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Regulation of the p53 cofactor JMY

A thesis presented by

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To

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Division of Biochemistry and Molecular Biology

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To Dad, Mum, Samira and Bilet

Abstract.

The anti-proliferative function of the p53 tumour suppressor depends primarily on its ability to act as a transcription factor and to coordinate the cellular response to various stress signals. p53 can induce several cellular responses, including cell-cycle arrest, DNA repair and apoptosis. The choice of the p53 response is thought to depend on many factors, including the action of p53-cooperating molecules.

The cofactor JMY has been demonstrated to cooperate with the p300 coactivator to augment p53 activity. The aim of this study was to investigate the mechanisms regulating JMY function to further understand its role in the stress response. Results presented here indicate that the subcellular localisation of JMY is regulated by the cellular environment. Whilst DNA damage stimulated the translocation of JMY from the cytoplasm to the nucleus, removal of proliferative and survival signals by serum deprivation induced rapid nuclear export of JMY and compromised its ability to affect cell cycle progression and stimulate apoptosis.

The Mdm2 oncoprotein negatively regulates p53 activity at multiple levels including regulation of the intracellular location of p53 and inhibition of its interaction with the transcriptional coactivator CBP. Mdm2 interacts with JMY in cells, and enhances its cytoplasmic localisation without promoting its degradation. Leptomycin B blocked Mdm2-dependent redistribution of JMY, implying the involvement of a nuclear export mechanism. DNA damage was found to abrogate the effect of Mdm2 on the subcellular localisation of JMY, and to reduce the

interaction between the two proteins. Mutations in the ubiquitin ligase domain of Mdm2, as well as removal of the proline-rich region in JMY abolished the Mdm2-mediated cytoplasmic translocation of JMY. These data suggest the existence of a functional relationship between the p53 cofactor JMY, and its major inhibitor Mdm2, which is likely to impact on p53 activity. Importantly, regulation of the intracellular localisation of JMY might provide an additional mechanism of modulating the p53 response.

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Abbreviations

AIP1	Apoptosis inducing protein 1
AP-1	Activating protein 1
Apaf-1	Apoptotic protease activating factor 1
APC	Adenomatous polyposis coli
ARF	Alternative reading frame
ASPP	Apoptosis stimulating protein of p53
ATM	Ataxia-telangiectasia mutated
ATR	Ataxia –telangiectasia and Rad3-related
Bax	Bcl-2 associated protein X
BCIP	5-Bromo-4-chloro-3-inodolyl phosphate
Bcl-2	B-cell lymphoma 2
β -gal	β -galactosidase
bp	Base pair
BRCA1	Breast cancer gene 1
BSA	Bovine serum albumin
c-Abl	Abelson virus cellular oncogene homolog
CBP	CREB-binding protein
Cdc2	Cell division cycle 2
Cdk	Cyclin dependent kinase
Chk	Checkpoint kinase protein
C/H	Cysteine/histidine rich domain
CHIP	Chromatin immunoprecipitation
CIP1	Cdk-interacting protein 1

CMV	Cytomegalovirus
CR2	Conserved region 2 (in the adenovirus E1A protein)
CRM1	required for chromosome region maintenance
CSD	Cytoplasmic sequestration domain
DR5	Death receptor 5
DAPI	4,6- Diamidino-2-phenylindole
DM	Double minute
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DNA-PK	Double-stranded DNA-activated protein kinase
DP	DRTF1 polypeptide
DTT	Dithiothreitol
E1A	Adenovirus early protein 1A
EDTA	Ethylene diamine tetra-acetic acid
ER	Estrogen receptor
ERK	Extracellular signal –regulated kinase
FCS	Foetal calf serum
G1	Gap phase 1
Gadd45	Growth arrest and DNA damage gene product
GDP	Guanidine di-phosphate
GR	Glucocorticoid receptor
GTP	Guanidine tri-phosphate
HA	Haemagglutinin peptide (derived from influenza virus)

HAT	Histone acetyltransferase
HBS	HEPES-buffered saline
Hdm2	Human homologue of murine double minute 2
HEPES	N-[2-Hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid]
HIF	Hypoxia inducible factor
HIV	Human immunodeficiency virus
Hn RNP	Heterogeneous ribonucleoprotein
HPV	Human papilloma virus
IB	Immunoblot
IGF BP	Insulin-like growth factor binding protein
I κ B	Inhibitor of NF κ B
IP	Immunoprecipitation
IPTG	Isopropyl- β -D-thiogalactopyranoside
IR	Ionising radiation
JAK	Janus kinase
JMY	Junction mediating and regulatory protein
JNK	c-Jun NH ₂ terminal kinase
KDa	Kilodalton
LMB	Leptomycin B
MAL	Megakaryocytic acute leukaemia
Mdm2	Murine double minute 2
MEF	Mouse embryonic fibroblasts
mRNA	Messenger ribonucleic acid
MyoD	Myogenic IILH transcription factor

NAP	Nucleosome assembly protein
NB	Nuclear body
NES	Nuclear export signal
NF AT	Nuclear factor of activated T cells
NFκ B	Nuclear factor of immunoglobulin κ locus in B cells
NLS	Nuclear localisation signal
NoLS	Nucleolar localisation signal
NPC	Nuclear pore complex
NS	Nucleocytoplasmic shuttling signal
P	Proline
PAGE	Polyacrylamide gel electrophoresis
Parc	p53-associated Parkin like cytoplasmic protein
PBS	Phosphate buffered saline
P/CAF	p300/CBP associated factor
PCIP	p300/CBP interacting protein
PCNA	Proliferating cell nuclear antigen
Pcg3	Paternally expressed gene 3
PIDD	p53 induced protein with death domain
PIGs	p53-induced genes
PI3K	Phosphatidylinositol 3 kinase
PML	Promyelocytic leukaemia
PMSF	Phenylmethylsulfonyl fluoride
PP2A	Protein phosphatase 2 A

PR	Progesterone receptor
PTEN	Phosphatase and tensin homolog
PUMA	p53 upregulated modulator of apoptosis
RAR	Retinoic acid receptor
Rb	Retinoblastoma tumour suppressor protein
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RTS	Rubinstein-Taybi syndrome
SDS	Sodium dodecyl sulphate
SH3	Src homology domain 3
SRC-1	Steroid receptor coactivator-1
SRE	Serum response element
SREBP	Sterol regulatory element binding protein
SRF	Serum response factor
STAT	Signal transducers and activators of transcription
S/T-P	Serine/Threonine-Proline
Strap	Stress induced activator of p300
SV40	Simian virus 40
TAF	TBP-associated factor
TBP	TATA binding protein
TPR	Tetratricopeptide motif
TR	Thyroid receptor
TSG101	Tumour susceptibility gene 101
sn RNA	Small nuclear RNA

UV	Ultraviolet light
v/v	Volume per volume
WAF1	Wild type p53-activated fragment 1
WH2	Wiscott-Aldrich homology domain 2
Wip1	Wild type p53 inducible phosphatase 1
ZF	Zinc finger
-/-	Homozygous mutant
+/-	Heterozygous 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.

A handwritten signature in blue ink, appearing to read 'Houda Boulahbel', with a stylized flourish at the end.

Houda Boulahbel

September 2003

Chapter 1. Introduction

Although cancer research and the study of evolution seem to be very distant branches of biological study, they focus on similar fundamental mechanisms. Cancer can be viewed as an evolutionary process at a micro-scale, not at the level of populations or whole organisms but at the cellular level. In fact, the two major forces of evolution, mutation and selection are also the driving forces in the development of cancer.

Cancer is a disease characterised by the progressive accumulation of genetic alterations which eventually lead to the transformation of normal cells to malignant cells with increasingly aggressive growth properties. Such cells must evade the cellular defence mechanisms that normally protect against malignancy, and this process is accelerated by lesions that target the cellular machinery involved in the control of cellular proliferation, and the maintenance of genomic integrity.

Human cancer mutations target two major classes of genes, the “caretaker” genes involved in the repair of DNA lesions, and the growth controlling oncogenes and tumour suppressor genes (Kinzler and Vogelstein, 1997).

Since tumour suppressor proteins act to restrain cellular growth, their inactivation confers a growth advantage to tumour cells and is a critical factor in tumourigenesis. In particular, the p53 protein, which possesses potent tumour suppressor functions, is targeted in the majority of human cancers. This resulted in the protein being the focus of intensive research that aims to understand its

function and dysfunction and to use the acquired knowledge in the development of novel cancer therapies.

1-1. Regulation of the p53 tumour suppressor protein

The p53 tumour suppressor is a short lived transcription factor with a pivotal role in the prevention of malignant disease. The function of the protein is tightly regulated by an intricate web of regulatory mechanisms which ensure its appropriate activation, and coordinated action in response to a variety of cellular insults. The ability of p53 to suppress tumourigenesis is mediated through its ability to cause cell cycle arrest, programmed cell death or DNA repair in response to cellular stress.

Mutation of the p53 tumour suppressor is one of the most common aberrations observed in tumours. It is estimated that at least 50% of human cancers carry mutations of the *p53* gene (Hollstein *et al.*, 1994), and accumulating evidence indicates that a large part of the remaining cancers bear mutations that target downstream or upstream effectors of the p53 pathway (Vousden and Lu, 2002). The most common mutations identified in the *p53* gene are missense mutations affecting one allele, and causing the production of a mutant protein that accumulates at high levels in cells. The remaining wild type allele is often mutated or lost later during tumourigenesis. Rare deletions or chain terminations of the *p53* gene have also been observed and cause a cancer-predisposing *p53* null phenotype (Levine, 1997).

Moreover, p53 is frequently targeted by virally encoded oncoproteins such as SV40 large T antigen, adenovirus E1A, and HPV E6, which inactivate its function (Prives and Hall, 1999).

The importance of p53 in the development of cancer is also highlighted by the phenotype of *p53* null mice. These mice develop normally but display a very high susceptibility to cancer (Donehower *et al.*, 1992). Furthermore, germline mutations of p53 are associated with the cancer-predisposition Li-Fraumeni syndrome. Affected individuals develop a wide variety of tumours often at a very early age (Malkin *et al.*, 1990).

1-1.1 Integration of cellular insults at p53

p53 is kept at low levels, in a latent form under normal conditions. However, a number of cellular stresses result in rapid stabilisation and activation of the transcription factor, which then mediates the cell response to the particular stress signal (Levine 1997) (figure 1.1).

Several types of stress have been shown to induce p53 activity, including DNA damage in the form of double strand breaks as well as DNA repair intermediates resulting from ultraviolet exposure and chemical damage to DNA (Levine, 1997).

In addition to DNA damage, hypoxia is able to stimulate p53 activity (Graber *et al.*, 1996). This is relevant to the biology of tumours as hypoxia occurs when tumours reach a critical size when the blood supply becomes rate-limiting. At this stage, tumour cell survival and continued growth depends on the formation of new blood vessels, a process termed angiogenesis. Activation of p53 by hypoxia will

eliminate such cells, and may also be involved in the prevention of angiogenesis, as suggested by its ability to regulate the gene encoding the anti-angiogenic factor thrombospondin (Dameron *et al.*, 1994).

Moreover, p53 is activated when the levels of ribonucleoside triphosphate, which are required to support DNA replication and repair, are low (Linke *et al.*, 1996). It also responds to aberrant growth signals such as those emanating from abnormal activation of oncogenes, including Ras and Myc. The oncogenic pathway of p53 activation involves the function of another tumour suppressor, ARF (Sherr and Weber, 2000).

1-1.2 The downstream effects of p53

Activation of p53 results in multiple cellular responses that are not only determined by the type and intensity of cellular stress, but also by the genetic and environmental context of the cell. The p53 response can manifest in the form of DNA repair, senescence and regulation of angiogenesis but the best understood activities of p53 are those that inhibit cell growth through cell cycle arrest and apoptosis (figure 1.1).

Cell cycle arrest

The ability of p53 to induce cell cycle arrest is correlated with its function as a transcription factor. A transcriptional target of p53 that is critical for this function is the *p21^{waf1/cip1}* gene (El Deiry *et al.*, 1993). The gene product is a cyclin dependent kinase inhibitor which can block the cell cycle at both the G1 and G2

phases. Further, p21 binds to the DNA polymerase processivity factor PCNA (proliferating cell nuclear antigen), and blocks its function in DNA replication but not its DNA repair function. Loss of p21 impairs G1 and G2 arrest and results in uncoupling of DNA synthesis and mitosis (Waldman *et al.*, 1995).

Gadd45, the product of another p53-responsive gene, can also cause G1 arrest in certain cells in culture. Gadd45 also interacts with PCNA and this interaction is also implicated in DNA repair (Smith *et al.*, 1994).

The cell cycle arrest functions of p53 also extend to the G2/M checkpoint. Members of the 14-3-3 family of proteins are involved in signal transduction and cell cycle control, and can mediate their effects by binding and sequestering proteins (Muslin and Xing, 2000). 14-3-3 sigma is induced by p53 and appears to mediate the role of p53 at the G2/M checkpoint (Hermeking *et al.*, 1997). 14-3-3 sigma deficient cells can still arrest at G2 but are unable to maintain this arrest (Chan *et al.*, 1999). Other p53 induced proteins which can contribute to G2 arrest include Gadd45 (Wang *et al.*, 1999), and Reprimo (Ohki *et al.*, 2000). Moreover, p53 may contribute to G2/M arrest by negatively regulating the cellular levels of cyclin B1, which is required for the initiation of mitosis (Innocente *et al.*, 1999).

Apoptosis

In addition to transient cell cycle arrest, p53 can irreversibly block cell growth by inducing programmed cell death through several apoptotic pathways. Both transcription dependent and independent mechanisms are believed to contribute to

p53-induced apoptosis, and the importance of p53-mediated transcription in this process has been controversial.

p53-dependent apoptosis was reported in experimental systems in the presence of actinomycin D, which blocks RNA synthesis, and cycloheximide, which prevents protein synthesis. In these systems, transcriptional activation or translation of p53-regulated genes is prevented and cannot account for the observed apoptotic cell death (Caelles *et al.*, 1994). In addition, introduction of p53 transcriptional activation mutants into cells induced apoptosis, albeit more slowly than the wild type protein (Haupt *et al.*, 1995).

Another line of evidence that questions the requirement for p53 transactivation for apoptosis comes from examination of the effect of Rb on the stability and activity of p53. The interaction of p53 with its negative regulator Mdm2 blocks its transactivation, cell cycle arrest and apoptotic activities and promotes its nuclear export and degradation (Vousden, 2002). Rb forms a trimeric complex with p53 and Mdm2, blocking Mdm2-dependent p53 degradation. This restores the apoptotic activity of p53 as well as its ability to repress transcription. However, Rb binding does not relieve Mdm2's inhibition of p53 transactivation (Hsieh *et al.*, 1999). Taken together, these data demonstrate that p53 dependent gene activation is not essential for the induction of apoptosis.

Nevertheless, this conclusion was challenged by a study in which homologous recombination was used to generate mice carrying a transactivation-defective p53 mutant (Jimenez *et al.*, 2000). Analysis of mouse embryo fibroblasts (MEFs)

from these mice revealed defects in cell cycle regulation and apoptosis and the cells were readily transformed by oncogenes. These data firmly establish the transactivation function of p53 as a crucial determinant of its apoptotic activity (Jimenez *et al.*, 2000).

Consistent with the transactivation function of p53 being necessary for the effective induction of apoptosis is the ability of p53 to activate genes involved in programmed cell death. Many of these encode proteins that mediate apoptosis via the mitochondrial pathway, the death receptor pathway, and there is evidence for inhibition of survival signalling by some of these gene products.

The mitochondrial pathway

One of the first p53 responsive genes to be involved in apoptosis is the *bax* gene (Miyashita and Reed, 1995), which encodes a Bcl-2 –family member. Apoptotic stimuli promote the translocation of Bax from the cytoplasm into the mitochondria, where it mediates cytochrome C release. p53 also activates the expression of *Peg3/Pw1* which encodes a protein that triggers Bax translocation to the mitochondria, an effect blocked by expression of *Peg3/Pw1* antisense (Deng and Wu, 2000).

Other proteins involved in the mitochondrial pathway that are encoded by p53 responsive genes include NOXA, PUMA and AIP1 which contribute to the loss of mitochondrial membrane potential, triggering cytochrome C release (Oda, E. *et al.*, 2000; Oda *et al.*, 2000; Nakano *et al.*, 2001; Yu *et al.*, 2001). Further down

the same pathway, the interaction of cytochrome C with the p53-inducible Apaf1 triggers the caspase degradation pathway thus committing the cells to apoptosis (Srinivasula *et al.*, 1998).

Another mechanism by which p53 activation targets the mitochondria involves PIGS, p53 inducible genes whose activation is associated with the generation of reactive oxygen species (ROSs). ROSs can cause damage to the mitochondria, which triggers apoptosis (Polyak *et al.*, 1997).

Furthermore, a transcription-independent role of p53 at the mitochondria has recently been proposed. p53 has been reported to localise at the mitochondria of tumour cells undergoing p53-mediated apoptosis, but not in cells undergoing p53-dependent arrest or p53-independent apoptosis (Marchenko *et al.*, 2000). Moreover, the mitochondrial localisation of p53 was correlated with its apoptotic activity (Dumont *et al.*, 2003). Another study showed the ability of p53 to localise to the mitochondria of ionising radiation-treated lymphocytes (Mihara *et al.*, 2003), and to antagonise the function of the anti-apoptotic molecules Bcl-2 and Bcl-xL in a transactivation-independent fashion (Mihara *et al.*, 2003).

Death receptor/death domain pathway

p53 may be involved in death receptor-mediated cell death. It activates the expression of genes encoding the death receptors DR5/KILLER and FAS/APO1 (Takimoto and El Deiry, 2000, Fuchs *et al.*, 1997, Bennet *et al.*, 1998).

PIDD (*p*53 induced protein with a death domain), is another p53-induced pro-apoptotic protein. PIDD has been proposed as a mediator of apoptosis as anti-sense inhibition of PIDD reduces apoptosis in response to p53 activation and DNA damage (Lin *et al.*, 2000).

Loss of survival signalling:

In addition to apoptotic signals, cell death can also be potentiated by inhibition of survival and anti-apoptotic signals. The IGF BP3 gene product blocks the interaction of IGF1 with its receptor, thus blocking the IGF mitotic signalling pathway (Buckbinder *et al.*, 1995). Similarly, the tumour suppressor PTEN blocks survival signals associated with PI3K/Akt signalling and contributes to the stabilisation of p53, thus promoting its growth inhibitory function (Mayo *et al.*, 2002).

1-1.3 Choice of response to p53

The outcome of p53 activation appears to be determined by a multitude of factors. Understanding these factors would provide important tools in the development of p53-based cancer therapies. As p53 is a stress responsive protein, it makes sense that the type and intensity of stress dictate the outcome of the response. Furthermore, tumourigenesis is associated with the accumulation of multiple cellular aberrations and with increased genomic instability. The extent of these aberrations could affect the way in which the cell responds to p53 activation (Vousden and Lu, 2002).

Other factors affecting the outcome of the p53 response include differential binding affinities for p53 apoptotic and cell cycle arrest genes. Apoptotic genes are thought to have a lower affinity for p53 binding, which may explain the correlation of apoptosis with high levels of p53 and cell cycle arrest with lower concentrations of the protein in certain experimental systems (Chen *et al.*, 1996). However, despite differences in binding affinity between certain p53-responsive promoters, the finding that the *PUMA* promoter is not different from the *p21* and *mdm2* gene promoters with respect to p53 binding indicates that binding affinity is not the sole factor affecting the choice of p53 response (Kaeser *et al.*, 2002).

Phosphorylation of p53 can contribute to promoter specificity as is exemplified by the *AIP1* gene promoter. Phosphorylation of p53 at serine 46 is required for activation of the gene, which encodes a pro-apoptotic molecule (Oda *et al.*, 2000).

Moreover, accumulating evidence implicates p53 cooperating proteins in the induction of apoptosis. The p53 homologues p63 and p73 may perform such functions since p53 is unable to bind promoters of apoptotic genes in *p63/p73* double null cells, and is compromised in its ability to induce apoptosis (Flores *et al.*, 2002).

Other p53 cooperating molecules that modulate the choice of p53 response include ASPP family members, which affect p53's ability to bind to responsive promoters. ASPP1 and 2 bind to p53, enhancing its interaction with promoters of apoptotic genes such as *bax*. ASPP proteins greatly stimulate p53-dependent

apoptosis and their inhibition blocks the apoptotic activity of p53 (Samuels-Lev *et al.*, 2001).

Similarly, the p300 cofactor JMY augments p53 dependent apoptosis by selectively enhancing p53's ability to activate apoptotic genes such as *bax* (Shikama *et al.*, 1999).

Interestingly, alternative splicing of the JMY mRNA results in a protein isoform JMY Δ P, which favours p53-dependent G1 arrest as opposed to apoptosis (Shikama *et al.*, 1999). Thus, it is an attractive speculation that expression of the different isoforms of JMY, potentially in response to different forms of stress, can direct the p53 response towards G1 arrest, apoptosis, or possibly favour other p53-mediated responses.

1-1.4 Regulation of p53 function

As described above, p53 activation triggers potent growth inhibitory processes which would be detrimental to normal cells. This is demonstrated by the adverse side effects of chemotherapeutic agents which are believed to be at least partly due to p53 (Komarov *et al.*, 1999). Moreover, loss of Mdm2-dependent regulation of p53 in *mdm2*^{-/-} mice has a lethal phenotype that is attributed to excessive p53-dependent cell death (Jones *et al.*, 1995). Therefore, p53 activity must be kept in check during normal growth and development. Various cellular processes feed into the p53 pathway, providing tight regulation of the tumour suppressor, and inducing its activity when necessary. p53 regulation has been described at the level of its transcription and translation but most importantly at

the level of its stability, its subcellular localisation and its activity as a transcription factor.

