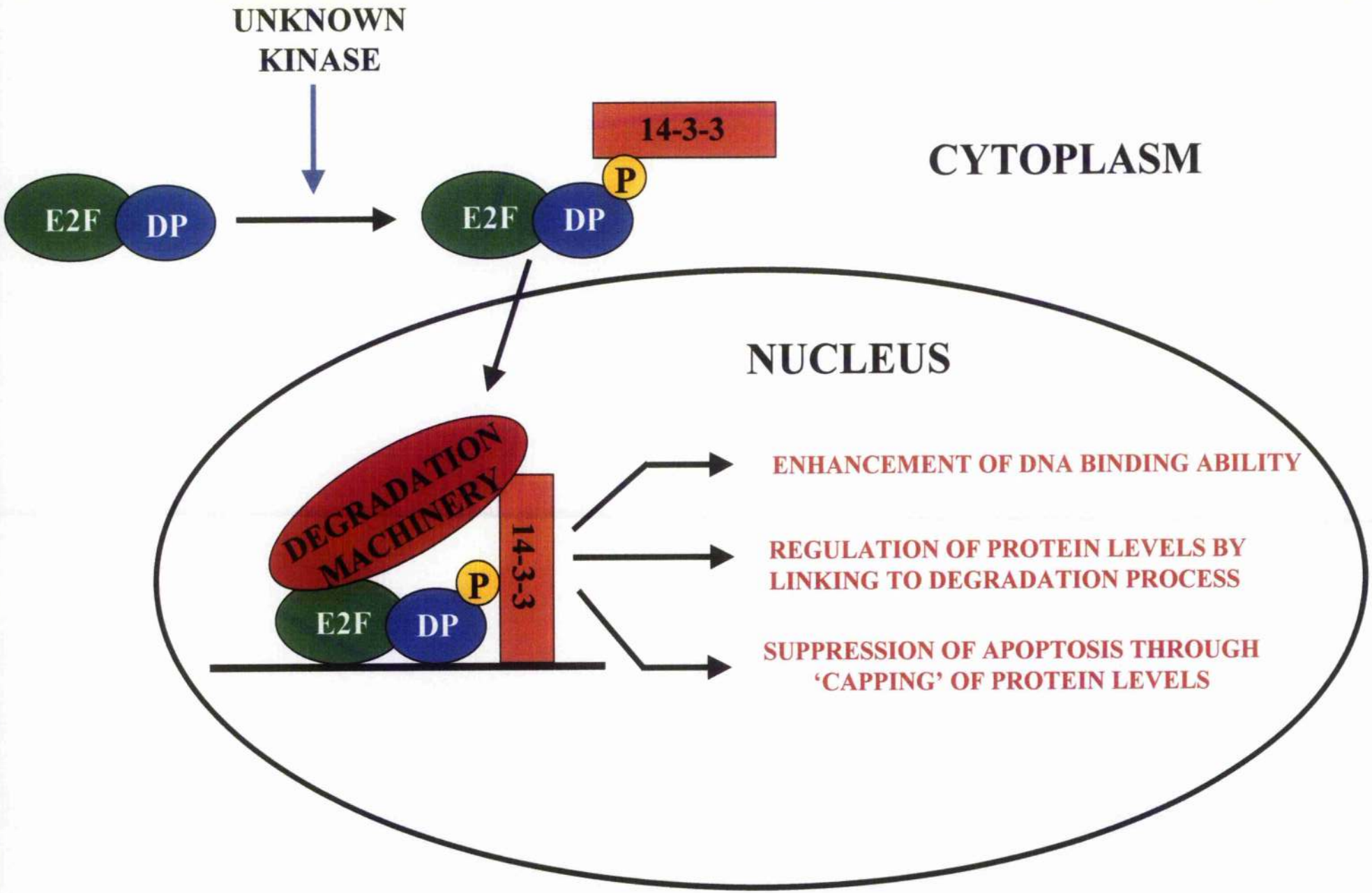
If the association between DP3 and 14-3-3 is mediated by phosphorylation this poses the question of the mechanism that switches the interaction off. Presumably the binding is dynamic in nature and 14-3-3 can be bound, or released, from the heterodimer depending on intra-cellular and extra-cellular signals. The association between p53 and 14-3-3 occurs after DNA damage following the de-phosphorylation of serine-376 (Waterman *et al.*, 1998). This creates a 14-3-3 binding site at serine-378. It may be that de-phosphorylation of the serine residues is also a critical step in the association between 14-3-3 and DP3. Presumably, de-phosphorylation would lead to the phenotypes observed with the 5S mutant heterodimer.

In some circumstances this may be desirable for the cell. For instance, after DNA damage, a loss of binding would result in a reduction in DNA binding, and an accumulation of the heterodimer. This would lead to an inhibition of cell cycle progression and a potent apoptotic response. This would allow the cell to positively regulate the apoptotic response following DNA damage. Given that 14-3-3σ is involved in the DNA damage response it is plausible that other isoforms such as 14-3-3ε may be as well. Figure 8.2A would certainly support this idea.

Given this, it would also be of interest to examine the role played by the various isoforms of 14-3-3 in the regulation of the E2F/DP3 association. It is possible that other isoforms of 14-3-3 can bind DP3, and perhaps regulate the activity of the heterodimer in other ways. Previous work in the laboratory suggested that the role played by 14-3-3 in the regulation of E2F5/DP3 differed when 14-3-3 associated with E2F1/DP3 (Cruickshank, 2000). Since the 'activating' and 'repressing' E2Fs have been shown to have functionally distinct characteristics, it may also be that the influence that 14-3-3 exerts on E2F is dependent on the member of the family that is in complex with DP3.

Figure 9.1: Model for the Regulation of E2F/DP3 by 14-3-3

If DP3 phosphorylation within the bi-partite NLS leads to the binding of 14-3-3 DP3 would be able to drive E2F5 and 14-3-3 into the nucleus. 14-3-3 may then be able to enhance the binding of the heterodimer to the DNA as well as link the E2F5/DP3/14-3-3 complex to the degradation machinery. This could lead to a more transcriptionally active E2F5/DP3 heterodimer due to the enhanced DNA binding, as well as the 'capping' of the levels of the complex. This tight regulation of protein levels may therefore be able to prevent the accumulation of E2F5/DP3/14-3-3 on the DNA, which could block the induction of an apoptotic response.


UNKNOWN
KINASE
14-3-3
P
E2F
DP
E2F
DP
CYTOPLASM
NUCLEUS
DEGRADATION
MACHINERY
14-3-3
P
E2F
DP
ENHANCEMENT OF DNA BINDING ABILITY
REGULATION OF PROTEIN LEVELS BY
LINKING TO DEGRADATION PROCESS
SUPPRESSION OF APOPTOSIS THROUGH
'CAPPING' OF PROTEIN LEVELS

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