Regulation of p53 stability

In addition to genes involved in cell cycle arrest and apoptosis, p53 activates the expression of genes involved in its own regulation. The most important of these is probably the *mdm2* gene. The Mdm2 protein is recognised as one of the major regulators of p53 and mediates its function by promoting the ubiquitination of p53, which allows its recognition and degradation by the 26S proteasome (Haupt *et al.*, 1997, Kubbutat *et al.*, 1997, Fang *et al.*, 2000). The resulting feedback loop, in which p53 induces the expression of its own negative regulator, is responsible for maintaining low levels of the tumour suppressor and needs to be interrupted to allow p53 accumulation in response to cellular stress.

p53 phosphorylation has been linked to its ability to stabilise in response to various forms of stress. Several potential phosphorylation sites have been identified in both the N-terminus and C-terminus of p53 including serines 15, 20, 33, 37 and 46 and threonine 18 and 81 (Buschmann *et al.*, 2001 and references within). Phosphorylation of some of these residues has been correlated with increased stability of p53 and attenuated interaction with Mdm2. Examples include phosphorylation of Serine 15 by the ATM (*Ataxia telangiectasia mutated*) kinase, and serine 20 by the Chk2 kinase (Chchab *et al.*, 2000). The importance of the ATM/Chk kinase cascade in activating the p53 response to certain types of genotoxic damage is highlighted by the finding that Chk2 deficient cells are

defective in their ability to stabilise p53 in response to ionising radiation (Hirao *et al.*, 2000).

Further evidence for the role of phosphorylation in regulating p53 stability comes from mouse embryonic stem cells in which serine 18 (equivalent to human serine 15) was mutated (Chao *et al.*, 2000). The p53 mutant accumulates less efficiently compared to wild type p53 and is compromised in its ability to induce *p21* gene expression and cell cycle arrest (Chao *et al.*, 2000).

Nevertheless, the requirement for phosphorylation in p53 stabilisation has been questioned by studies of mutants in which all potential N-terminal phosphorylation residues were altered into non phosphorylatable residues (Ashcroft *et al.*, 1999; Blattner *et al.*, 1999). These mutants are stabilised in a comparable fashion to the wild type protein indicating that phosphorylation is not essential for p53 stabilisation.

The ability of p53 to accumulate in the absence of phosphorylation suggests that other mechanisms are involved in its stability. Since Mdm2 is a major regulator of p53 stability, regulation of Mdm2 activity is likely to be one such mechanism, and can occur at the level of its transcription, protein levels as well as by posttranslational modification (described below).

In addition to Mdm2, other proteins that target p53 for degradation include the Jun NH2 terminal kinase, JNK. JNK binding to p53 promotes its ubiquitination and

subsequent proteolysis (Fuchs *et al.*, 1998). JNK can also phosphorylate p53 at threonine 81, leading to its stabilisation and enhanced growth arrest and apoptosis by p53 (Buschmann *et al.*, 2001), providing another example of the positive effect of p53 phosphorylation on its protein levels.

p53 phosphorylation does not always correlate with accumulation of the protein. Phosphorylation of threonine 155 by the COP9 signalosome enhances its degradation (Bech-Otschir *et al.*, 2001) and may contribute to maintaining low levels of p53 in the absence of stress. Similarly, protein kinase C-dependent phosphorylation of p53 in the C-terminus also enhances its ubiquitination and degradation in unstressed cells (Chernov *et al.*, 2001).

Regulation of the subcellular localisation of p53

As discussed above, the ability to act as transcription factor is important for p53's growth inhibitory function. Since transactivation is a nuclear process, the nuclear localisation of p53 is crucial for its function and, like every aspect of p53 function, is subject to regulation.

Three nuclear localisation signals (NLS) have been identified in the C-terminal domain of p53 (figure 1.1.B), the first of which appears to be the most active in mediating p53 nuclear import (Liang *et al.*, 1999). The C-terminus of p53 also contains a cytoplasmic sequestration domain (CSD) that can reduce the efficiency of p53 nuclear import (Liang *et al.*, 1998,1999), and a nuclear export signal (NES) that allows leptomycin B sensitive p53 export (Stommel *et al.*, 1999). Another

NES sequence has more recently been identified in the N-terminal transactivation domain of p53 (Zhang and Xiong, 2001).

The N-terminal leucine rich NES of p53 has been implicated in stress-regulated control of p53 localisation. DNA damage-induced phosphorylation within this domain (serine 15) blocks export of p53 and may contribute to the nuclear accumulation of the protein in the presence of stress (Zhang and Xiong, 2001). Thus, in addition to contributing to the stability of p53, phosphorylation can also regulate its localisation within the cell.

The C-terminal localisation signals are close to, and in the case of the NES, contained within the oligomerisation domain of p53. Mutations in the C-terminal NES were reported to affect nuclear export as well as tetramer formation which suggested a possible role for tetramerisation in the regulation of p53 localisation (Stommel *et al.*, 1999). This hypothesis was supported by studies in neuroblastoma cells in which p53 localises mainly to the cytoplasm as a result of enhanced nuclear export (Moll *et al.*, 1995; Stommel *et al.*, 1999). Ectopic expression of the tetramerisation domain restores the nuclear localisation of p53, implying that tetramerisation can mask the NES, and block p53 nuclear export (Stommel *et al.*, 1999).

The Mdm2 protein is a major regulator of p53 function and it has been shown to affect p53 localisation. The ring finger domain of Mdm2, which harbours its ubiquitin ligase activity, is essential for its ability to relocalise p53 to the cytoplasm

(Boyd *et al.*, 2000, Geyer *et al.*, 2000). Moreover, ubiquitination-deficient p53 mutants are resistant to Mdm2-mediated nuclear export (Lohrum *et al.*, 2001). Taken together, these data suggest that ubiquitination of p53 can affect its cellular localisation and enhance its export.

In addition to nuclear export mechanisms, cytoplasmic sequestration of p53 has also been proposed to affect its intracellular localisation. Interaction of p53 with the glucocorticoid receptor (GR) results in the cytoplasmic sequestration of p53 and this is accompanied by inactivation of p53's transactivation and growth inhibitory functions (Sengupta *et al.*, 2000). In neuroblastoma cells, where p53 localises mainly to the cytoplasm, disruption of the p53-GR complex with GR antagonists restores the nuclear localisation of p53 as well as its ability to activate transcription, and to promote growth arrest and apoptosis (Sengupta *et al.*, 2000).

Another molecule that can retain p53 in the cytoplasm is Parc (p53-associated parkin-like protein). Parc forms part of a cytoplasmic mega-complex, and binds to p53 in the cytoplasm, preventing its transport into the nucleus (Nikolaev *et al.*, 2003). Interestingly, Parc is expressed at particularly high levels in neuroblastomas. Taken together, these data provide support for the involvement of cytoplasmic sequestration of p53 in tumourigenesis.

The intracellular localisation of p53 is also regulated by PML. PML is a tumour suppressor famous for its ability to promote the formation of nuclear structures or foci referred to as nuclear bodies (NBs). PML co-recruits p53 and the

transcriptional regulators p300/CBP to the NBs where p53 is acetylated by the HAT activity of p300/CBP. This correlates with increased p53 activity (Wei *et al.*, 2003).

Regulation of p53 transcription activation functions

Another level of p53 regulation relates to the modulation of its transcriptional activation function. p53's old friend Mdm2 masks its transactivation domain through direct interaction and reduces its ability to activate transcription (Chen *et al.*, 1993; Oliner *et al.*, 1993).

A number of post-translational modifications have been implicated in the regulation of p53's transactivation functions. First, phosphorylation at the N-terminus which is associated with increased stability also enhances p53-dependent transcriptional activation. Moreover, phosphorylation at the C-terminus stimulates the activity of the protein. For example, phosphorylation of serine 392, and serine 315 by casein kinase II and cyclin dependent kinases, respectively, upregulates the transcriptional activity of p53, potentially through stimulation of DNA binding (Blaydes *et al.*, 2001; Hupp *et al.*, 1992). Moreover, phosphorylation can confer promoter specificity to p53 transactivation as illustrated by phosphorylation at serine 46 which is required for the induction of *AIP1* expression (Oda *et al.*, 2000).

N-terminal phosphorylation of p53 has been suggested to facilitate its acetylation by p/CAF and to increase its interaction with p300 (Sakaguchi *et al.*, 1998, Dumaz and Meek, 1999). As p53 acetylation is induced by DNA damage, and

inhibited by Mdm2 (Ito *et al.*, 2001), it is not unreasonable to postulate an important role for this modification in p53 activity. However, although there is some evidence for the ability of acetylation to increase the transcriptional activity of p53, its significance and mechanisms of action remain unclear and controversial (Prives and Manley, 2001).

Finally, SUMO modification of p53 at lysine 386 has also been reported to positively regulate p53 transactivation. However, this modification does not seem to be absolutely necessary for p53 activity and only affects a small proportion of cellular p53 (Rodriguez *et al.*, 1999).

1-1.5 Feedback loops in the regulation of p53

An emerging theme in p53 research is the involvement of feedback loops in the regulation of p53 activity. p53-inducible genes appear to cluster into different categories according to the function of the proteins they encode. A particular class of these genes seems to be involved in the regulation of p53, the most famous example being the *mdm2* gene. The negative feedback loop formed between Mdm2 and p53 is responsible for maintaining low levels of p53 under normal conditions, and is regulated by several cellular mechanisms that allow the release of p53 and its activation in response to stress (Alcaron-Vargas and Ronai, 2002).

Moreover, p53 also induces the expression of proteins that regulate Mdm2 function. Examples include cyclin G (Okamoto and Beach, 1994), which recruits the serine/threonine phosphatase PP2A to Mdm2, resulting in its

dephosphorylation, and increasing its ability to promote p53 degradation (Okamoto *et al.*, 2002). Conversely, the tumour suppressor PTEN inhibits PIK3/Akt signalling, thus blocking the ability of AKT to promote nuclear entry of Mdm2. This restores the transactivation function of p53 and allows its accumulation (Mayo *et al.*, 2002).

In addition to regulation of Mdm2, p53 can induce, or repress mechanisms involved in its own regulation. 14-3-3 sigma, whose expression is induced in response to γ irradiation in a p53 dependent manner (Hermeking *et al.*, 1997), can bind the C-terminus of p53 and increase its sequence specific DNA binding activity (Waterman *et al.*, 1998). Conversely, a negative feedback loop has been suggested between p53 and Chk1 and Chk2, two kinases implicated in the phosphorylation and stabilisation of p53 in response to DNA damage. p53 expression has been suggested to downregulate the mRNA levels of both Chk1 and Chk2 (Gottifredi *et al.*, 2001; Tominaga *et al.*, 1999).

Furthermore, a negative feedback loop has been proposed between p53 and ARF, the alternative product encoded by the mammalian *CDKN2A* locus. Whereas ARF promotes p53 stabilisation by interfering with the Mdm2-mediated degradation of p53, p53 has been shown to negatively affect ARF expression and an inverse correlation has been demonstrated between ARF expression and p53 status in tumour cells (Pomerantz *et al.*, 1998; Stott *et al.*, 1998).

The advantage conferred by feedback loops is that they allow tight and appropriate regulation of p53 under normal conditions. Whereas positive feedback loops can contribute to the amplification of the response, negative feedback loops ensure that the p53 response is only induced when necessary, i.e. in the presence of stress.

1-1.6 Conclusions

The p53 tumour suppressor plays a critical role in the prevention of tumourigenesis by restraining the proliferation of cells with potentially oncogenic lesions. This function of p53 relates to its ability to respond to diverse stress signals and to translate these into appropriate cellular responses.

The importance of p53 function is reflected in the complexity of mechanisms regulating its function. The oncoprotein Mdm2 plays a central part in p53 regulation and the relationship between the two proteins is far from being a simple linear mechanism as it is modulated by diverse signalling pathways which act through multiple protein-protein interactions and post-translational modifications.

Due to its inactivation in a large subset of tumours, p53 is a very attractive target for the development of cancer therapies. Consequently, a fuller understanding of p53 function, and the mechanisms controlling its activity would provide invaluable tools for the treatment of cancer.

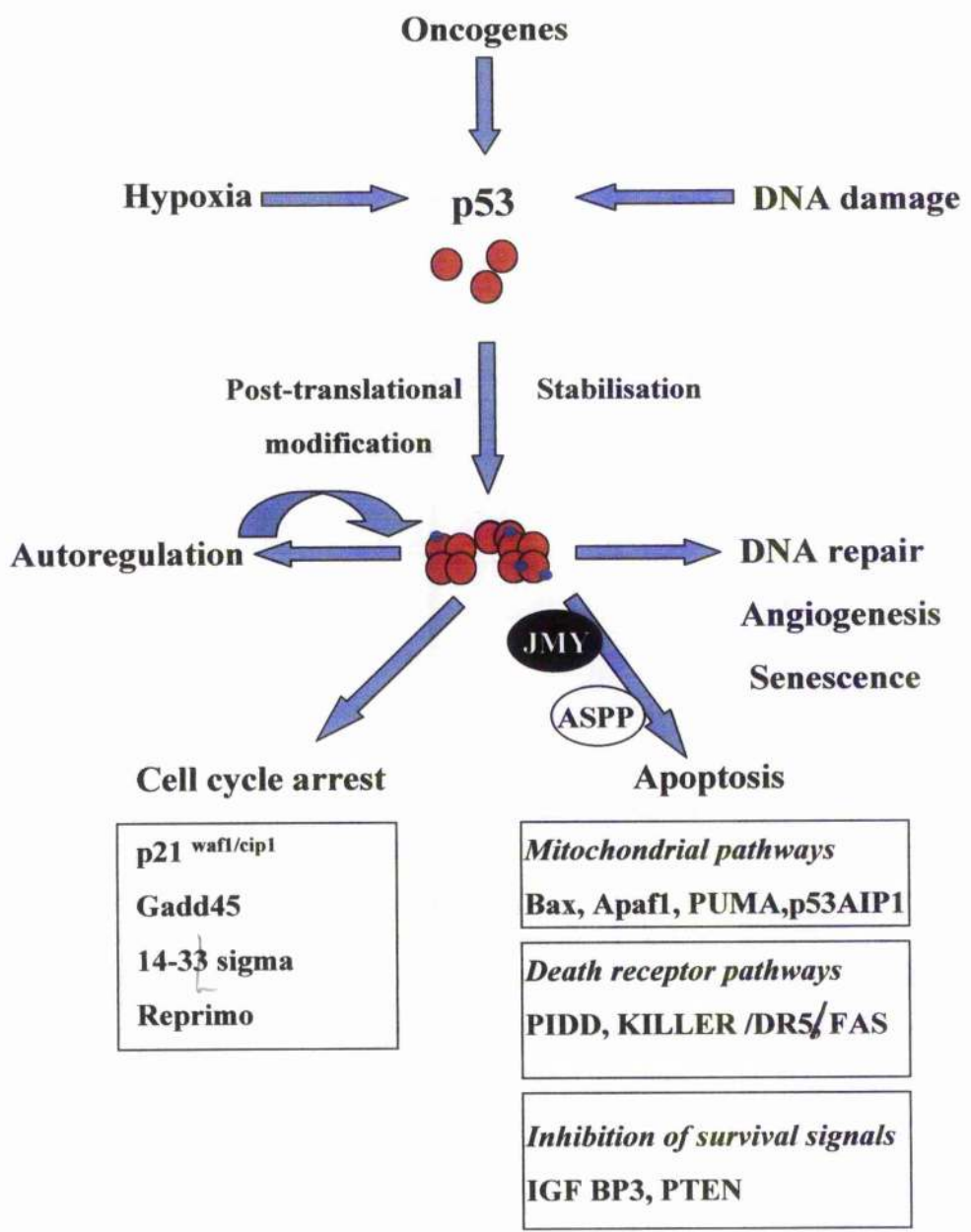
1.1. The p53 response

p53 mediates the cellular response to various stress signals. These signals commonly result in post-translational modification of the protein and cause its stabilisation.

p53 activation generally inhibits cell growth, either by cell cycle arrest or apoptosis. The choice of response is influenced by cofactor molecules such as JM_Y and ΔSPP.

Other outcomes of p53 activation have been reported, including DNA repair, senescence and regulation of angiogenesis. The level of the p53 response is regulated by stress signals as well as autoregulatory loops involving p53 and products of its target genes.

The diagram was adapted from Lu and Vousden, 2000.



1-2. Mdm2, master regulator of p53:

Tumour development occurs as a result of multiple events that cause the deregulation of cellular pathways governing normal cell proliferation, as well as pathways that repair and prevent the propagation of potentially harmful lesions.

Inappropriate expression of oncogenes is one such event and can occur via many mechanisms, including mutation, translocation and gene amplification.

One of the cytogenetic manifestations of gene amplification is in the form of paired, acentric chromatin bodies referred to as double minutes (DMs). These regions often contain amplified genes involved in the control of cell proliferation and tumorigenesis (Cahilly-Snyder *et al.*, 1987).

A search for genes located in double minutes in the spontaneously transformed 3T3 DM mouse cell line led to the identification of the *mdm2* gene (murine double minute), which was amplified in these cells (Cahilly-Snyder *et al.*, 1987). The oncogenic abilities of *mdm2* were demonstrated by its contribution to cell immortalisation and transformation in several independent studies. For example, overexpression of the *mdm2* gene in rodent cells led to their transformation (Fakharzadeh *et al.*, 1991). Also, whilst overexpression of *mdm2* alone was sufficient to immortalise primary fibroblasts, co-expression of activated *ras* led to a transformed phenotype (Finlay, 1993).

The early nineties were an exciting time in the Mdm2 field. The human gene was mapped to chromosome 12q13-14 and was shown to be amplified in sarcomas

(Oliner *et al.*, 1992). More crucially, Mdm2 was shown to interact with the tumour suppressor p53 and to inhibit its transcriptional activation and growth inhibitory functions (Mommand *et al.*, 1992; Finlay,1993; Barak *et al.*, 1993; Oliner *et al.*, 1993). The ability of Mdm2 to regulate p53 activity provided an explanation for its oncogenic properties and it was not long before Mdm2 became the focus of intensive research which highlighted its key role in p53 regulation.

1-2.1 Mdm2 gene structure and protein domains

Mdm2 gene structure and protein domains

The *mdm2* gene has two different promoters P1 and P2, which lead to transcripts initiating at different AUG codons. The upstream promoter P1 is constitutively active and allows low levels of *mdm2* expression. P2 is downstream from p53 binding sites located in the first intron of the gene, and is induced by p53. (Freedman *et al.*, 1999). The ability of p53 to induce *mdm2* transcription is the basis of the autoregulatory feedback loop which is crucial for regulation of p53 activity.

The regulation of *mdm2* gene expression is rather complex and multiple mRNA splice forms are expressed to give rise to different Mdm2 proteins (Momand and Zambetti, 1997). For example, in the 3T3 DM cell line, from which *mdm2* was originally identified, five Mdm2 polypeptides have been identified with sizes ranging from 57 to 90 kilodaltons (kDa) (Olson *et al.*, 1993) Nevertheless, the distinctive functions of the multiple forms of Mdm2 remain elusive.

Mdm2 protein domains

The largest Mdm2 protein consists of 489 amino acids in the mouse and 491 amino acids in humans and contains several evolutionarily conserved domains (figure 1.2).

The first conserved domain resides in the amino-terminus (residues 19-102), which is necessary for p53 binding, and sufficient to inhibit its transcriptional activation and growth arrest functions (Chen *et al.*, 1993, Wu *et al.*, 1996).

Mdm2 also contains a domain rich in acidic residues located between amino acids 221-472, which allows its interaction with the ribosomal protein L5 (Marcechal *et al.*, 1994), as well as with the ARF tumour suppressor protein (Pomerantz *et al.*, 1998). This region is also important in the regulation of p53 stability. Deletion mutants in this region promote p53 stabilisation in a dominant negative fashion, indicating the importance of this region in the degradation function of Mdm2 (Argenitni *et al.*, 2001).

Additional structural features of Mdm2 include a zinc finger motif between residues 305-322, and a ring finger domain at amino acids 438-478 (Lozano and Montes Oca Luna, 1998). Ring finger motifs are protein-protein interaction motifs with an ability to interact with DNA and RNA in some cases. The Mdm2 ring finger has been shown to bind specific RNA sequences or structures *in vitro* (Flenbaas *et al.*, 1996). Importantly, the ring finger domain of Mdm2 possesses E3 ubiquitin ligase activity (Fang *et al.*, 2000). E3 ligases catalyse the final step of ubiquitination, which involves the transfer of an activated ubiquitin molecule

onto their substrate. This function of Mdm2 is crucial for the regulation of p53 and Mdm2 stability (Fang *et al.*, 2000).

Other key features of Mdm2 include sequences involved in its sub-cellular localisation. The conserved basic nuclear localisation signal (NLS) is in close proximity to a nuclear export signal (NES) and these sequences allow the shuttling of Mdm2 between the nucleus and the cytoplasm (Roth *et al.*, 1998). Moreover, a nucleolar localisation signal (NoLS) has been identified in the C-terminus of Mdm2, which is not functional in the absence of stress. However, this signal cooperates with the NoLS of the tumour suppressor p14^{ARF} to allow the relocalisation of both proteins to the nucleolus (Lohrum *et al.*, 2000).

1-2.2 Mdm2 negatively regulates p53 function

The most important known function of Mdm2 is the negative regulation of p53. Mdm2 binds to p53 and inhibits its activity at multiple levels; namely, transcriptional activation, stability and sub-cellular localisation.

Inhibition of p53-dependent gene expression

Mdm2 inhibits p53-dependent transcription by directly interacting with, and masking its transactivation domain (Mommand *et al.*, 1992; Oliner *et al.*, 1993). This region of p53 is required for its interaction with components of the transcriptional machinery, including the TATA-binding protein (TBP), and transcription associated factors such as TAF_{II}32 and TAF_{II} 70 (Thut *et al.*, 1995; Lu and Levine, 1995). The partial overlap of interaction sites of Mdm2 and

components of the transcriptional machinery on the transcriptional activation domain of p53 provides a biochemical mechanism for Mdm2-mediated inhibition of p53-dependent transcription.

In addition to interacting with components of the transcriptional machinery, p53's transactivation function depends on the presence of the transcriptional coactivators p300/CBP (Avantaggiati *et al.*, 1997; Lill *et al.*, 1997, Thomas and White, 1998). p300/CBP interact with p53, promoting its acetylation, which potentially enhances its function as a transcription factor (Prives and Manley, 2001). Overexpression of Mdm2 has been shown to reduce p53 acetylation by p300/CBP, a function that requires binding to both p53 and p300/CBP (Inoue *et al.*, 2001).

Interestingly, Mdm2 also interacts with p300/CBP, and has been proposed to inhibit the association of the p53 N-terminus and p300/CBP, thereby down-regulating p53 transactivation (Wadgaonkar and Collins, 1999).

Finally, fusion of Mdm2 to a heterologous DNA-binding domain revealed its ability to repress p53-dependent and -independent transcription in an *in vitro* transcription system. This suggested an additional mechanism of inhibiting p53 transactivation, involving intrinsic repressor activities of Mdm2 (Thut *et al.*, 1997).

Regulation of p53 stability

One of the ways in which Mdm2 regulates p53 function is by targeting it for degradation by the 26S proteasome pathway (Haupt *et al.* 1997; Kubutat *et al.*, 1997). Mdm2 acts as a ubiquitin ligase (E3) for p53 and this function depends on the ring finger (Fang *et al.*, 2000), as well as the central acidic domain of Mdm2 (Meulmeester *et al.*, 2003, Kawai *et al.*, 2003).

Another requirement for Mdm2-dependent p53 degradation is the p300 coactivator. It has been demonstrated that most of the endogenous Mdm2 is in complex with p300 (Grossman *et al.*, 1998), and that this interaction is essential for p53 degradation. Overexpression of a C/H1 polypeptide, comprising the p53 and Mdm2 binding regions on p300 acts in a dominant negative manner to increase p53 stability, whereas Mdm2 mutants that are defective in p300 binding are compromised in their ability to promote p53 degradation but not its ubiquitination (Zhu *et al.*, 2001).

Regulation of the subcellular localisation of p53

Mdm2-directed ubiquitination of p53 has been implicated in p53 export. This is supported by studies in which mutation of the ring finger domain abrogates the ability of Mdm2 to stimulate p53 export (Boyd *et al.*, 2000; Geyer *et al.*, 2000).

Moreover, Mdm2 is also unable to promote p53 export at the restrictive temperature in a cell line containing a temperature-sensitive mutation in the E1 ubiquitin charging enzyme. Re-introduction of wild type E1 enzyme restores

Mdm2-driven p53 export, highlighting the importance of Mdm2-mediated ubiquitination for p53 export (Boyd *et al.*, 2000).

Finally, the acidic region of Mdm2 has also been implicated in its ability to ubiquitinate p53 and this was correlated with nuclear export (Kawai *et al.*, 2003). These observations support a model in which ubiquitination of p53 by Mdm2 activates its nuclear export signal (possibly by unmasking), thus allowing its transport into the cytoplasm.

1-2.3 Regulation of the p53-Mdm2 feedback loop

The functional interaction of Mdm2 and p53 forms a negative feedback loop wherein p53 upregulates Mdm2 and Mdm2 downregulates p53. This loop serves to keep p53 function under tight control, and its physiological relevance was demonstrated by the early embryonic death of *mdm2* null mice. This phenotype is completely rescued by deletion of the *p53* gene, indicating that embryonic lethality is the result of excessive, uncontrolled p53 activity, and pointing to Mdm2 as a principal regulator of p53 (Jones *et al.*, 1995).

However, for p53 to respond to stress signals, the feedback loop has to be interrupted. This is thought to result from the many cellular signals that feed into the p53-Mdm2 circuit, and involves post-translational modification of p53 and Mdm2, regulation of Mdm2 levels, as well as the action of a number of cooperating proteins (figure 1.3).

Post-translational modification of p53

The major phosphorylation events thought to impinge on the p53-Mdm2 interplay involve phosphorylation sites in the N-terminus of p53, within and near the Mdm2 binding site. DNA damage-induced phosphorylation of different sites in p53 is well documented and has been proposed to abrogate its interaction with Mdm2, thus relieving inhibition by Mdm2 (Alarcon-Vargas and Ronai, 2002).

Nevertheless, mutation of all the phosphorylation sites on p53 to non-phosphorylatable residues yields a protein that is comparable to wild type p53 in terms of Mdm2 binding, sensitivity to Mdm2-mediated degradation, and stabilisation by DNA damage (Ashcroft *et al.*, 1999, Blattner *et al.*, 1999). On the other hand, mutation of serines 15 and 37 to aspartic acid, which mimics the phosphorylated state resulted in slight resistance to Mdm2-dependent degradation. These observations suggest that phosphorylation can contribute to p53 stabilisation without being absolutely essential (Ashcroft *et al.*, 1999).

Post-translational modification and down-regulation of Mdm2

The dissociation of p53 phosphorylation from its stabilisation by DNA damage raised the possibility that DNA damage results in the modification of other molecules involved in p53 regulation. Being a major regulator of p53, Mdm2 was an attractive candidate and has been shown to be a phosphorylation target for a number of kinases (Hay and Meek, 2000).

Mdm2 phosphorylation on serine 395 by the ATM kinase has been reported to occur prior to p53 accumulation in response to ionising radiation (IR) (Khosravi *et al.*, 1999, Maya *et al.*, 2001). An Mdm2 mutation that mimics phosphorylation at this site does not affect the binding of Mdm2 to p53 but compromises its ability to destabilise p53 and promote its nuclear export (Maya *et al.*, 2001).

Similarly, c-Abl mediated phosphorylation of Mdm2 on tyrosine 394 was suggested to reduce Mdm2's ability to promote p53 degradation, as well as its ability to inhibit p53 transcription and apoptosis (Goldberg *et al.*, 2002). As both ATM and c-Abl have been implicated in p53 stabilisation in response to DNA damage, it is conceivable that regulation of both components of the p53-Mdm2 feedback loop by the same kinase ensures a well-coordinated p53 response (Maya *et al.*, 2001; Goldberg *et al.*, 2002).

Mdm2 phosphorylation is not exclusively associated with down-regulation of its effect on p53. Phosphorylation of at least two residues, serines 166 and 186 is mediated by the Akt serine/threonine kinase in response to mitogenic signals (Mayo and Donner, 2001; Zhou *et al.*, 2001; Ashcroft *et al.*, 2002). This allows nuclear entry of Mdm2 and enhances its ability to interact with p300 (Mayo and Donner, 2001; Zhou *et al.*, 2001). Consequently, Mdm2's ability to inhibit p53 is enhanced, an effect reversed by blockade of the PI3/Akt signalling pathway (Mayo and Donner, 2001).

Regulation of Mdm2 levels has also been linked to p53 stabilisation in response to certain DNA damaging agents. Alkylating agents and UV radiation have been reported to reduce the levels of Mdm2 mRNA and protein, and to decrease p53 ubiquitination, providing an additional mechanism of p53 activation (Inoue *et al.*, 2001).

Regulation of the p53-Mdm2 loop by interacting proteins

ARF

The ARF tumour suppressor contributes to the stabilisation of p53 in response to oncogene activation. The ARF protein binds directly to Mdm2 and inhibits Mdm2-dependent p53 ubiquitination (Pomerantz *et al.*, 1998; Honda and Yasuda, 1999). Furthermore, at least when expressed at high levels, ARF can sequester Mdm2 in the nucleolus, thus physically preventing its interaction with p53 (Weber *et al.*, 1999).

Rb

Mutations or inactivation of both p53 and Rb are very commonly found in tumours. p53 is involved in the regulation of Rb phosphorylation through activating the expression of the cyclin kinase inhibitor p21. Moreover, Rb can affect p53 levels and function through its interaction with Mdm2. The binding of Rb to Mdm2 prevents its ability to promote p53 degradation, and inhibits its anti-apoptotic functions (Hsieh *et al.*, 1999).

HIF-1 α

HIF-1 α is the regulated component of the hypoxia responsive HIF transcription factor, and has been implicated in the stabilisation of p53 in response to hypoxia. This is thought to involve the interaction of HIF-1 α and Mdm2, which prevents Mdm2-mediated ubiquitination, degradation and nuclear export of p53. Furthermore, co-expression of HIF-1 α in a reporter assay abrogated the ability of Mdm2 to repress transcription in a transient transfection assay. These data provide a potential mechanism for the regulation of the p53-Mdm2 loop under conditions of hypoxia (Chen *et al.*, 2003).

TSG101

TSG101 is a ubiquitin conjugase (E2)-like protein whose levels were found to decrease in the presence of high levels of Mdm2. Conversely, TSG101 can prevent Mdm2 degradation by the proteasome, and thus may form a negative feedback loop with Mdm2. This regulatory circuit might impinge on the p53-Mdm2 loop since the lethal phenotype of *tsg101*^{-/-} mice is partially rescued by the concomitant deletion of *p53* (Li *et al.*, 2001, Ruland *et al.*, 2001).

1-2.4 p53-independent functions of Mdm2

Overexpression of Mdm2 contributes to human tumour formation, and this is largely attributed to its ability to inhibit p53 function. However, as the list of Mdm2-interacting proteins is becoming progressively longer (figure 1.2), several lines of evidence point to additional p53-independent functions of Mdm2, which could potentially contribute to tumour formation.

First, if the oncogenic potential of Mdm2 were restricted to the down-regulation of p53, inappropriate expression of Mdm2 in tumours carrying p53 mutations would be a redundant event. However, *mdm2* gene amplifications and *p53* mutations have been reported to occur in a rare subset of tumours. These tumours are more aggressive than tumours carrying one of the two alterations (Cordon-Carlo *et al.*, 1994). Moreover, splice variants of Mdm2 lacking the p53-binding domain have been found in certain human tumours, and these appear to retain their transforming abilities (Sigalas *et al.*, 1996).

Another line of evidence comes from transgenic mice with targeted overexpression of Mdm2 in the mammary epithelium (Lundgren *et al.*, 1997). High levels of Mdm2 resulted in hyperplasia of the ducts of breast tissue, as well as lack of terminal differentiation and polyploidy. This phenotype was maintained despite crossing into a *p53* null background, but was not observed in *p53* null mice that do not overexpress Mdm2. These observations suggest that the tumorigenic activities of Mdm2 in this situation do not involve p53 inhibition (Lundgren *et al.*, 1997).

Several characteristics of Mdm2 could contribute to its p53-independent oncogenic potential. As discussed below, Mdm2 forms an interacting partner for a number of proteins involved in the control of cellular proliferation and/or differentiation (figure 1.2.A). Some of these interactions are summarised below.

p73

In addition to structural similarities, the p53 homologue p73 shares some functional properties with p53, namely induction of cell cycle arrest and apoptosis. Mdm2 interacts with p73 and inhibits its transactivation and apoptotic function without promoting its degradation, or nuclear export. Moreover, Mdm2 disrupts the interaction of p73 and p300 by competing with p73 for binding to the N-terminus of p300 (Zeng *et al.*, 1999). It can also promote the redistribution of p73 to nuclear aggregates, indicating divergent regulatory mechanisms for p53 and its homologue (Gu *et al.*, 2001).

E2F-1

An exciting candidate for regulation by Mdm2 is E2F-1. Mdm2 interacts with the E2F1/DP-1 heterodimer and increases E2F-1-mediated transactivation in an overexpression assay (Martin *et al.*, 1995). Moreover, Mdm2 cooperates with E2F-1/DP-1 to stimulate DNA synthesis but also promotes the degradation of the heterodimer. This has been proposed to reduce the abundance of the heterodimer to levels appropriate for the G1-S transition but that are insufficient for the induction of apoptosis (Loughran and LaThangue, 2000).

PML

The tumour suppressor PML is a nuclear phosphoprotein that characteristically localises to distinct nuclear foci referred to as PML nuclear bodies (NBs).

PML^{-/-} mice have an increased susceptibility to carcinomas following treatment with tumour promoting agents, and the cells derived from these mice display

resistance to apoptosis (Wei *et al.*, 2003, and references therein). The mechanisms of tumour suppression by PML remain unclear but apparently depend on its crucial role in the formation of NBs. Furthermore, PML can stimulate p53 activity by recruiting it, together with the transcriptional coactivators CBP/p300 to the PML NBs, where p53 acetylation by its coactivators is believed to enhance its function as a transcription factor (Pearson *et al.*, 2000).

Mdm2 interacts with PML and promotes its relocalisation to the cytoplasm, and reduces its ability to stimulate the transcriptional activity of a Gal4-CBP fusion protein (Wu *et al.*, 2003). As the proper localisation of PML is important in its function, it has been proposed that Mdm2-mediated export could contribute to the inhibition of PML function (Wei *et al.*, 2003).

Furthermore, Mdm2's contribution to tumourigenesis could also involve the inhibition of tissue/cell type transcription factors:

MyoD

Amplification of the *mdm2* gene has been described in certain rhabdomyosarcoma cell lines displaying a non-differentiating phenotype (Fiddler *et al.*, 1996). In such cells, transfection of an anti-sense *mdm2* induced muscle specific gene expression, suggesting that Mdm2 is responsible for the lack of muscle specific gene expression and also for the lack of differentiation of these cells. One possible explanation for this effect of Mdm2 is its ability to block the

transcriptional activity of the muscle-specific transcription factor Myo D (Fiddler *et al.*, 1996).

Numb

Numb is involved in cellular signalling and is believed to act as a determinant of neural cell differentiation and/or cell fate (Juven-Gershon *et al.*, 1998).

Mdm2 interacts with and promotes the ubiquitination and degradation of the mammalian Numb protein. Moreover, elevated levels of Mdm2 can also promote the nuclear export of Numb (Juven-Gershon *et al.*, 1998; Yogosawa *et al.*, 2003). Thus, the ability of Mdm2 to regulate Numb levels may indicate a role of Mdm2 in cell differentiation or cell fate.

1-2.5 Mdm2 as a target for cancer therapy

Mdm2 is an attractive target for cancer therapy because of its pivotal role in p53 regulation. Blocking the Mdm2-p53 interaction would relieve Mdm2-imposed inhibition and allow the accumulation and activation of p53. This has been shown by the use of a synthetic protein which blocks p53 binding (Böttger *et al.*, 1997). This protein greatly elevates the levels of p53, and induces its transactivation and cell cycle arrest functions in a comparable manner to DNA damage. Moreover, micro-injection of a monoclonal antibody against the p53 binding domain of Mdm2 blocks the association of p53 and Mdm2 and allows the activation of p53 reporter genes (Böttger *et al.*, 1997).

Another mechanism of relieving inhibition of p53 by Mdm2 would be to reduce the levels of Mdm2 in cell lines that overexpress it. The use of anti-sense oligonucleotides for the *mdm2* message induces p53-dependent gene transcription and apoptosis in cell lines overexpressing Mdm2 and carrying wild type p53 (Chen *et al.*, 1998).

Although these and other results are encouraging, and show the therapeutic potential of targeting Mdm2 to interrupt the p53-Mdm2 regulatory circuit, the transition from experimental systems to *in vivo* drug administration has proved difficult due to physio-chemical properties of certain inhibitors, as well as the incredible diversity displayed by tumours (Chène, 2002)

1-2.6 Conclusion

The Mdm2 oncoprotein is a fascinating molecule, not only because of its ability to closely control p53 activity, but also because of its potential partnership with other regulators of cell growth and differentiation.

The relationship between p53 and Mdm2 provides a well established example of the relationship between a tumour suppressor and an oncoprotein. The ability of Mdm2 to regulate p53 is coordinated with cellular conditions, thus ensuring that p53 inactivation is restricted to normal conditions, and allowing p53 to break free in response to stress.

An emerging pattern in the regulation of the p53-Mdm2 circuit is the simultaneous regulation of both partners by the same kinase. This adds precision to the coordinated regulation of the two proteins.

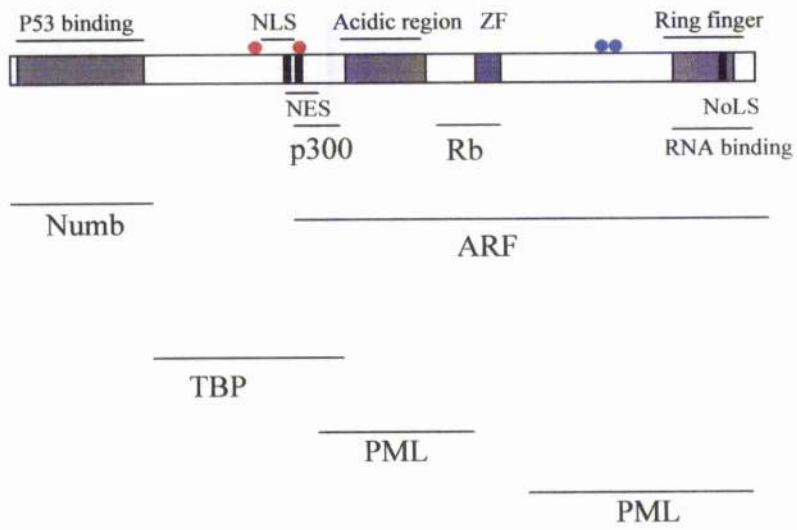
In addition to its critical role in p53 regulation, the p53-independent properties of Mdm2 are likely to contribute to its oncogenic potential. Therefore, elucidating the physiological significance of these properties would further our understanding of the role of Mdm2 in cellular transformation.

1.2. Structural features of Mdm2

The functional domains of Mdm2 are shown. NLS, nuclear localisation sequence; NES, nuclear export sequence; ZF, zinc finger domain; NoLS, nucleolar localisation signal. Some Mdm2 - interacting proteins and their sites of interaction are shown (Dajjat *et al.*, 2001, Wei *et al.*, 2003).

- Phosphorylation events enhancing Mdm2's inhibition of p53
- Phosphorylation events inhibiting Mdm2's inhibition of p53

Mdm2

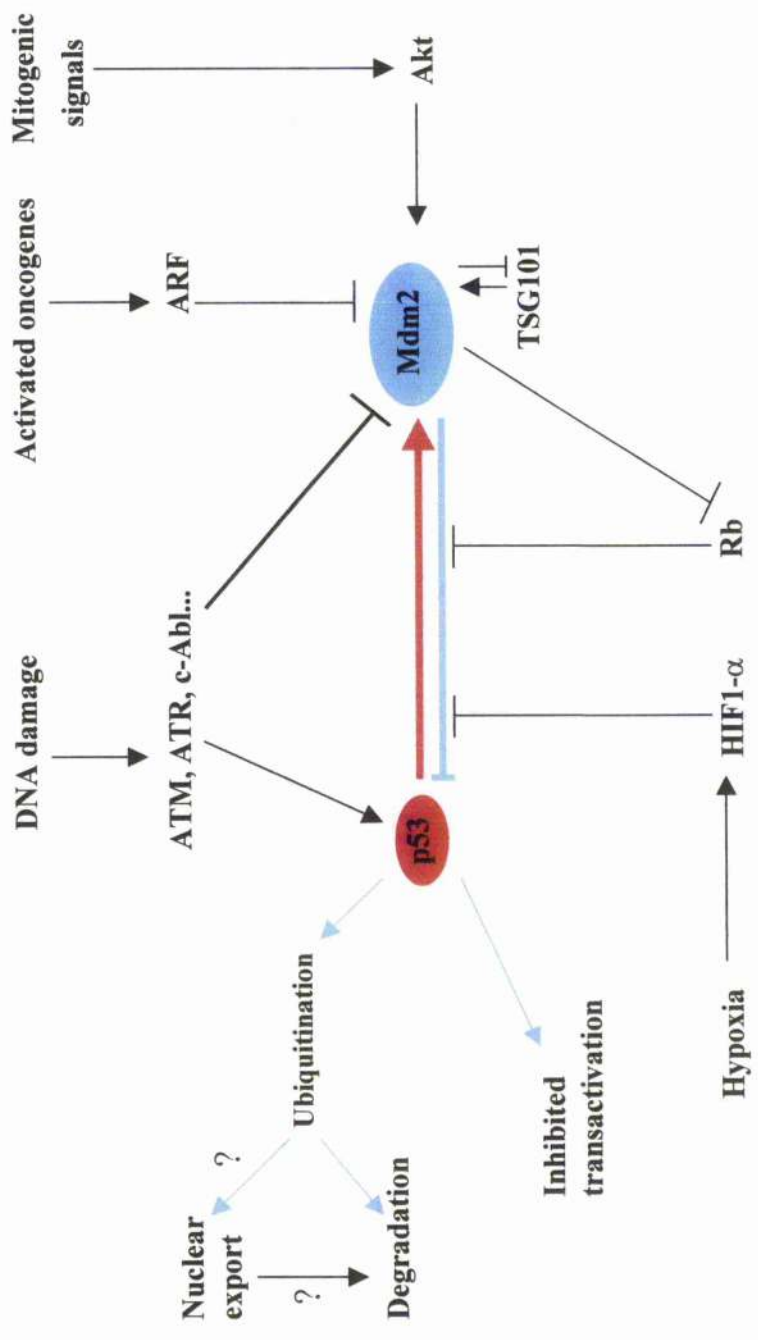


1.3. Regulation of the p53-Mdm2 feedback loop

The interplay between Mdm2 and p53 forms a negative feedback loop in which p53 upregulates Mdm2 expression (red arrow) whereas Mdm2 downregulates p53 (Blue arrows).

The loop is interrupted by stress signals, allowing the activation of p53. DNA damage activates a number of kinases with opposing effects on the activity and stability of p53 and Mdm2. Other types of stress may involve the activity of p53-cooperating molecules such as ARF, HIF1 α , and Rb.

Conversely, Akt-mediated phosphorylation of Mdm2 enhances its ability to inhibit p53 function.



1-3. Regulation of p53 by p300/CBP

Many protein-protein interactions have been implicated in the regulation of p53 activity. One of the most important and most intensively studied of these interactions is that with Mdm2 which was discussed in the previous section. Another major regulator of p53 is the p300/CBP family of proteins. These proteins act as coactivators for p53. p300/CBP regulation of p53 is more complex than simple stimulation of transcriptional activity and involves cooperation with both positive and negative regulators of p53.

1-3.1 p300/CBP

The p300/CBP proteins are members of a family of proteins that, through contributing to various signal dependent-transcription events, participate in many physiological processes, including proliferation, differentiation and apoptosis (Shikama *et al.*, 1997).

The cellular functions of p300/CBP are compromised in a number of diseases affecting the *p300/cbp* genes. Haplo-insufficiency of CBP causes Rubinstein-Taybi syndrome (RTS), a condition characterised by mental retardation, various physical abnormalities and increased predisposition to cancer (Petrij *et al.*, 1995). Similar abnormalities are observed in mice heterozygous for a disruption in the *cbp* gene and these mice have an increased incidence of leukaemias and certain types of sarcomas (Tanaka *et al.*, 1997; Kung *et al.*, 1999). These data indicate that gene dosage of CBP, and therefore, the level of the protein, is important for

its function and that despite sharing extensive homology, p300 cannot substitute for CBP function.

Mutation of the *p300/cbp* genes has been observed in a number of human tumours. Somatic mutations in the *p300* gene have been observed in breast, gastric and colon cancer and are often associated with loss of heterozygosity of the second allele (Gayther *et al.*, 2000). Furthermore, chromosomal translocations involving the *cbp* and *p300* genes are associated with certain forms of leukaemia, indicating a role in the proliferation and differentiation of haematopoietic cells. Therefore, p300 and CBP display some classical features of tumour suppressors (Gayther *et al.*, 2000).

p300/CBP function as coactivators in that they assist transcription by sequence-specific DNA binding transcription factors. A considerable number of transcription factors have been reported to interact with p300/CBP and to respond to its coactivating properties (figure 1.4)(reviewed in Shikama *et al.*, 1997). These include ligand-dependent nuclear receptors, transcription factors involved in myogenesis, and in mitogenic and serum-responsive signalling. Moreover, signal transduction by the JAK/STAT pathway in response to interferon stimulation utilises the coactivating properties of p300/CBP (Shikama *et al.*, 1997).

Competition among various transcription factors for limiting levels of p300/CBP has been proposed as a mechanism of regulating transcription in response to

different cellular signals. This is supported by studies demonstrating that the engagement of p300/CBP in transcription by various hormone receptors inhibits AP-1-dependent transcription. Hormone signalling is often associated with differentiation and inhibition of proliferation whilst AP-1 is involved in mitogenic signalling. Thus, it is possible that p300/CBP function is targeted to a specific subset of transcription factors in response to various signal transduction pathways (Kamei *et al.*, 1996).

The ability of p300/CBP to interact with a multitude of transcription factors and with components of the basal transcription machinery allows it to connect these molecules, allowing transcriptional activation (Shikama *et al.*, 1997).

Furthermore, the central domain of p300/CBP contains a domain with histone acetyltransferase activity (HAT), which allows the acetylation of lysine residues in histone tails, a process associated with transcriptional activity. The HAT activity of p300/CBP also extends to the transcription factors themselves, enhancing their transcriptional activity (reviewed by Chan and LaThangue, 2001). In fact, many of the tumour associated mutations of the *p300* gene result in loss of the acetyltransferase activity, indicating that the ability to acetylate one or more cellular proteins may be critical for the control of cell growth (Gayther *et al.*, 2000).

p300/CBP are also believed to mediate their function by acting as a platform for the assembly of multi-component coactivator complexes, which can also contain other HATS such as SRC-1 and P/CAF (Chan and La Thangue, 2001). The

formation of these multi-component complexes may serve to increase the relative concentration of transcription regulatory proteins in the local transcription environment and facilitate protein-protein and protein-DNA interactions. Since many proteins interact with p300/CBP in response to various signals, the ability to nucleate the formation of multi-protein complexes would also facilitate the integration of a variety of cellular signals and would allow the coordination of these signals with transcription. Finally, by forming different combinations of these cofactors, the cell can generate a very large number of regulatory complexes through combinatorial specificity and synergism (Chan and LaThangue, 2001).

1-3.2 p300/CBP and p53 regulation

The adenovirus E1A targets p300/CBP and can generally repress the activation of promoters that utilise the coactivating properties of p300/CBP, including p53-target promoters. The inability of p300/CBP binding defective E1A mutants to repress p53-dependent transcription provided one of the first clues to the role of p300/CBP in p53 activity (Somasundaram and El-Deiry, 1997). Since then, numerous studies have proved the importance of p300/CBP in the regulation of p53. The transfection of E1A was shown to inhibit p300/CBP and block p53-dependent transcriptional activation and apoptosis. Cotransfection of full length p300 restores both functions of p53 (Lill *et al.*, 1997). Moreover, p53-dependent apoptosis was abrogated by dominant-negative derivatives of p300/CBP proteins. These, and many more studies have established p300 and CBP as critical regulators of p53 function.

Acetylation of p53 by p300/CBP

In addition to histones, the HAT activity of p300/CBP targets p53 for acetylation at the C-terminus (Gu and Roeder, 1997, Ito *et al.*, 2001). Acetylation of p53 by p300/CBP, and also by P/CAF has been shown to be induced by a variety of cellular stresses (Ito *et al.*, 2001), and to be stimulated by phosphorylation at the N-terminus (Sakaguchi *et al.*, 1998). The initial studies suggested that acetylation enhances sequence specific DNA binding by p53 (Gu and Roeder, 1997; Sakaguchi *et al.*, 1998). However, this view has been challenged by many studies showing lack of effect of acetylation on DNA binding by p53. For example, comparison of p53 acetylation mutants with the wild type protein in CHIP assays showed equal binding of both forms to the p21 promoter in U2OS cells (Barlev *et al.*, 2001). The functional significance of p53 acetylation is still ambiguous and the best current guess is that it might have a positive effect on transcriptional activity of p53 without being absolutely and ubiquitously essential (Prives and Manley, 2001).

p53 stability and the Mdm2 connection

The multifunctional nature of p300/CBP is highlighted by their dual role in the regulation of p53. In contrast to their positive effect on p53-dependent transcription, p300/CBP are essential for the degradation of p53 by its negative regulator Mdm2 (Grossman *et al.*, 1998). In fact, most of the endogenous Mdm2 is believed to be in complex with p300 and Mdm2 mutants that are defective in p300 binding lose their ability to promote p53 degradation (Grossman *et al.*, 1998, Zhu *et al.*, 2001)

p300/CBP contain three highly conserved cysteine and histidine-rich domains referred to as C/H1, C/H2 and C/H3. These domains are believed to be important in mediating protein-protein interactions and interact with a number of cellular and viral proteins. The most N-terminal of these, C/H1, has been shown to bind p53 as well as Mdm2. Overexpression of this domain in osteosarcoma cells led to the stabilisation of endogenous p53. This was dependent on C/H1 binding to Mdm2 but not to p53 (Grossman *et al.*, 1998).

Further, p300 was shown to cooperate with Mdm2 in the polyubiquitination of p53, thus acting as an E4 enzyme for p53 ubiquitination (Grossman *et al.*, 2003).

Recruitment of cofactors

As discussed above, p300/CBP can act as a scaffold for the formation of multi-component coactivator complexes which are appropriate for the particular transcription event. Because the molecular composition of such complexes dictates their function, identification of the individual components is crucial for a better understanding of p300/CBP regulation and function. Cofactors that interact with p300/CBP have been identified such as NAP and P/CAF (Shikama *et al.*, 1997; Schiltz and Nakatani, 2000).

Consistent with its role in p53 transactivation, p300 may also be involved in the recruitment of cofactors that enhance the p53 response. Two such cofactors, JMY and Strap, have been identified recently and have been shown to impact on p53 function.

1-3.3 JMY and Strap, novel cofactors for the p53 response

JMY and the p53 response

JMY (*Junction mediating and regulatory protein*) and p300 cooperate to augment the p53 response. This cooperation results from the ability of the two proteins to interact directly with each other, and to be recruited to the p53 complex in response to DNA damage (Shikama *et al.*, 1999).

JMY is a 110 kDa protein with a number of interesting features (figure 1.5). The N-terminal part of the protein contains a cluster of potential phosphorylation sites for S/T-P-directed kinases, including three consensus sites for cyclin-dependent kinases. A central region contains a domain with homology to the adenoviral CR2 conserved region. The C-terminal half of JMY contains a WH2 (*Wiscott Aldrich homology*) domain which in other proteins is associated with actin regulation, as well as a proline-rich domain (Shikama *et al.*, 1999, and unpublished observations).

Spliced variants of JMY mRNA occur in certain types of cells. One of these variants is translated into a protein isoform of JMY, termed JMY Δ P, that neatly lacks the proline rich region found in the C-terminus of the wild type protein. The physiological significance of this variant became clear when its effect on the p53 response was compared to that of the wild type protein.

Co-expression of JMY and p300 results in enhanced p53-dependent activation of the *bax* gene. However, the effect of JMY on other p53-responsive promoters, namely *waf1/cip1* and *mdm2* is less pronounced. In contrast, JMY Δ P is less able to enhance transcription from the *bax* promoter. Given the role of Bax in

apoptosis, these data prompted the examination of the role of JMY and JMY Δ P in p53-dependent apoptosis and growth arrest (Shikama *et al.*, 1999).

Wild type JMY efficiently enhances p53 dependent apoptosis whereas JMY Δ P has a negligible effect on apoptosis. In contrast, JMY Δ P is more active at inducing cell cycle arrest relative to JMY (figure 1.6). These data suggest a potential role for the expression of different JMY isoforms in the modulation of the p53 response.

Strap: stress responsive activator of p300

Strap, another component of the p300 coactivator complex, was isolated as a JMY-interacting protein in a yeast two-hybrid screen. Strap is composed of six tetratricopeptide repeat (TPR) motifs, which are generally involved in mediating protein-protein interactions (figure 1.5). This property of Strap is reflected in its ability to interact with both JMY and p300 and to facilitate their interaction (Demonacos *et al.*, 2001).

As the name indicates, Strap is a stress responsive protein and is induced in response to a variety of DNA-damaging stress signals, with a marked response to etoposide treatment. Stabilisation of Strap further stimulates complex formation between JMY and p300, and also increases the association of Strap with the p53 complex.

Consistent with its ability to interact with two p53 cofactors, JMY and p300, Strap also increases p53 activity on p53-responsive promoters, and augments p53-dependent apoptosis. These functions of strap correlate with its ability to override the inhibitory effect of Mdm2 on p53 levels and transcriptional activity. The effect of Strap on p53 activity is less pronounced in the absence of Mdm2, suggesting that modulation of the activity of Mdm2 contributes to this function.

Taken together, these data point to an important role for Strap in the p53 response, which may involve the formation of multi-cofactor complexes as well as modulation of Mdm2 activity and of p53-dependent transcription (Demonacos *et al.*, 2001).

1-3.4 Conclusions

The p300/CBP coactivators occupy a central position in the complex cellular network controlling cell growth and differentiation. The formation of p300/CBP-containing multi-protein complexes contributes to the cross-talk between diverse cellular signals and to the translation of these signals into patterns of gene expression.

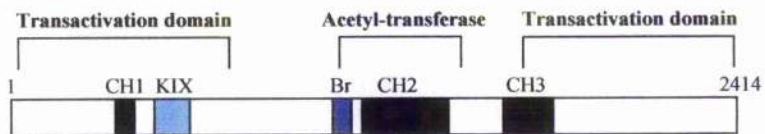
p300/CBP are important regulators of p53 activity and have opposing effects on the tumour suppressor. The dual function displayed by p300/CBP in p53 regulation reflects their pleiotropic nature.

p53 regulation involves multiple layers of control which regulate every aspect of its function. The identification of p53 cofactors that can affect the outcome of the response (as shown for JM1 isoforms), or that can modulate p53 activity through modulating its regulators (illustrated by the relationship between Strap and Mdm2) adds another level of complexity to p53 regulation. In order to ensure a coordinated response, such cofactors would be expected to be under regulation. Understanding of the regulatory mechanisms that impact on coactivator function is, therefore, crucial for better understanding of the p53 response, and can potentially provide novel targets for cancer therapy.

1.4. The functional domains of p300.

The cysteine/histidine-rich domains CH1, CH2 and CH3; the KIX domain and the bromodomain, Br, of p300 are shown. CBP shows high sequence homology to p300. The N- and C-terminal domains of p300/CBP can function as transactivation domains, and the central region accommodates the acetyl-transferase domain.

The pleiotropic nature of p300/CBP is reflected in their ability to interact with proteins involved in various cellular processes. The regions involved in protein-protein interactions and some of the interacting proteins are shown (Chan and LaThangue, 2001). The binding domains in p300 for JMY and Strap were taken from Shikama *et al.*, 1999 and Demonacos *et al.*, 2001; respectively.



611 1257 1572 2283
 JMY JMY

1 595 1572 2283
 Strap Strap

RXR Stat2 CREB
 ER E2F-1 Tax
 RAR p53 RelA
 GR Mdm2 MYB
 TR SYT JUN
 PR HIF1 SREBP-2

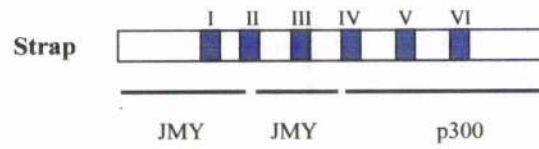
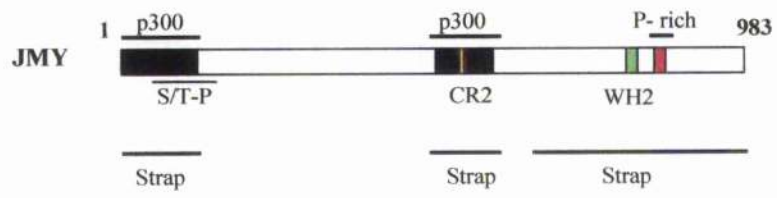
TBP
 p73

E1A
 SV40 T antigen
 E2F1
 p53
 P/CAF
 MyoD
 FOS
 SYT
 p73

TBP
 JUN
 TF_{II}B

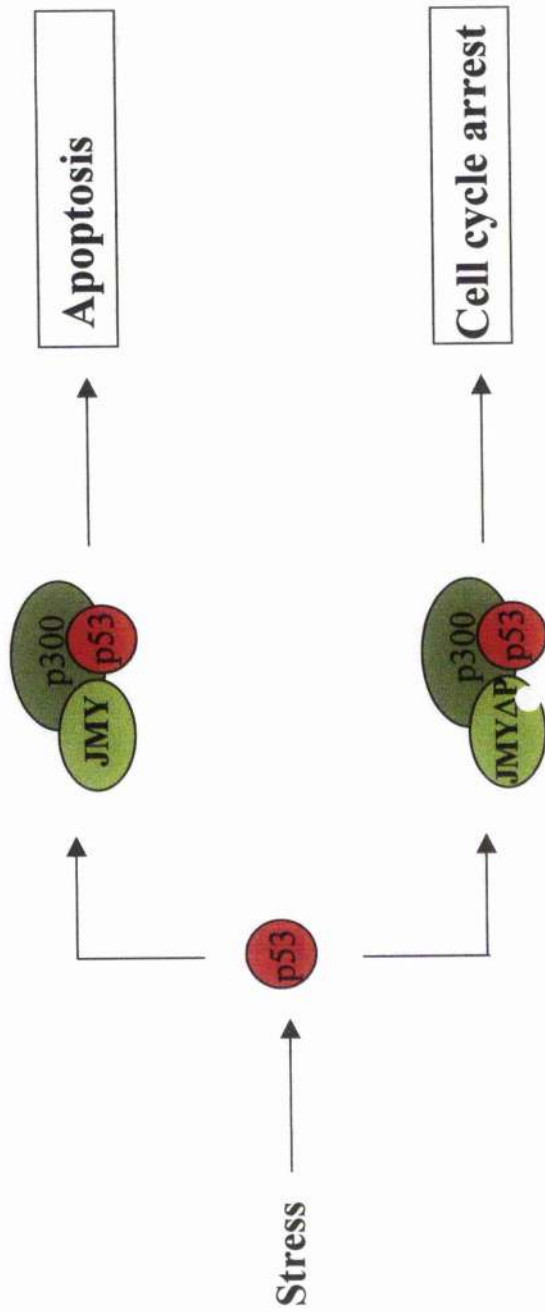
1.5. Structural features of JMY and Strap

JMY :S/T-P region with a cluster of potential phosphorylation sites for S/T-P directed kinases; CR2 : region with sequence homology to the adenoviral CR2 region; WH2, Wiscott-Aldrich syndrome homology domain 2; P-rich, proline rich domain; *Strap*: I-VI, tetratricopeptide(TPR) motifs in Strap. Interaction domains for Strap, JMY and p300 are shown (Demonacos *et al.*,2001).



1.6. Protein isoforms of JMY alter the outcome of the p53 response

JMY cooperates with p300 to enhance the p53 response and augment p53-dependent apoptosis. In contrast, the isoform JMY Δ P has compromised apoptotic activity and possesses greater cell cycle arrest activity than JMY.



1-4. Control of protein function by regulation of nuclear transport

The exchange of molecules between the nucleus and the cytoplasm is tightly regulated by the nuclear envelope. This ensures that genetic information is protected and transmitted correctly, and facilitates the coordination of nuclear and cytoplasmic processes (Corbett and Silver, 1997). Access across the nuclear envelope involves the nuclear pore complexes (NPCs) (Keldherr *et al.*, 1984), which are large protein structures composed of approximately 100 different proteins referred to as nucleoporins. Metabolites, ions and molecules smaller than 40kDa can diffuse passively through the NPC, while larger molecules tend to be actively transported (Doye and Hurt, 1997).

Nuclear import and export mechanisms exhibit considerable diversity, and are, in certain cases, regulated rather than constitutive. The ability to regulate the nuclear transport of certain molecules allows the integration of intracellular and extracellular input signals, and the tailoring of the cellular response to particular signals.

1-4.1 Basic steps of nuclear transport

Nuclear transport reactions (import or export) involve the same basic steps:

First, the transport cargo is recognised by, and associates with its transport receptor. This involves recognition of nuclear transport signals in the cargo molecule. The next step involves docking of the receptor-cargo complex at the nuclear envelope through interaction of the transport receptor with nuclear pore

proteins. This is followed by the translocation of the transport complex through the nuclear pore complex (NPC), and, finally, transport is terminated by irreversible dissociation of the cargo-receptor complex, and transport receptors are recycled for use in an another round of transport (Corbett and Silver, 1997).

1-4.2 Nuclear transport signals

Identification of cargos by the transport machinery involves inherent *cis*-acting signals within the cargo molecule. These show a great level of diversity and include:

Nuclear localisation signals (NLS)

The classical NLS was the first transport signal to be identified and comprises a short stretch of positively charged residues, generally lysine or arginine residues (Corbett and Silver, 1997). The bi-partite NLS contains two basic clusters of positively charged residues separated by a spacer region (Corbett and Silver, 1997). Other nuclear localisation signals include ankyrin repeats in the $\text{NF-}\kappa\text{B}$ inhibitor, $\text{I}\kappa\text{B}\alpha$ (Sachdev *et al.*, 1998.; Lee and Hannink, 2002) and the trimethyl-guanosine cap used in snRNA transport (Huber *et al.*, 1998).

Nuclear export signals (NES)

Nuclear export signals were first identified by mutational analysis of proteins that are rapidly translocated from the nucleus to the cytoplasm. The best understood nuclear export signal (NES) is the leucine-rich NES in proteins such as the HIV Rev protein (Fischer *et al.*, 1995), and the protein kinase A inhibitor (Wen *et al.*, 1995). Similarly to NLS signals, nuclear export signals show great variability but

are generally characterised by a cluster of hydrophobic residues; generally leucines but isoleucine and valine residues are also present in certain NESs (Corbett and Silver,1997).

Nucleocytoplasmic shuttling signals (NS)

Nucleocytoplasmic shuttling signals are bi-directional transport signals (Michael 2000). All proteins currently known to have a nucleocytoplasmic sequence are mRNA-interacting proteins.

The M9 motif is an example of an NS signal. It is much larger than a classical NLS (38 amino acids) and does not contain clusters of basic residues. M9 has been implicated in both the import and export of the RNA binding protein hnRNP A1, and these two functions proved to be inseparable (Michael/2000). In other, less extreme cases, nuclear import and export functions of the NS overlap but are spatially separable, and can be independent of transport receptor binding, involving direct interaction with the NPC (Michael, 2000).

1-4.3 Nuclear transport receptors

Nuclear localisation signals are recognised by members of the nuclear import receptor family. These receptors are generally large acidic proteins with an N-terminal RanGTP binding domain and an N-terminal cargo binding domain. Another important feature of these receptors is their ability to interact with components of the nuclear pore complex (Adam, 1999).

Different types of NLS appear to associate with distinct receptors (Nadler *et al.*, 1997). The importin α/β complex imports "classical NLS"-containing proteins. While both subunits of the import receptor can interact with NLS sequences, the α subunit displays stronger binding and is believed to be responsible for interaction with the NLS-bearing substrate while importin β mediates binding to the nuclear pore complex (Gorlich *et al.*, 1995).

Other importin α/β -like proteins have also been identified. For example, import of the M9-containing hnRNP A1 protein is mediated by the import receptor transportin, a distant relative of importin β (Pollard *et al.*, 1996), and the adaptor protein snurportin-1 has been shown to interact with importin β and mediate snRNA import (Huber *et al.*, 1998).

Studies of nuclear export receptors have been greatly facilitated with the use of the antibiotic Leptomycin B. Leptomycin B was found to inhibit the export of the HIV Rev protein, and to inhibit Rev-mediated RNA export (Fornerod *et al.*, 1997; Wolff *et al.*, 1997). The ability of leptomycin B to block export of other NES-containing proteins indicated that it was an inhibitor of the nuclear export machinery and identification of the cellular target of leptomycin B revealed the function of CRM1 as a nuclear export receptor. CRM1 shows sequence similarity with importin β suggesting that nuclear transport receptors form a family of proteins (Fornerod *et al.* 1997; Wolff *et al.*, 1997).

1-4.4 Cargo-receptor interaction

The directionality of nuclear transport depends on the distribution of transport receptors and the regulated association of these receptors and their cargos.

The small GTPase Ran plays a crucial role in the directionality of transport as it regulates the assembly and disassembly of nuclear transport complexes.

Ran exists in two nucleotide-bound states: Ran GTP and Ran GDP. The distribution of proteins that control the GTP/GDP-bound states of Ran creates a steep gradient across the nuclear envelope such that Ran GTP localises mainly to the nucleus whereas Ran GDP is cytoplasmic (Izaurralde *et al.*, 1997, Nachury and Weiss, 1999).

The association of export receptors with their cargo requires interaction with RanGTP in the nucleus (Izaurralde *et al.*, 1997) and their dissociation occurs in the cytoplasm where Ran GTP is rapidly hydrolysed into the GDP-bound form (figure 1.7).

On the other hand, Ran GDP forms a stable association with cargo-import complexes in the cytoplasm (Hopper *et al.*, 1990, Mahajan *et al.*, 1997). The cargo is released in the nucleus upon binding of Ran GTP to the nuclear import receptor (figure 1.7). The importance of the Ran GTP/GDP gradient is highlighted by *in vitro* experiments showing that when the Ran GTP/GDP gradient is inverted across the nuclear membrane, the direction of the traffic is reversed (Nachury and Weis, 1999). Furthermore, depletion of Ran GTP levels

by sodium azide has been reported to block nuclear transport (Schwoebel *et al.*, 2002).

1-4.5 Regulation of nuclear transport

The multi-step nature of nuclear transport mechanisms provides several potential points of regulation. These include regulation of components of the transport machinery and regulation of the association between the receptor and cargo molecules.

Regulation of the transport machinery

The physiological state of the cell has been suggested to dictate the rate of nuclear transport and the maximum aperture of the nuclear pore complex (Feldherr *et al.*, 1998). For instance, the passage of cells from a quiescent to a proliferating, to a transformed cell increases the rate of nuclear transport as well as the aperture of the nuclear pore complex (Feldherr *et al.*, 1998).

Moreover, modification of the nuclear pore complex may contribute to the regulation of nucleocytoplasmic traffic. Kehlenback *et al.*, have recently shown that phosphorylation of a component of the nuclear pore complex inhibits importin β and transportin-dependent import, without affecting CRM1-dependent export (Kehlenback *et al.*, 2000).

Another mechanism of regulation involves preferential use of transport factors. Mammalian cells contain three families of importin α proteins. Although there may be considerable functional redundancy among these, some evidence suggests

the existence of non-overlapping functions and specificity as illustrated by the import of the transcription factor STAT1 which specifically requires importin $\alpha 5$ (Sekimoto *et al.*, 1997).

Regulation of transport through regulation of cargo molecules

a) Post-translational modification

Post-translational modification of nuclear transport signals can affect the accessibility of these sequences to the transport machinery and alter the affinity of the cargo for receptors. Examples that illustrate the regulation of nuclear transport by post-translational modification are summarised below.

Phosphorylation

Phosphorylation of the yeast transcription factor Pho4 allows its interaction with the export factor Msn5 (Komeili *et al.*, 1999). Phosphate starvation provides the stimulus that triggers dephosphorylation of a serine residue in the NLS. This relieves inhibition and allows the association of Pho4 with the import receptor Pse1 (Kaffman *et al.*, 1998).

In contrast, phosphorylation of signal transducers and activators of transcription (STATs) activates their transactivation function. STATs are involved in the interferon-mediated cellular response to viral infection (O'shea *et al.*, 2002). Interferon stimulation activates the JAK kinases which in turn, phosphorylate tyrosine residues of STATs, allowing their dimerisation and subsequent nuclear transport and DNA binding (O'shea *et al.*, 2002). Dephosphorylation of STATs

and their dissociation from DNA is believed to unmask their NES signal, allowing CRM1-driven nuclear export (O'shea *et al.*, 2002).

Methylation

Methylation of arginines has been proposed to affect the export of some yeast hnRNPs (Shen *et al.*, 1998).

Ubiquitination

The oncoprotein Mdm2 negatively regulates the p53 tumour suppressor at multiple levels, including subcellular localisation. Early studies suggested the function of Mdm2 as a shuttling protein that can transport p53 out of the nucleus (Roth *et al.*, 1998). However, more recent findings demonstrated that the ubiquitin ligase activity of Mdm2 rather than its nucleocytoplasmic shuttling properties is critical in regulating the nuclear exclusion of p53 (Boyd *et al.*, 2000; Geyer *et al.*, 2000). Since Mdm2 promotes p53 ubiquitination at lysine residues near the C-terminal NES, it was speculated that ubiquitination may reveal the p53 NES and allow its nuclear export. This is supported by the inability of p53 ubiquitination mutants to undergo Mdm2-directed export (Lohrum *et al.*, 2001).

b) Protein-protein interaction

Masking

Masking involves the competitive binding of an accessory protein to the NLS/NES signal, which masks the signal from the import/export machinery.

A well characterised example of transport regulation by domain masking is the interaction between NF κ B and its negative regulators, the I κ B proteins. Association of NF κ B with its inhibitor masks one or both NLSs of the NF κ B subunits, and prevents its nuclear import (Huxford *et al.*, 1998, Malek *et al.*, 2001). Disruption of the NF κ B/I κ B complex allows the import and nuclear localisation of NF κ B (Beg *et al.*, 1993).

Nuclear/cytoplasmic anchoring and sequestration

This involves the immobilisation of a protein within a specific cellular compartment. The tumour suppressor APC associates with cytoskeletal microtubules which might be important for anchoring APC in the cytoplasm (Smith *et al.*, 1994). Also, p53 contains a cytoplasmic sequestration domain (CSD) which inhibits the association of importin α with the main NLS signal in the C-terminus of p53 (Liang *et al.*, 1999).

Piggy Back

In certain cases, nuclear transport of a particular protein is mediated by binding to another protein which bears a nuclear transport signal. The cyclin component of cdk2/cyclin E and cdc2/cyclin B acts as a nuclear targeting unit for its partner kinase. Cyclin E binds to the import adaptor importin α while cyclin B1 can interact directly through its NLS with the import receptor importin β . In addition to the NLS cyclin B1 also provides an NES for its associated kinase (Yang *et al.*, 1998).

1-4.6 Nuclear transport and regulation of cell proliferation and oncogenesis

A considerable body of evidence supports the importance of factors such as protein modification, protein stability and protein-protein interactions in the regulation of protein function. In particular, proteins that control cellular proliferation are tightly regulated at multiple levels to prevent abnormal cell growth and propagation of potentially deleterious mutations. Regulated nuclear transport is emerging as an important mechanism regulating the function of a variety of proteins and this property is highlighted by deregulation of the cellular distribution of certain proteins in some cancers.

Cell cycle regulatory proteins

The intracellular localisation of cyclin B1 is regulated throughout the cell cycle. It is believed to shuttle between the nucleus and cytoplasm during interphase. However, phosphorylation of several serine residues at the beginning of mitosis blocks CRM1 binding, allowing nuclear accumulation of cyclin B1 (Yang *et al.*, 1998).

Signal transduction

Regulated translocation of components of signal transduction cascades can be an efficient mechanism of coordinating fast and adequate cellular responses to a variety of stimuli. Signal-induced translocation of signaling proteins to their site of action not only regulates their access of the downstream components of the cascade, but also allows the signal to be rapidly amplified.

The extracellular signal- regulated kinases (ERKs) 1 and 2 are cytoplasmic in unstimulated cells. Stimulus-dependent phosphorylation is thought to trigger the dimerisation, and nuclear localisation of these kinases. An NES-dependent export mechanism is presumed to relocate the unphosphorylated forms of ERKs to the cytoplasm (Khokhlatchev et al., 1998).

Tumour suppressors

The importance of nuclear transport in regulating the function of tumour suppressors is highlighted by the observation that it is deregulated in certain tumours.

For example, BRCA1 was reported to mislocalise to the cytoplasm in breast cancer derived cell lines and in certain breast carcinomas (Taylor *et al.*, 1998). Further, abnormal cytoplasmic localisation of p53 has been reported in breast carcinoma (Moll *et al.*, 1992), neuroblastomas (Moll *et al.*, 1995), and in colorectal cancers (Bosari *et al.*, 1995). As discussed earlier, several mechanisms appear to regulate the subcellular localisation of p53, including phosphorylation, ubiquitination, domain masking (tetramerisation) and cytoplasmic sequestration. The existence of such a variety of regulatory mechanisms supports the importance of localisation for p53 function.

1-4.7 Conclusions

Recent developments of the nucleocytoplasmic transport field have highlighted the complexity of mechanisms governing the movement of molecules between the nuclear and cytoplasmic compartments. The regulation of nuclear transport

involves the regulation of the multiple components involved and the specificity of their action.

Subcellular distribution is increasingly recognised as an important aspect of the function of numerous cellular factors involved in cell proliferation and tumourigenesis. Therefore, better understanding of the mechanisms regulating subcellular localisation would provide important clues to the function of such proteins.

1.7. Nuclear transport and the Ran cycle

Model for import and export of molecules through the nuclear pore complex.

Import complexes assemble in the cytoplasm in the presence of Ran GDP, and are translocated into the nucleus. In the nucleus, binding of RanGTP to the import complex causes its dissociation and cargo release.

Nuclear export complexes assemble in the nucleus in the presence of RanGTP. Following translocation into the cytoplasm, GTP hydrolysis results in the release of the cargo.

- ◉ Leptomycin B site of action
- ◉ Azide site of action

1-5. Objectives

The ability of p53 to induce apoptotic cell death in response to stress signals is crucial for its function as a tumour suppressor. This property of p53, frequently compromised in tumours, depends primarily on its function as a transcription factor and on its ability to form complexes with other transcriptional regulators that modulate its transcriptional activity.

The p300-associated cofactor JMY selectively augments p53-mediated apoptosis, and thus forms part of a growing family of cofactors that are required for p53-dependent apoptosis. The involvement of such cofactors in the p53 pathway indicates the existence of additional mechanisms regulating the apoptotic response, the understanding of which would prove invaluable in the development of new therapies that restore p53 mediated apoptosis.

The work presented here focuses on the control of JMY function, and aims to describe the mechanisms that allow it to co-operate with p53 under the appropriate conditions.

Previous studies have demonstrated that JMY is recruited to p53 complexes in response to stress. Therefore, it was of interest to determine if the intracellular localisation of JMY was affected by cellular stress, and to examine the effect of different cellular conditions on the cell cycle function of JMY.

The activity of p53 is partly regulated by its sub-cellular localisation and involves the activity of its main regulator, Mdm2. Mdm2 promotes the export of p53 into

the cytoplasm, thus inhibiting its ability to activate gene expression. Considering the involvement of both Mdm2 and JMY in p53 regulation and the ability of Mdm2 to act as a shuttle protein, another objective of this research relates to the ability of Mdm2 to affect the cellular localisation of JMY. The requirement for the ubiquitin ligase domain of Mdm2 for such function led to questions about its ability to regulate the cellular levels of JMY.

JMY Δ P, a protein isoform of JMY that lacks a proline-rich domain in the C-terminal region of the protein, is also capable of enhancing the p53 response. However, this isoform of JMY favours p53-dependent G1 arrest as opposed to apoptosis. Considering that JMY Δ P possesses functional properties that distinguish it from wild type JMY, it was of interest to assess whether the differences between these two isoforms were reflected in the control of their subcellular localisation.

Chapter 2. Materials and Methods

2-1. Plasmids

The following plasmids have previously been described: pcDNA3 HAJMY (Shikama *et al.*, 1999), pCHDMIA, pCHDMA58-89, pCHDM C464A, pCHDM1-440 (Chen *et al.*, 1993; 1996).

pCHDM Δ 58-89;C464A was generated by restriction digest of pCHDMC464A and pCHDMA58-89 with SalI. The fragments containing the C464A and the Δ 58-89 mutations were ligated to generate a plasmid with both mutations.

2-2. Antibodies

Primary antibodies

HA	Mouse monoclonal HA11 (Babco)
	Rabbit polyclonal Y-11 (Santa Cruz)
JMY	Rabbit polyclonal 1289 (generated in the laboratory)
Mdm2	Mouse monoclonal Ab-1 (Oncogene)
	Mouse monoclonal SMP-14 (Santa Cruz)
	Rabbit polyclonal H-221 (Santa Cruz)
p53	Mouse monoclonal DO-1 (Santa Cruz)
	Sheep polyclonal anti-p53 (Scottish Antibody Production Unit)
CD20	L27 (Becton Dickinson)

Secondary antibodies

Anti-Ig AP	Alkaline phosphatase-conjugated (Promega)
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Na₂HPO₄), followed by gentle mixing. The mixture was incubated for 20 minutes at room temperature before being added drop-wise to the cells.

The backbone vector pcDNA3 was used to maintain a constant amount of transfected DNA among samples, and pCMVβgal was included as an internal control for transfection efficiency.

12-18 hours post transcription, cells were washed 3 times with PBS to remove excess precipitate and fresh media was added. Cells were harvested 24-72 hours post transfection.

2-5. Immunofluorescence

Cells seeded onto 35mm glass coverslips were washed three times in cold PBS and fixed in a solution of 4% paraformaldehyde at room temperature for 15 minutes. Following three washes in PBS, permeabilisation of the cells was performed at 4°C for 5 minutes with a PBS solution containing 0.5% Triton X-100. Fixed cells were then incubated in a blocking 10% FCS PBS solution for 10 minutes at room temperature. Primary antibodies diluted in 1% FCS PBS were incubated with the cells for 30 minutes at room temperature. Cells were then extensively washed in a 1% FCS PBS solution, and the secondary antibody was added in a 10% FCS PBS solution for 30 minutes. Anti-mouse or anti-rabbit IgG conjugated to Alexa Fluor[®]488 (green), or Alexa Fluor[®] 594 (red) (Molecular Probes) was used for detection. Coverslips were washed extensively, with addition of DAPI (4,6-Diamino-2-phenylindole) to the final wash. Finally, cells were mounted on microscope slides using Citifluor

(Citifluor Ltd) and immunofluorescence was viewed under a fluorescence microscope (Olympus).

Where cellular compartmentalisation was quantified, the percentage of cells with each cellular localisation was defined relative to the population of transfected cells.

2-6. Immunoprecipitation

Cells were washed twice in PBS and incubated in trypsin for 2 minutes at 37°C. Media supplemented with 10% FCS was then added and the cells collected and centrifuged at 1000 rpm for 5 minutes. The cell pellets were washed three times in cold PBS, re-suspended in TNN buffer (50 mM Tris-HCl pH7.5, 150mM NaCl, 0.5% NP-40, 5mM EDTA, 0.1mM DTT, 0.5µg/ml leupeptin, 0.5µg/ml aprotinin, 40µg/ml bestatin), and after a 30 minute incubation on ice, they were centrifuged at 13000 rpm for 30 minutes at 4°C. The cell extract was pre-cleared by incubating with protein G agarose for 30 minutes and then harvested and rotated on a wheel at 4°C with 35µl protein A agarose and 1-4µl primary antibody. The reaction was left for 6-16 hours. The agarose beads were subsequently washed three times in TNN reaction buffer. Bound proteins were released into 3x SDS loading buffer (375mM Tris-HCl pH6.8, 30% glycerol, 6%SDS, 0.015% Bromophenol Blue). The sample was boiled for 3 minutes and separated by SDS-PAGE and the proteins of interest were detected by western blot with the appropriate antibody.

2-7. Western blot analysis

Protein expression was detected by immobilisation of cell extract onto a nitrocellulose membrane (Inverclyde Biologicals), blocking in a 5% milk PBS solution and detection with specific antibodies. All antibodies were diluted in a 5% milk PBS solution. Primary antibodies were used at a 1:500-1:2000 dilution and secondary antibodies were diluted 1:5000. Two detection systems were used to amplify the signal. Horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences UK Ltd) were used with the Super Signal® West Pico solutions (Pierce); and substrate tablets (Sigma Fast™ 5-Bromo-4-chloro-3-indoyl phosphate/nitro blue tetrazolium) were used for detection of calf alkaline phosphatase conjugated secondary antibodies (Promega).

2-8. Phosphatase treatment of cell extracts

Phosphatase treatment of cellular extracts was performed by addition of 1µl lambda protein phosphatase (New England Biolabs). The extracts were incubated with the phosphatase (final concentration 2000 U/ml) at 30°C for 1 hour prior to SDS-PAGE and western blot analysis.

2-9. Flow cytometry

U2OS cells were transfected with 5µg pCMV-HAJMY or pcDNA3 empty vector, and 8µg of CD20 expression vector. Cells were serum starved for 16 hours, exposed to ultraviolet light 6 hours before harvesting, or harvested without treatment. In all cases, cells were harvested 48 hours post-transfection. For flow cytometry analysis the cells were captured by monitoring the expression of the

cell surface marker CD20. After transfection cells were harvested by treatment with cell dissociation solution (Sigma) for 30-45 minutes at 37°C. Cells were washed in PBS by centrifugation at 2000 rpm for 3 minutes, and resuspended in 200µl of PBS containing 20µl of a mouse anti-CD20 antibody (Becton Dickinson) conjugated to fluorescein isothiocyanite (FITC). The cell suspension was incubated on ice for 30 minutes followed by washing in cold PBS. Cell pellets were subsequently fixed by the drop-wise addition of 50% ethanol/PBS solution and left overnight at 4 °C.

Cells were then washed in PBS and re-suspended in 500µl of PBS containing propidium iodide (20µg/ml) and RNase (125 U/ml). Flow cytometry was performed on a Becton Dickinson fluorescence activated cell sorter. All cell populations were analysed at a rate of 100-200 cells/second to prevent mis-read. To determine the cell cycle profiles of transfected cells the intensity of propidium iodide staining was analysed in cells positive for FITC staining. Using Cell Quest Pro software, the cell cycle profile of 10000 transfected cells was analysed.

Chapter 3. The subcellular localisation of JMY is regulated by cellular conditions

3-1. Introduction

Many cancer-related molecules are regulated at multiple levels to ensure tight control of cellular proliferation. Defects in these regulatory mechanisms can affect the cell's response to external insults and to internal aberrations, and eventually lead, or contribute to the development and progression of cancer.

Recent developments in the field of nucleocytoplasmic transport have provided insight into the mechanisms governing the cellular distribution of proteins, and highlighted the importance of subcellular localisation in protein function (Hood and Silver, 2000). Aberrant intracellular localisation of cancer-related molecules has been reported in certain cancers. Examples include the mis-localisation of BRCA1 to the cytoplasm in breast cancer cells but not normal cells (Taylor *et al*, 1998), and cytoplasmic sequestration of p53 in neuroblastomas, breast and colon cancers (Moll *et al*, 1992; 1995; Bosari *et al*, 1995).

The potent apoptotic and growth inhibitory functions of p53 are tightly controlled by diverse mechanisms. These not only regulate the stability of the protein and its ability to act as a transcription factor, but also impact on its intracellular distribution (Vousden, 2002).

In addition to direct regulation of the p53 protein, a growing number of cofactors have been reported to modulate the outcome of p53 activation (Lu and Vousden 2002). Such cofactors could potentially provide additional regulation points for the p53 response, and include the p300 cofactor JMY. JMY cooperates with p300 to enhance p53-dependent transcription and apoptosis (Shikama *et al.*, 1999). While JMY has the ability to enhance p53-dependent apoptosis, JMY Δ P, a protein isoform of JMY that arises through alternative splicing of the mRNA, has a more pronounced effect on p53-mediated cell cycle arrest, suggesting that the different isoforms of JMY can modulate the functional outcome of p53 activation (Shikama *et al.*, 1999).

Considering the involvement of JMY in the p53 response, it was of interest to investigate whether it was regulated by cellular stress. The intracellular localisation of JMY was of particular interest because previous studies had reported variation in the localisation of the protein, and demonstrated that DNA damage results in enhanced interaction of JMY/p300 with the p53 complex (Shikama *et al.*, 1999).

3-2. The subcellular localisation of JMY in unstressed cells

The subcellular localisation of JMY was assessed in the absence of stress. Due to difficulty in generating suitable antibodies for the analysis of endogenous JMY, this study focused on the use of ectopially expressed JMY.

U2OS cells were transfected with the HAJMY expression vector pCMV-HAJMY and the subcellular localisation of HAJMY was examined by indirect immunofluorescence (Figure 3.1.A).

To quantify the results, cells were categorised as having exclusively cytoplasmic, nuclear, or uniform (both nuclear and cytoplasmic) fluorescence staining and the percentage of cells in each category was graphically represented (Figure 3.1.C).

In U2OS cells, JMY localised to both the nucleus and the cytoplasm in the majority of cells. A considerable proportion of transfected cells (42%) also displayed exclusively cytoplasmic staining and very few cells (1%) showed nuclear staining (Figures 3.1.A and C).

A similar pattern was observed with Flag-tagged JMY and the data were not affected by the transfection method nor the amount of plasmid transfected since titration of the plasmid produced similar results (not shown).

SAOS2 cells, which lack endogenous p53, were also examined for the intracellular distribution of HAJMY. A similar distribution pattern to that seen in U2OS cells was observed, with the majority of cells displaying uniform staining

(73%), and smaller populations exhibiting cytoplasmic and nuclear staining (22% and 6%, respectively) (Figures 3.1.B and C).

Interestingly, U2OS cells appear to contain a relatively higher proportion of cytoplasmic-staining cells than SAOS2 cells (43% and 22 %, respectively), suggesting a degree of cell type variation in the subcellular localisation of JMY.

3-3. DNA damage causes the translocation of JMY into the nucleus

The above experiment showed an important level of cytoplasmic localisation for JMY. JMY cooperates with p300 to stimulate p53-dependent transcription, and has been shown to interact more efficiently with p53 under DNA damage conditions (Shikama *et al*,1999). Considering that DNA damage induces the p53 response, and that nuclear localisation is required for transcriptional coactivation, it was speculated that the sub-cellular localisation of JMY might also be regulated by DNA damage.

Three types of DNA damaging treatments, actinomycin D, etoposide and exposure to UV, were used to investigate whether JMY was regulated by DNA damage. These cause damage to DNA via different mechanisms. Actinomycin D blocks the movement of RNA polymerase along the DNA template by binding tightly to DNA whilst UV irradiation causes the formation of pyrimidine dimers, and etoposide triggers double strand breaks through its ability to target topoisomerase II (Friedberg *et al.*, 1995).

U2OS cells were transfected with pCMV-HAJMY and were treated with 20nM actinomycin D for 16 hours, 10 μ M Etoposide for 7 hours, or exposed to UV (60 J/m²) and harvested 6 hours later. Changes in the sub-cellular localisation of JMY compared to untreated control cells were examined by indirect immunofluorescence.

Actinomycin D resulted in an almost twofold reduction in the population of cells with exclusively cytoplasmic JMY (42% to 24%). This was paralleled by an increase in the population of cells with nuclear JMY (1.7% to 17%). A similar effect was observed with UV which caused a sharp decrease in the percentage of cytoplasmic-staining cells (42% to 4.5%) and an increase in cells with nuclear (1.7% to 27%), or uniform (56.4 to 68.5%) staining, implying translocation of the protein into the nucleus (Figures 3.2. A, B, D). In contrast, etoposide treatment did not significantly alter the localisation of JMY (Figures 3.2.C and D). Taken together, these data suggest that, under certain conditions of DNA damage, the nuclear localisation of JMY is enhanced, possibly by enhanced import or reduced nuclear export, which would facilitate its interaction with other components of the p53 complex, and potentially enhance its transcriptional coactivation properties.

3-4. Serum withdrawal enhances the cytoplasmic localisation of JMY

The above data show that DNA damaging conditions enhance the nuclear localisation of JMY. Considering that DNA damage activates cellular checkpoints which prevent cell-cycle progression to allow repair of the damage, it

was of interest to examine the subcellular localisation of JMY under conditions which halt cell cycle progression without causing damage to DNA. In particular, the effect of serum deprivation on the intracellular distribution of JMY was examined.

Conditions of low serum (0.2% serum for 16 hours) did not alter the subcellular localisation of p53 (figure 3.3.A) and, in contrast to actinomycin D treatment, did not cause its stabilisation (Figure 3.3.B).

To investigate the response of JMY to serum withdrawal, U2OS cells were transfected with pCMV-HAJMY, and 24 hours following transfection, they were incubated for 16 hours in media supplemented with 0.2% serum.

The sub-cellular localisation of JMY was observed by indirect immunofluorescence. Under such conditions, JMY localised exclusively to the cytoplasm in the majority of cells (65%), implying reduced import, or enhanced nuclear export of the protein (Figures 3.3 C, 3.5.B).

Western blot analysis of HAJMY under these conditions showed the appearance of a faster migrating band following serum withdrawal (Figure 3.3.D, lane 2). The possibility that this band is a phospho-form of the protein was confirmed by phosphatase treatment of the cell extracts which reduced the appearance of the band (Figure 3.3.D lane 3).

3-5. Rapid cytoplasmic translocation of JMY in response to serum withdrawal and nuclear re-entry following serum stimulation

The kinetics of the cytoplasmic translocation of JMY in response to serum withdrawal were investigated. U2OS cells were transfected as above and incubated in low serum media (0.2% FCS) 24 hours following transcription. The subcellular localisation of JMY was examined following 1, 2, 4 and 16 hours of serum withdrawal. At 16 hours, cells were divided into two groups. The first group was maintained in low serum media whilst the second group was incubated in 10% FCS media. The subcellular distribution of JMY was examined 4 hours later. The data are summarised in figure 3.4.

An increase in the proportion of cells with cytoplasmic JMY was observed after one hour of serum deprivation (40% to 46%), and this continued throughout the time course (Figure 3.4). After 16 hours of serum withdrawal, approximately 67% of cells displayed nuclear exclusion of JMY. Examination of the subcellular localisation of JMY after 20 hours revealed a decrease in this population in cells stimulated with serum (48%) but not in cells that were maintained in low serum (70%) (Figure 3.4).

These data suggest that the cytoplasmic relocalisation of JMY is an early event during serum deprivation, and hint that growth factor signalling may be involved in regulating the intracellular distribution of JMY.

3-6. The cytoplasmic localisation of JMY in serum starved cells is the result of nuclear export

The enhanced cytoplasmic localisation of JMY in serum-starved cells could be the outcome of enhanced nuclear export, or reduced import of the protein. To distinguish between these two events, U2OS cells were transfected with the expression vector pCMV-HAJMY, and were subsequently serum starved in the presence of the antibiotic leptomycin B. Leptomycin B is a specific inhibitor of nuclear export which targets CRM1-dependent export and causes the accumulation of CRM1-substrates in the nucleus (Fornerod *et al.*, 1997; Wolff *et al.*, 1997).

Confirming the nuclear export hypothesis, leptomycin B treatment reversed the cytoplasmic relocalisation of HAJMY since there was an increase in the percentage of cells with nuclear HAJMY (7% to 60%), paralleled by a decrease in the population of cells exhibiting cytoplasmic staining (64.5% to 28.5%) (compare 3.5.A(i) and (ii) and 3.5.B).

To further confirm that the cytoplasmic localisation of JMY is the result of active transport into the cytoplasm, HAJMY-overexpressing U2OS cells were incubated in 0.2% serum media supplemented with sodium azide. Sodium azide depletes the levels of RanGTP, which is essential for the assembly the nuclear export complex (Schwoebel *et al.*, 2002). Sodium azide treatment reduced the cytoplasmic localisation of JMY (64.5% to 27%) and increased uniform and nuclear staining, further demonstrating that NES dependent export is involved in JMY regulation (Figures 3.5.A (iii) and 3.5.B).

3-7. Effect of UV and serum deprivation on the cell cycle function of JMY

JMY has been demonstrated to stimulate both p53-dependent G1 arrest and apoptosis (Shikama *et al.*, 1999). Considering that the intracellular distribution of JMY is regulated by DNA damage and serum deprivation, it was of interest to examine the effect of these conditions on the cell cycle arrest and apoptotic properties of JMY.

To this end, U2OS cells were transiently transfected with an empty vector, or with an expression vector for HAJMY. The cells were exposed to UV, or incubated in 0.2% FCS media, and the cell cycle profiles were examined and compared to profiles of untreated cells (Figure 3.6).

Overexpression of JMY in unstressed cells caused a two-fold increase in apoptosis relative to mock transfected cells (Figure 3.6.A), consistent with the ability of JMY to induce apoptosis in the presence of p53 (Shikama *et al.*, 1999). Surprisingly, a different effect was observed in UV treated cells as a slight increase in G1 and decrease in G2/M were observed (Figure 3.6.B). A more dramatic effect of JMY was expected under these conditions due to the UV-induced nuclear translocation of JMY. Nevertheless, it cannot be excluded that JMY participates to p53-mediated transactivation of non-apoptotic genes in response to UV.

Serum starvation completely abolished the effect of JMY on the cell cycle and the cell cycle profiles are identical in the absence or presence of overexpressed JMY (Figure 3.6.C). This may be explained by the cytoplasmic translocation of

JMY under these conditions which could prevent it from contributing to transcriptional events in the nucleus.

3-8. The proline rich region of JMY is dispensable for response to serum withdrawal

The JMY isoform ΔP , which lacks the proline rich region in the C-terminus (Figure 3.7.A), has distinct properties that distinguish it from the wild type protein. The two proteins have the ability to augment the p53 response, albeit with different outcomes. While wild type JMY preferentially augments apoptosis, JMY ΔP appears to favour p53-dependent cell cycle arrest and is compromised in its ability to facilitate apoptosis (Shikama *et al.*, 1999).

To determine whether the different properties of JMY and JMY ΔP are reflected in their subcellular localisation, HAJMY ΔP was overexpressed in U2OS cells and changes in its intracellular localisation were assessed in the presence of DNA damage, or following serum starvation.

Similarly to the wild type protein, the majority of cells displayed uniform JMY ΔP staining in the absence of stress. Nevertheless, despite the presence of HAJMY ΔP in both the nucleus and the cytoplasm, the majority of these cells exhibited strong staining in the nucleus relative to the cytoplasm. In other words, most JMY ΔP -overexpressing cells exhibited strong nuclear and weaker cytoplasmic staining. Moreover, the proportion of cells with exclusive nuclear staining (18%) was higher than that observed with the wild type protein (1.6%) (Figures 3.7.B and

E). These observations suggest that HAJMY Δ P can more readily localise to the nucleus compared to wild type JMY, implying possible differences in the rates of import and/or export between the two isoforms.

When JMY Δ P-overexpressing cells were treated with actinomycin D (20nM for 16 hours), etoposide (10 μ M for 7 hours) or UV (60J/m²), there was a very slight change in the intracellular localisation of JMY Δ P, and no further nuclear accumulation of the protein was observed (Figures 3.7. C and E).

In contrast, serum starvation resulted in an increase in the population of cells with exclusively cytoplasmic JMY Δ P (18.5% to 61%), paralleled by a decrease in the populations with nuclear or uniform staining (Figures 3.7. D and E). This indicates that, in a similar fashion to the wild type protein, JMY Δ P can be exported out of the nucleus and that its nuclear export is enhanced under conditions of serum starvation.

Given the different responses of JMY and JMY Δ P to DNA damage, and the ability of both proteins to undergo nuclear export under low serum conditions, it is conceivable that JMY utilises different properties under these conditions and that different cellular pathways impinge on the regulation of JMY.

3-9. Conclusions

Through its function as a p53 coactivator, JMY is involved in the regulation of the p53 response. The data presented here suggest that JMY is itself a regulated protein, and that it is regulated at the level of its sub-cellular localisation.

Under normal growth conditions, transfected cells were heterogeneous with respect to the localisation of JMY, suggesting that the intracellular distribution of JMY might be regulated. Although the majority of cells exhibited both nuclear and cytoplasmic localisation, JMY was excluded from the nucleus in a considerable proportion of cells, which was not consistent with its function as a transcriptional cofactor.

However, DNA damage, as a result of actinomycin D treatment, or exposure to UV light, reduced the cytoplasmic localisation of JMY, and caused its translocation into the nucleus, the cellular compartment where it is required for transcriptional coactivation. This is consistent with the enhanced ability of JMY to interact with p53 and p300 under such conditions, and with its role in stimulating p53 activity (Shikama *et al*, 1999). Nevertheless, although it slightly enhanced G1 arrest, JMY was unable to augment apoptosis in UV-treated cells. It is speculated that nuclear translocation of JMY following UV-treatment might regulate its function in processes other than apoptosis.

In contrast to UV and actinomycin D, etoposide treatment did not significantly alter the distribution of JMY, suggesting that its relocalisation to the nucleus

might involve particular cellular signals that are not triggered by etoposide treatment.

Furthermore, JMY also responds to signals related to cellular growth. Whereas withdrawal of serum did not alter the stability or subcellular distribution of p53, it promoted rapid cytoplasmic translocation of JMY which was reversed by serum stimulation of starved cells.

The cytoplasmic relocalisation of JMY was blocked by inhibiting CRM1-dependent export with leptomycin B, and sodium azide, implicating nuclear export in the regulation of the intracellular localisation of JMY.

Moreover, serum deprivation abolished the effect of JMY on the cell cycle, suggesting that cytoplasmic relocalisation under these conditions may serve to limit the growth arrest or apoptotic functions of JMY.

Besides changes in intracellular localisation, serum starvation resulted in a mobility shift of HAJMY which was abrogated by phosphatase treatment, suggesting that a phosphorylation event may also be involved in JMY regulation under these conditions.

The JMY isoform JMY Δ P, which lacks the proline rich region in the C-terminus, displayed stronger nuclear staining relative to the wild type protein. The nuclear localisation of JMY Δ P was not enhanced by actinomycin D, etoposide or UV-induced DNA damage, suggesting different mechanisms of regulation for the two

isoforms. In contrast, serum withdrawal augmented the cytoplasmic localisation of JMY Δ P in a similar fashion to the wild type protein.

Taken together, these data indicate that the intracellular distribution of JMY is regulated, and may impact on its ability to induce apoptosis or enhance cell cycle arrest. Similar mechanisms appear to regulate the localisation of JMY and JMY Δ P in response to serum withdrawal but not in the presence of DNA damage.

3.1. The subcellular localisation of HAJMY in unstressed cells

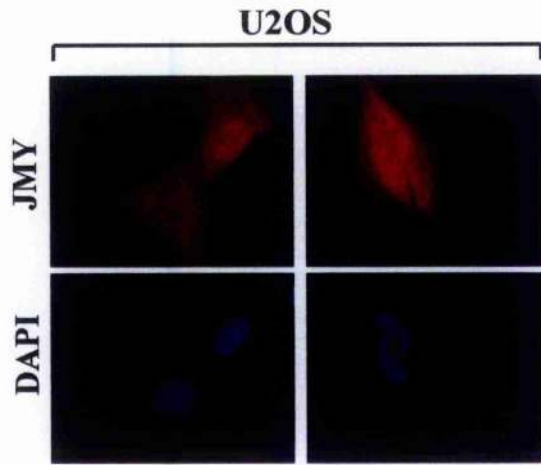
A. U2OS

U2OS cells were transfected with 2 μ g of pCMV-HAJMY. Thirty six hours following transfection, the localisation of JMY was determined by indirect immunofluorescence using a rabbit anti HA antibody (Y-11) and a red fluorescent secondary antibody. The nuclear stain DAPI was used to determine the position of nuclei.

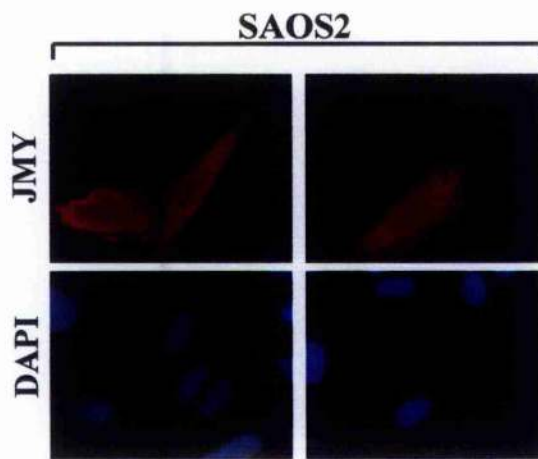
B. SAOS2

SAOS2 cells were transfected with 3 μ g of pCMV-HAJMY and fixed 36 hours post-transfection. JMY was detected with the anti-HA Y-11 antibody and a red fluorescent secondary antibody.

A.



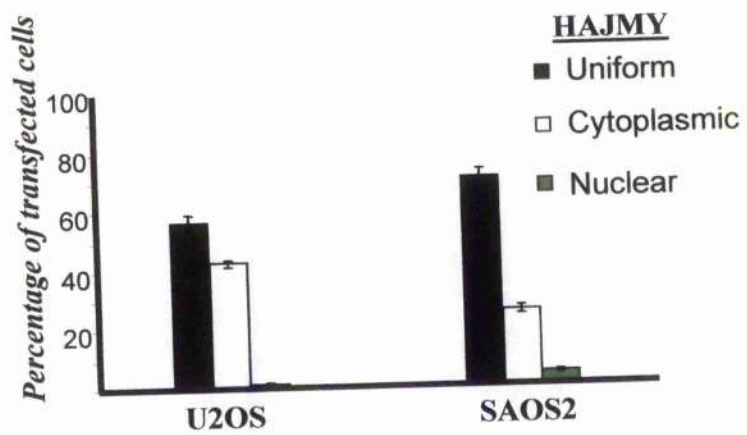
B.



C. Quantification of the intracellular distribution of overexpressed JMY in U2OS and SAOS2 cells:

The data were quantified by scoring 100-150 cells with respect to the localisation of JMY. The data represent average values from 3 separate experiments.

C.



3.2. The subcellular localisation of JMY in response to DNA damage

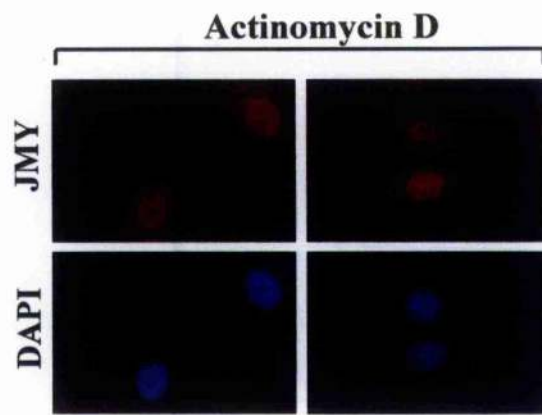
A. Actinomycin D treatment

U2OS cells were transfected with 2µg pCMV-HAJMY. Twenty four hours following transfection, the cells were incubated for 16 hours in actinomycin D-containing media (20nM) before being fixed and examined for the subcellular localisation of JMY. JMY was detected with the anti-HA Y-11 antibody and a red fluorescent secondary antibody. The nuclear stain DAPI was used to determine nuclei position.

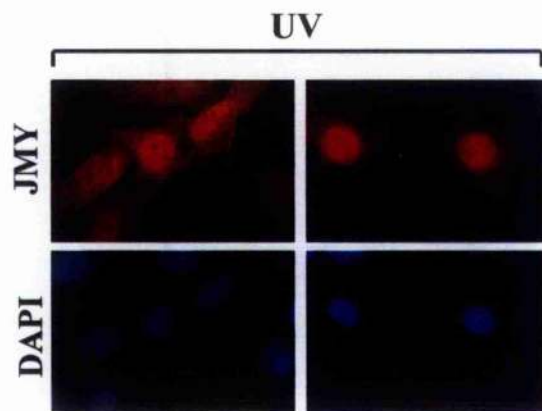
B. UV treatment

U2OS cells were transfected with 2µg pCMV-HAJMY. Thirty hours following transfection, the cells were exposed to UV (60 J/m²) for 6 hours. JMY was detected by immunofluorescence using the anti-HA Y-11 antibody and a red fluorescent secondary antibody.

A.



B.

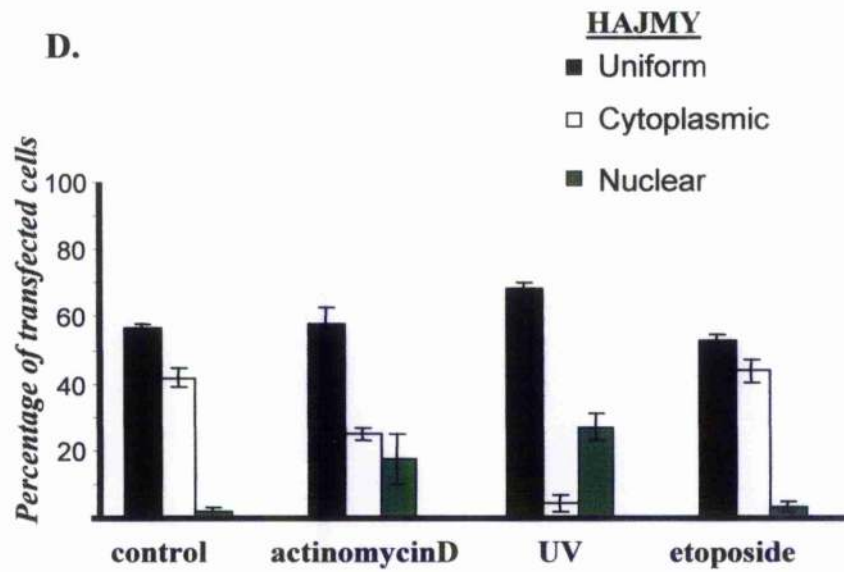
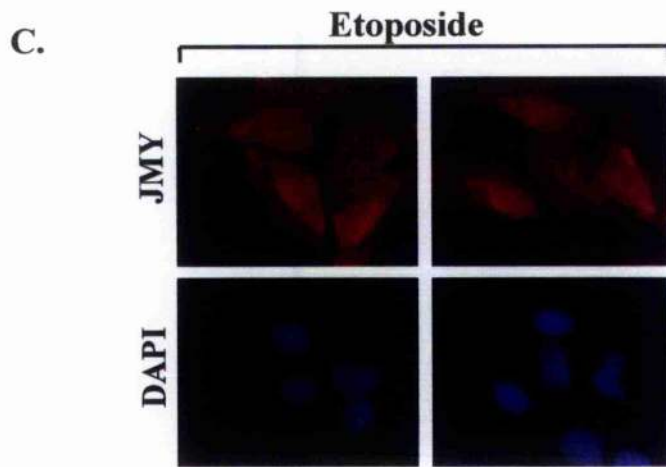


C. Etoposide treatment

U2OS cells were transfected with 2 μ g pCMV-HAJMY. Thirty hours following transfection, the cells were treated with etoposide (10 μ M) for 7 hours. JMY was detected by immunofluorescence using the anti-HA Y-11 antibody and a red fluorescent secondary antibody. DAPI staining shows the position of nuclei.

D. Quantification of the effect of DNA damage on JMY localisation

The data were quantified by scoring 100-150 cells with respect to the subcellular localisation of JMY. The data represent average values from 3 separate experiments.



3.3. The subcellular localisation of JMY in response to serum withdrawal

A. The subcellular localisation of p53 is not affected by low serum conditions

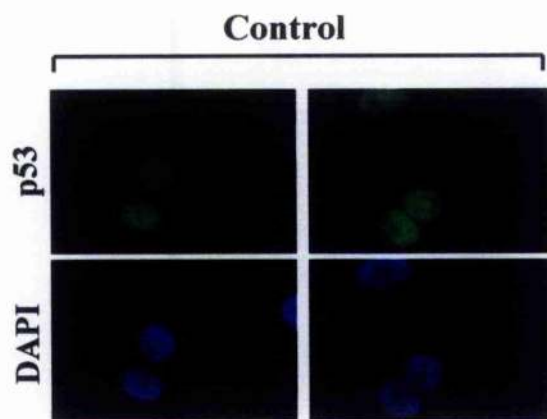
Endogenous p53 was visualised in U2OS cells that were grown under normal growth conditions (i), or following a 16 hour incubation in 0.2% serum media (ii). p53 was detected with the anti-p53 antibody DO-1 and a green fluorescent secondary antibody, and DAPI was used for nuclei identification.

B. Serum starvation does not cause the accumulation of p53

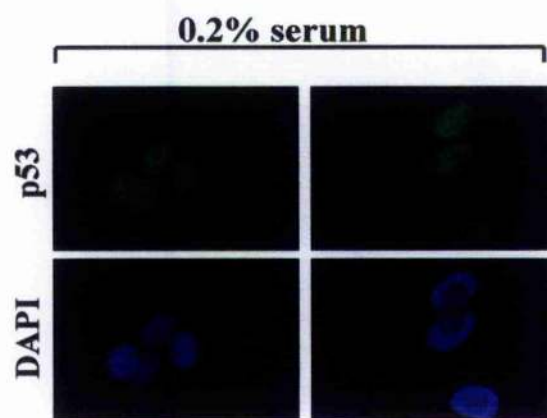
Endogenous p53 levels in U2OS cells were examined under normal conditions (lane 1), 16 hours post-treatment with actinomycin D (20nM, lane 2), or after 16 hours of serum withdrawal (lane 3). Sheep anti-p53 serum was used to detect p53.

A.

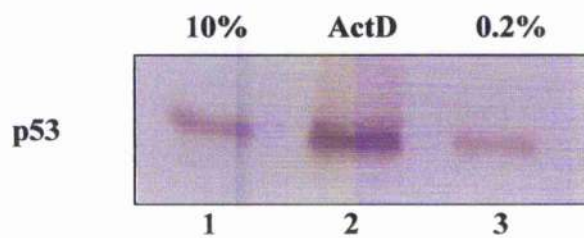
i)



ii)



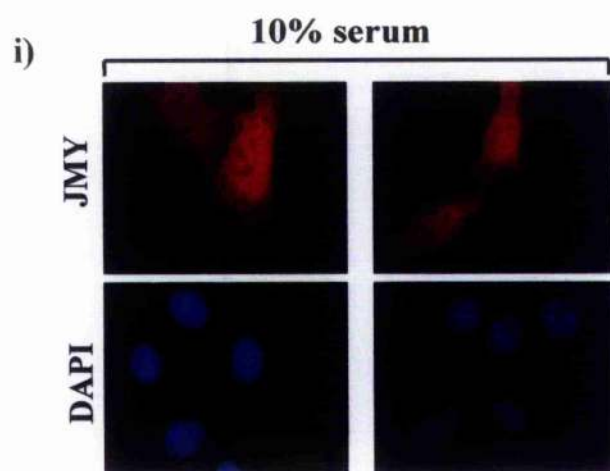
B.



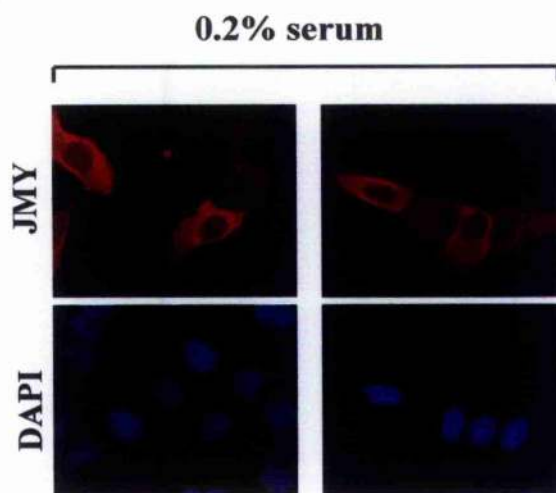
C. Serum starvation causes the translocation of JMY into the cytoplasm

U2OS cells were transfected with 2 μ g of pCMV-HAJMY and incubated in media supplemented with 10% (i) or 0.2%(ii) foetal calf serum. JMY was detected by indirect immunofluorescence with Y-11 and a red fluorescent secondary antibody.

C.



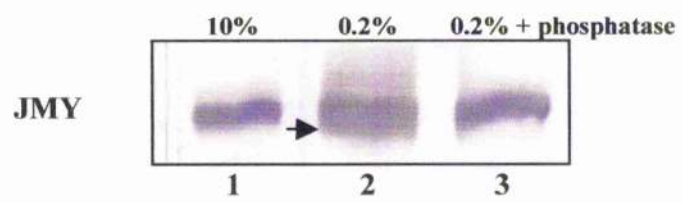
ii)



D. JMY phosphorylation in response to serum withdrawal.

HAJMY levels were examined in serum-starved cells. U2OS cells were transfected with 4 μ g pCMV-HA-JMY and were incubated in media with 10% serum (lane1), or serum starved (0.2% FCS) for 16 hours (lanes 2 and 3). Half the cell lysate was used in a phosphatase reaction using λ phosphatase (lane 3). The arrow indicates the position of the faster migrating phosphoform of JMY.

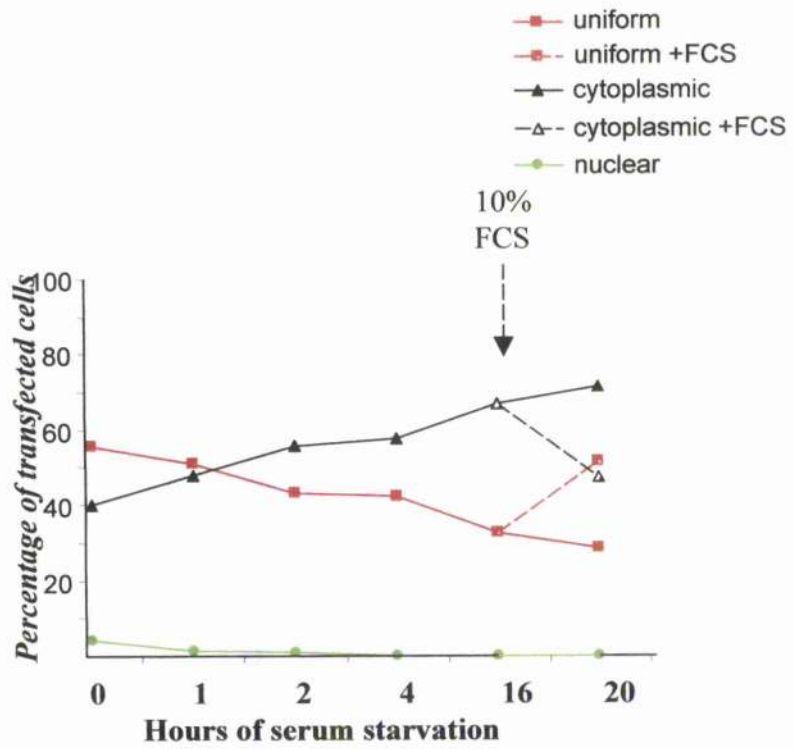
D.



3.4. Time course of the effect of serum deprivation on the subcellular localisation of JMY

24 hours following transfection with pCMV-HAJMY, U2OS cells were incubated in 0.2% serum media for 1, 2, 4, and 16 hours. After 16 hours, the cells were maintained in low serum media (solid lines), or incubated in serum supplemented with 10% FCS (dotted lines) for a further 4 hours. The subcellular localisation of JMY was examined by indirect immunofluorescence with the anti-HA antibody Y-11 and a red fluorescent secondary antibody. The graph represents the average of three experiments.

HJMY



3.5. Regulation of the localisation of JMY by nuclear export

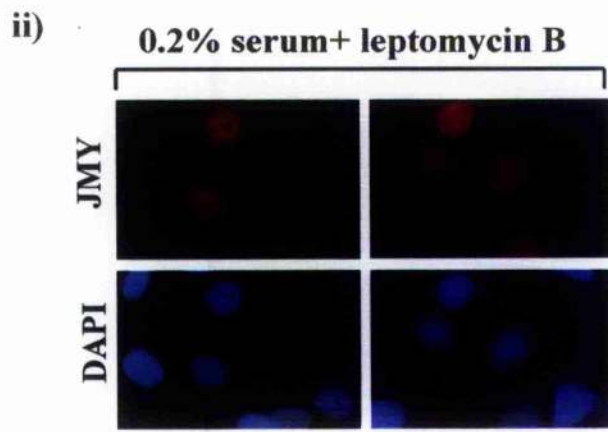
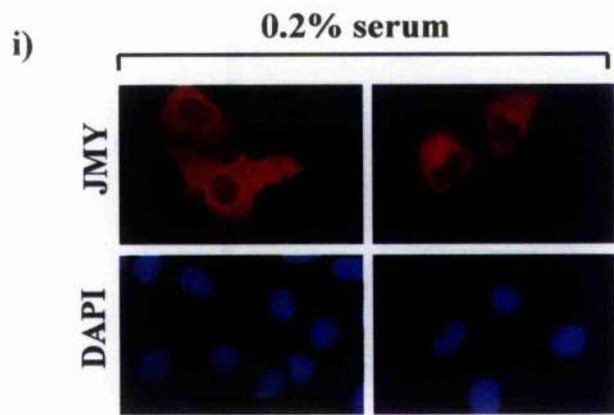
A.(i) Serum starved cells

Twenty four hours following transfection with 2 μ g of pCMV-HAJMY, U2OS cells were incubated for 16 hours in media with 0.2% FCS. JMY was detected by immunofluorescence with an anti HA antibody (Y-11) and a red fluorescent secondary antibody. The position of nuclei was determined using DAPI stain.

(ii) Leptomycin B inhibits JMY's cytoplasmic relocation

7
Cells transfected and serum starved as described above. Five hours prior to fixation, the nuclear export inhibitor leptomycin B was added at a final concentration of 1 μ M. JMY was detected with the anti-HA Y-11 antibody and a red fluorescent secondary antibody.

A.



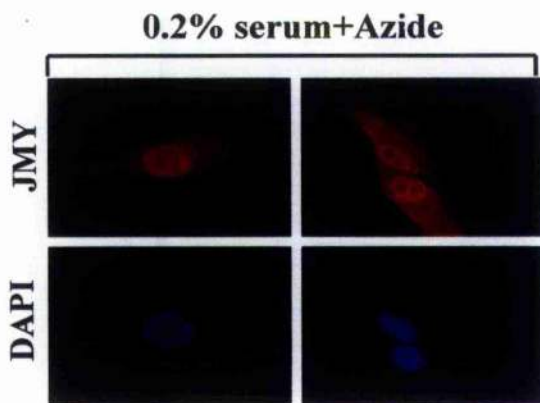
(iii). Inhibition of nuclear export with sodium azide inhibits the cytoplasmic relocalisation of JMY

U2OS cells were transfected with 2 μ g of pCMV-HAJMY and, 24 hours after transfection, were serum starved for 16 hours. Seven hours before fixation, sodium azide was added to the low serum media at a final concentration of 10 mM. JMY was detected with the anti-HA Y-11 antibody and a red fluorescent secondary antibody.

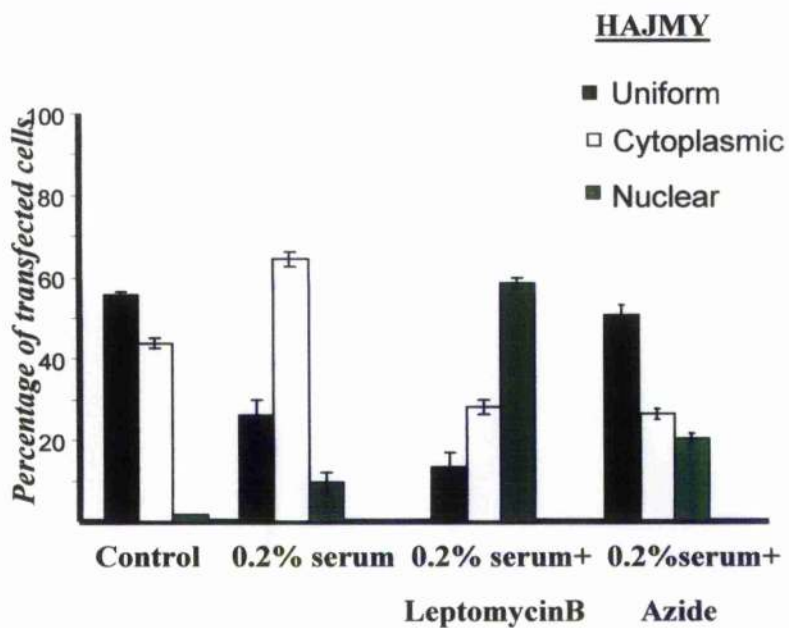
B. Quantification of the effect of leptomycin B and sodium azide on the localisation of JMY in serum starved cells

The data were quantified by scoring 100-150 cells with respect to the subcellular localisation of JMY. The data represent average values from 3 separate experiments.

iii.



B.



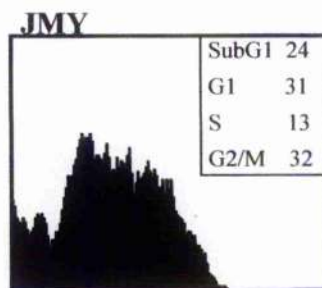
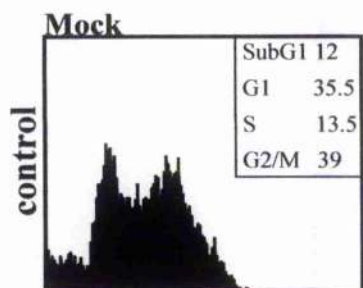
3.6. Cell cycle effects of JMY in response to UV and serum withdrawal

A. U2OS cells were transfected with 8 μ g of the cell surface expression marker CD20 together with 5 μ g pcDNA3 empty vector, or with 5 μ g pCMV-HAJMY. Transfected cells grown in 10% FCS were identified by staining with the anti-CD20 fluorescein-conjugated antibody and their DNA content observed using the DNA inter-chelater propidium iodide. Cell cycle profiles were examined with the Cell Quest Pro software.

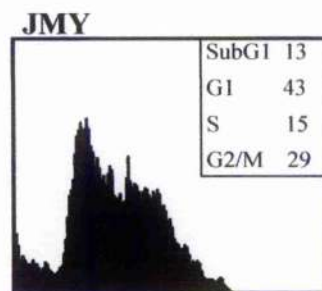
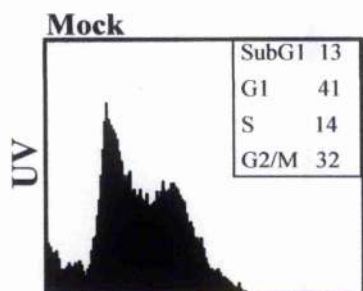
B. U2OS cells were transfected as described in (A). Twenty four hours following transfection, cells were incubated in low serum media (0.2% FCS) for 16 hours before examination of cell cycle profiles with the Cell Quest Pro software.

C. U2OS cells were transfected as described in (A). Thirty hours post-transfection, cells were exposed to UV (60 J/m²) and were incubated for 6 hours prior to cell cycle analysis.

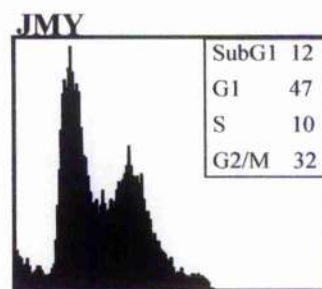
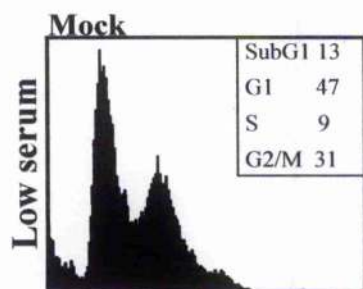
A.



B.



C.

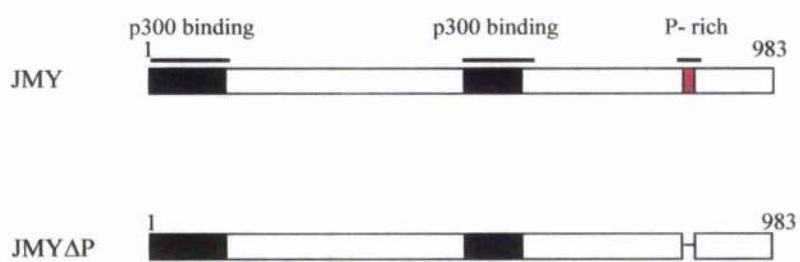


3.7. The proline rich region of JMY is not necessary for nuclear export in response to serum withdrawal

A. Schematic representation of JMY and JMY Δ P

The p300 binding domains (black) and the proline rich region (red) are shown. P-rich, proline rich region.

A.



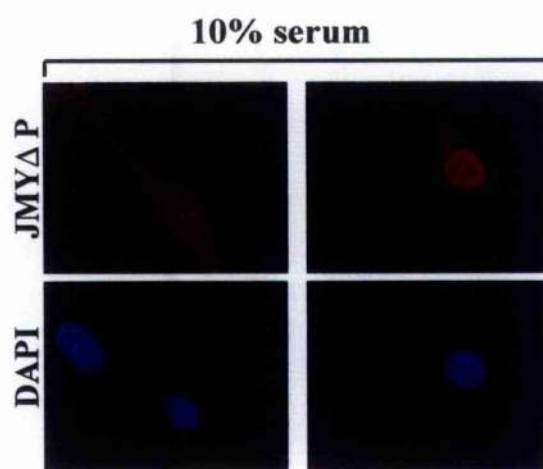
B. Subcellular localisation of JMY ΔP in unstressed cells

U2OS cells were transfected with 2 μ g of pCMV- HA-JMY ΔP . JMY ΔP was detected by indirect immunofluorescence with the anti-HA antibody Y-11 and a red fluorescent secondary antibody.

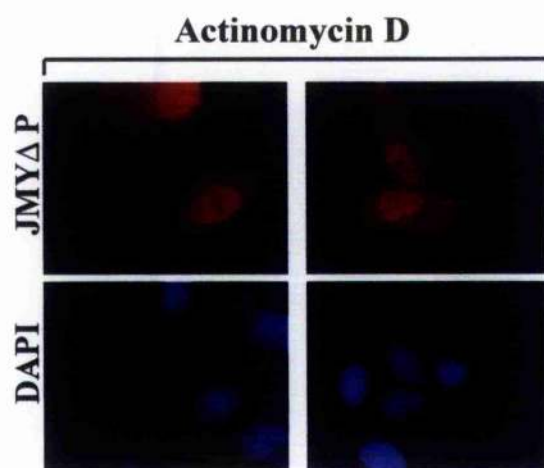
C. Subcellular localisation of JMY ΔP in actinomycin D treated cells

U2OS cells were transfected with 2 μ g pCMV-HAJMY ΔP . Twenty four hours following transfection, the cells were incubated for 16 hours in actinomycin D-containing media (20nM) before being fixed and examined for the subcellular localisation of JMY. JMY was detected with the anti-HA Y-11 antibody and a red fluorescent secondary antibody. The nuclear stain DAPI was used to determine nuclei position.

B.



C.



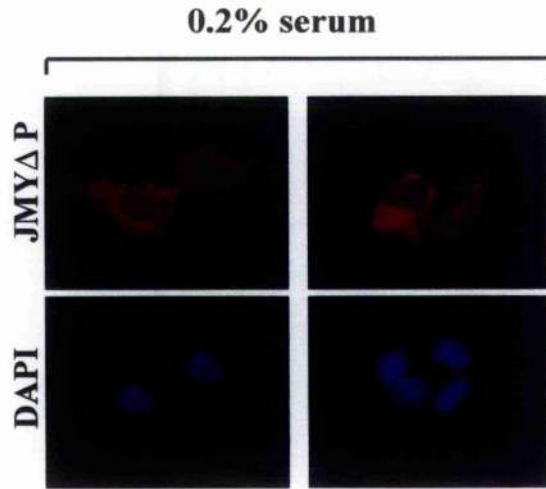
D. Subcellular localisation of JMY ΔP serum-starved cells

Twenty four hours after transfection with pCMV-HAJMY ΔP , U2OS cells were incubated for 16 hours in media supplemented with 0.2% foetal calf serum. JMY ΔP was detected by indirect immunofluorescence with the anti-HA antibody Y-11 and a red fluorescent secondary antibody.

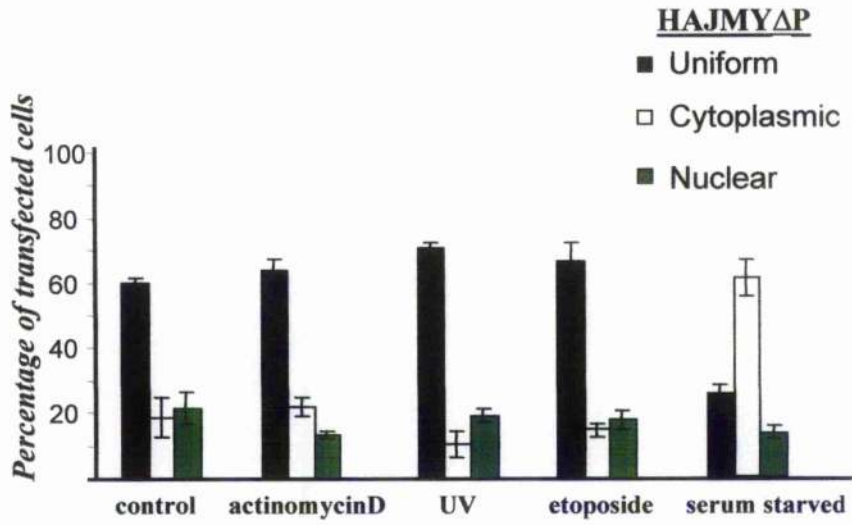
E. Quantification of the effect of serum starvation, and DNA damage on the subcellular localisation of HAJMY ΔP

Cells overexpressing HAJMY ΔP were treated with actinomycin D (20nM for 16 hours), UV (60 J/m² and fixed following 6 hours), or etoposide (10 μ M for 7 hours). The data were quantified by scoring 150 cells with respect to the intracellular localisation of JMY ΔP . The data represent average values from 3 separate experiments.

D.



E.



Chapter 4. Hdm2 regulates the subcellular localisation of

JMY

4-1. Introduction

Nuclear localisation is essential for the tumour suppressor activity of p53 as evidenced by the impaired response to stress signals in certain tumours where cytoplasmic sequestration of p53 occurs (Stommel *et al.*, 1999; Moll *et al.*, 1992; 1995). A considerable body of evidence has shown that p53 activity is controlled partly by regulation of its localisation within the cell, and the molecular mechanisms involved have been intensively studied.

The oncoprotein Mdm2 enhances the cytoplasmic relocalisation of p53. This function of Mdm2 is dependent on its ability to interact with p53, and to promote its ubiquitination. The importance of Mdm2-directed ubiquitination in p53 export is highlighted by studies in which loss of Mdm2-mediated ubiquitination of p53 blocks p53 export (Boyd *et al.*, 2000, Geyer *et al.*, 2000, Lohrum *et al.*, 2001).

The data presented in the previous chapter suggest that the subcellular distribution of JMY is regulated. Considering that both JMY and p53 are regulated at the level of intracellular localisation, and that Mdm2 has nuclear export properties, it was of interest to investigate whether Mdm2 could also affect the localisation of JMY within the cell.

4-2. Hdm2 promotes the nuclear export of JMY

To test the hypothesis that Mdm2 regulates the nuclear export of JMY, the effect of its co-expression on the intracellular localisation of JMY was assessed.

U2OS cells were transfected with the HAJMY expression vector pCMV-HAJMY, together with an empty vector, or a plasmid encoding the human homologue of Mdm2, Hdm2 (pCHDM1A).

The data were quantified by scoring the cells into three categories based on whether HAJMY was cytoplasmic, nuclear, or detected in both compartments (referred to as uniform). The majority of cells displayed nuclear Hdm2 and only these were included in the study.

In the absence of exogenous Hdm2, HAJMY localised to both the nucleus and the cytoplasm, as well as to the cytoplasm in a proportion of cells (Figures 4.1.A(i) and 4.1.C). When Hdm2 was co-expressed with HAJMY, an increase in cytoplasmic HAJMY (44% to 77%), and decrease in uniform (54% to 23% of transfected cells) and nuclear (1.5% to 0% of transfected cells) HAJMY staining were observed (Figures 4.1.A (ii) and C).

To determine whether the effect of Hdm2 on HAJMY was the result of nuclear export, HAJMY and Hdm2 were co-expressed in the presence of the nuclear export inhibitor leptomycin B. Leptomycin B reversed the effect of Hdm2 on HAJMY, increasing its nuclear localisation and confirming that the relocalisation of JMY in the presence of elevated levels of Hdm2 involves CRM1-dependent export (Figures 4.1.B, C).

A possible explanation for the cytoplasmic relocalisation of JMY by Hdm2 is that, being part of the p53 complex, JMY is being relocalised as an indirect result of Hdm2-mediated nuclear export of p53. 07

To test this hypothesis, the effect of Hdm2 on the localisation of HAJMY was assessed in the *p53*-null cell line SAOS2. Hdm2 caused the cytoplasmic relocalisation of HAJMY in these cells in a similar way to that observed in U2OS cells, suggesting that p53 is not necessary for JMY relocalisation (Figures 4.1.D and E).

4-3. Hdm2 does not mediate the degradation of JMY

In addition to promoting p53 export, Mdm2/Hdm2 also targets it for proteasome-dependent degradation (Haupt *et al.*, 1997; Kubbutat *et al.*, 1997). Given that Hdm2 can enhance the cytoplasmic localisation of JMY, it was of interest to determine whether it can also promote its degradation in a similar manner to p53.

To this end, U2OS cells were transfected with pCMV-HAJMY and an empty vector (Figure 4.2. lane 1) or increasing amounts of the Hdm2 expressing plasmid pCHDM1A (lanes 2 and 3). The levels of endogenous p53 were examined as a positive control.

Hdm2 overexpression did not result in degradation of JMY and JMY levels remained stable as opposed to p53 levels, which were efficiently reduced by Hdm2 (Figure 4.2). Therefore, Hdm2 does not affect the protein levels of JMY.

4-4. Effect of Hdm2 on JMY localisation is abolished following DNA damage

As UV and actinomycin D-induced DNA damage increased the nuclear localisation of JMY, it was of interest to see how Hdm2 could affect the ability of JMY to move into the nucleus following DNA damage.

U2OS cells over-expressing HAJMY and Hdm2 were treated with actinomycin D (20 nM) for 16 hours, or exposed to UV (60J/m²) and fixed 6 hours later. The subcellular localisation of both HAJMY and Hdm2 were examined under these conditions and in the absence of stress.

Both treatments attenuated the effect of Hdm2 on HAJMY localisation as indicated by the decrease in the percentage of cells exhibiting cytoplasmic staining (from 77% in untreated cells to 30% in actinomycin D-treated, and 17% in and UV-treated cells), and the increase in uniform and nuclear-staining cells compared to untreated cells (Figures 4.3. A-C).

4-5. The proline rich region of JMY is necessary for export by Hdm2

The subcellular localisation of JMY Δ P is regulated in a similar fashion to JMY under conditions of serum starvation, but not in the presence of DNA damage. Considering that both isoforms can be regulated by nuclear export, it was speculated that Hdm2 could affect the localisation of JMY Δ P in a similar manner to wild type JMY.

To examine the effect of Hdm2 on JMY Δ P, U2OS cells were transfected with a vector for HAJMY Δ P expression, pCMV-HAJMY Δ P, together with an empty vector or with the Hdm2 expression vector pCHDM1A. The localisation of Hdm2 and HAJMY Δ P was examined by indirect immunofluorescence as before, and cells were scored with respect to the localisation of HAJMY Δ P.

In the absence of overexpressed Hdm2, HAJMY Δ P exhibited both nuclear and cytoplasmic staining in the majority of cells, with a lower percentage of cells with exclusively cytoplasmic, or nuclear staining (figures 4.4.A(i) and B).

Hdm2 overexpression did not significantly alter this profile, indicating that Hdm2 fails to relocalise the Δ P isoform of JMY (4.4.A (ii) and B).

4-6. Mapping of the functional domains in Hdm2 that are required for JMY export

Since the ability of Hdm2 to export p53 requires intact p53 binding and ring finger domains (Boyd *et al.*, 2000, Geyer *et al.*, 2000), it was of interest to assess the possibility that these domains are also involved in the cytoplasmic relocalisation of JMY.

A panel of Hdm2 mutants with deficient p53-export functions was used to address this question (Figure 4.5.A). The Hdm2 mutants 1-440 and C464A have deficient ubiquitin ligase domains generated by a deletion, and a single residue substitution, respectively. Hdm2 Δ 58-89 contains a deletion in the p53 binding domain, and Hdm2 Δ 58-89;C464A, has the same deletion as well as a cysteine to

alanine substitution in a zinc coordinating residue in the ubiquitin ligase domain (Figure 4.5.A).

The effect of these mutants on the sub-cellular localisation of HAJMY was assessed by immunofluorescence in U2OS cells (summarised in figure 4.5.F).

In contrast to wild type Hdm2, overexpression of the ring finger mutants Hdm2 C464A and Hdm2 1-440 did not increase the population of cells with exclusively cytoplasmic HAJMY. Interestingly, these mutants allowed the nuclear accumulation of HAJMY in a small population of cells (figures 4.5.B, C, F).

The proportion of cells with exclusively cytoplasmic HAJMY increased (44% to 68%) following overexpression of Hdm2 Δ 58-89 in a similar fashion to wild type Hdm2 (figure 4.5.D, F), confirming that p53 is dispensable for Hdm2-driven JMY export. However, additional mutation of the ring finger domain in Hdm2 Δ 58-89; C464A abolished the cytoplasmic relocalisation of HAJMY as indicated by the increase in nuclear and uniform staining of HAJMY, and the decrease in cytoplasmic localisation (Figure 4.4.E) relative to Hdm2 Δ 58-89 (27% of transfected cells with exclusively cytoplasmic JMY compared to 68% following overexpression of Hdm2 Δ 58-89).

Taken together, these results show that, in a similar fashion to p53, an intact ring finger is necessary for Hdm2 to promote the cytoplasmic translocation of JMY. In contrast, the N-terminal region involved in p53 binding is only required for p53 export but not for the relocalisation of JMY.

4-7. Conclusions

The ability of Hdm2 to regulate the intracellular distribution of JMY was investigated. The data presented suggest that overexpression of Hdm2 increases the cytoplasmic localisation of exogenous JMY in a leptomycin B-sensitive manner suggesting a nuclear export mechanism. This effect of Hdm2 is independent of p53 since it was observed in a p53-null context in SAOS2 cells. Examination of JMY levels in the presence of elevated levels of Hdm2 indicates that, in contrast to p53, Hdm2 does not target JMY for degradation.

Actinomycin D and UV-induced DNA damage was shown to increase the nuclear localisation of JMY. This property of JMY is not affected by Hdm2 since Hdm2-mediated relocalisation of JMY was compromised under these conditions. This suggests that the effect of Hdm2 on JMY is regulated by cellular conditions.

Hdm2 failed to promote the cytoplasmic relocalisation the JMY isoform JMY Δ P, indicating that the C-terminal proline-rich region of JMY is essential for its relocalisation by Hdm2.

Moreover, examination of the domains involved in Hdm2-directed nuclear export of JMY revealed the importance of the ring finger of Hdm2. Deletion, or point mutation of this domain abrogated its ability to export JMY. In contrast, deletion of the p53 binding domain did not affect the ability of Hdm2 to enhance the

cytoplasmic localisation of JMY, further confirming that p53 is not required for this function.

4.1. HDM2 affects the subcellular localisation of JMY

A.(i) JMY in U2OS cells

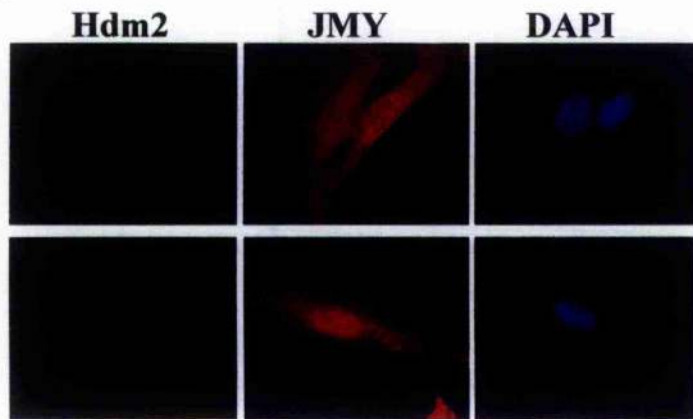
U2OS cells were transfected with 3 μ g of pCMV-HAJMY and 9 μ g pcDNA3 empty vector. The subcellular localisation of JMY was determined by indirect immunofluorescence 30-36 hours post-transfection. JMY was detected with the anti-HA antibody Y-11 and a red fluorescent secondary antibody. Nuclei were visualised with the nuclear stain DAPI.

(ii) Hdm2 enhances the cytoplasmic localisation of JMY

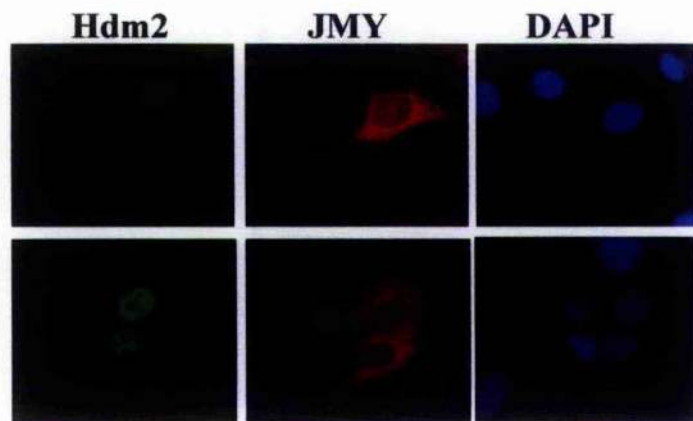
U2OS cells were transfected with 3 μ g of pCMV-HAJMY and 9 μ g of the Hdm2 expression vector pCHDMIA. The subcellular localisation of JMY and Hdm2 was determined by indirect immunofluorescence 30-36 hours following transfection. JMY was detected with the anti-HA antibody (Y-11) and red fluorescent secondary antibody. Hdm2 was detected with the monoclonal antibody Ab-1 and a green fluorescent secondary antibody. DAPI staining shows the position of nuclei.

A.

(i)



(ii)



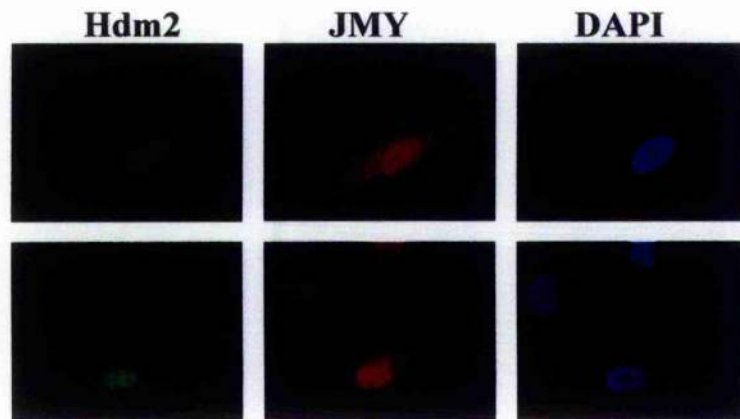
B. Leptomycin B blocks the relocalisation of JMY by Hdm2

U2OS cells were transfected with 3 μ g of pCMV-HAJMY and 9 μ g of the Hdm2 expression vector pCHDM1A, and the subcellular localisation of JMY and Hdm2 was determined 36 hours following transfection. Five hours prior to fixation, the nuclear export inhibitor leptomycinB was added at a final concentration of 1 μ M. JMY and Hdm2 were visualised by immunostaining as described in (A).

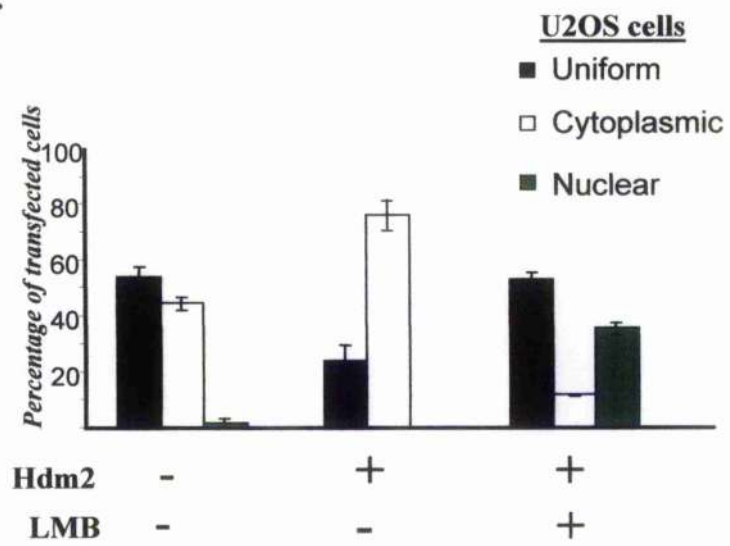
C. Quantification of the effect of Hdm2 on the subcellular localisation of JMY

100-150 cells were scored with respect to the subcellular localisation of JMY. The data represent average values from 3 separate experiments. Only cells with nuclear Hdm2 staining (which represent the majority of cells) were included.

B.



C.



D. Hdm2 promotes the cytoplasmic relocalisation of JMY in SAOS2 cells

(i) JMY in SAOS2 cells

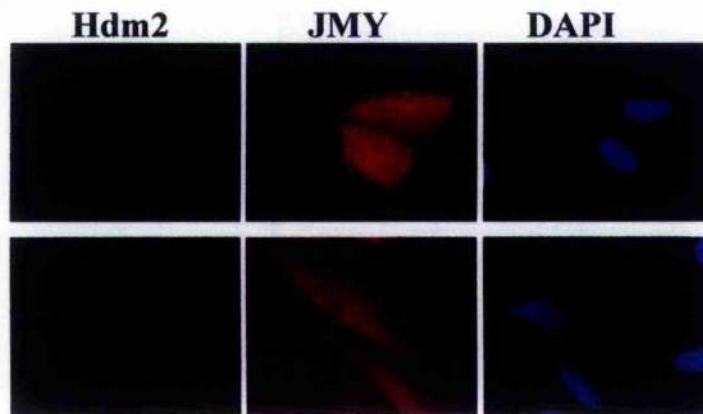
SAOS2 cells were transfected with 3 μ g of pCMV-HAJMY and 9 μ g pcDNA3 empty vector. The subcellular localisation of JMY was determined by indirect immunofluorescence 30-36 hours post-transfection. JMY was detected using an anti HA antibody (Y-11) and a red fluorescent secondary antibody.

(ii) Hdm2 enhances the cytoplasmic localisation of JMY in SAOS2 cells

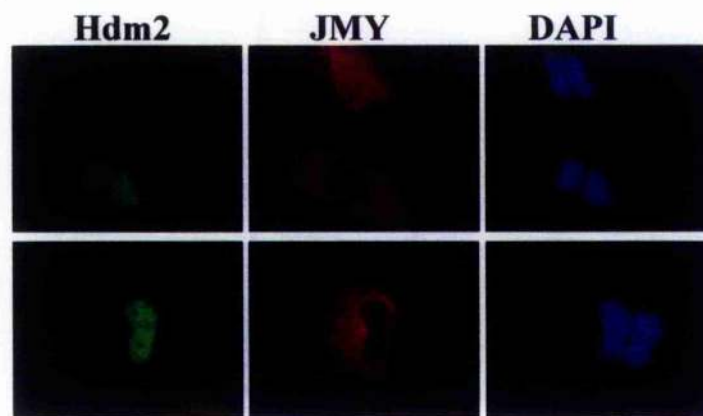
SAOS2 cells were transfected with 3 μ g of pCMV-HAJMY and 9 μ g of the Hdm2 expression vector pCHDM1A. Thirty six hours following transfection, JMY was detected using the polyclonal anti-HA antibody Y-11 and a red fluorescein-conjugated antibody. Hdm2 was detected with the monoclonal antibody Ab-1 and a green fluorescent secondary antibody. DAPI staining shows the position of nuclei

D.

(i)



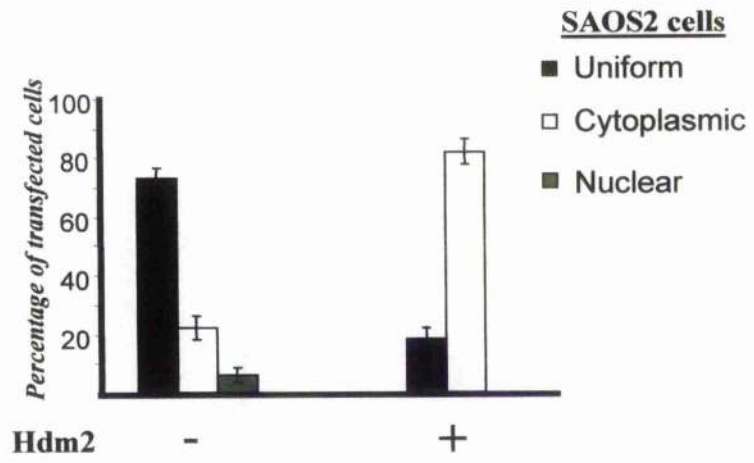
(ii)



E. Quantification of the cytoplasmic redistribution of JMY in Hdm2-overexpressing SAOS2 cells

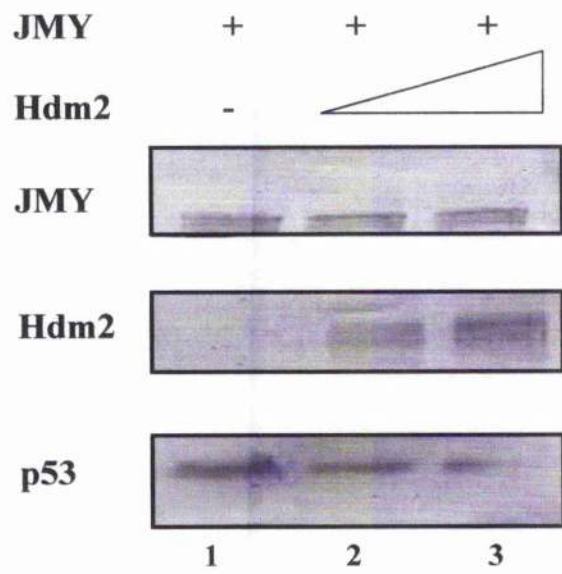
70-100 cells were scored with respect to the subcellular localisation of JMY. The data represent average values from 3 separate experiments and only include data from cells with nuclear Hdm2 staining (which represent the majority of cells).

E.



4.2. Hdm2 does not promote JMY degradation

U2OS cells were transfected with 1 μ g of pCMV-HAJMY and 6 μ g of an empty vector (lane 1), or 3 and 6 μ g Hdm2 expression vector (lanes 2 and 3; respectively). The levels of JMY were examined with a mouse anti-HA antibody (HA11). Hdm2 was detected with the monoclonal antibody Ab-1. p53 levels were examined as a positive control, and p53 was detected with the monoclonal antibody DO-1.



4.3. DNA damage reduces the effect of Hdm2 on the subcellular distribution of JMY

A. Actinomycin D treatment

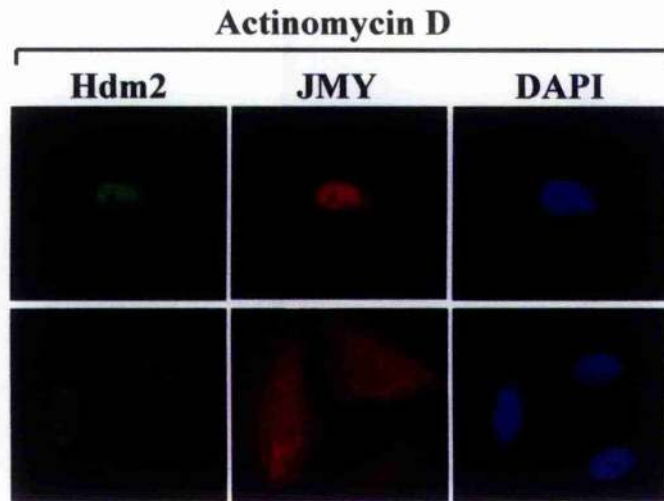
U2OS cells were transfected with 3 μ g of pCMV-HAJMY and 9 μ g of pCHDMIA. Twenty four hours following transfection the cells were treated with 20nM actinomycin D for 16hrs.

The subcellular localisation of JMY was visualised by indirect immunofluorescence with the anti-HA antibody Y-11 and a red fluorescent secondary antibody. Hdm2 was detected with the monoclonal antibody Ab-1 and a green fluorescent secondary antibody. DAPI staining shows the position of nuclei.

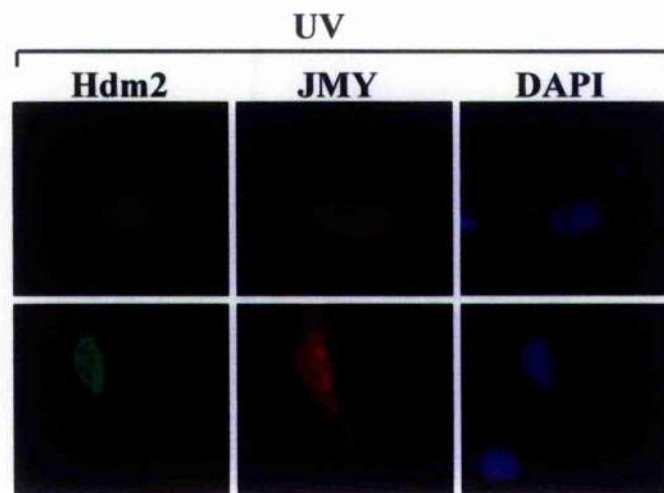
B. UV treatment

Cells were transfected with 3 and 9 μ g of pCMV-HAJMY and pCHDMIA, respectively. Thirty hours following transfection, cells were exposed to UV (60J/m²) and fixed 6 hours later. JMY and Hdm2 were detected with the Y-11 and Ab-1 antibodies as described in (A).

A.



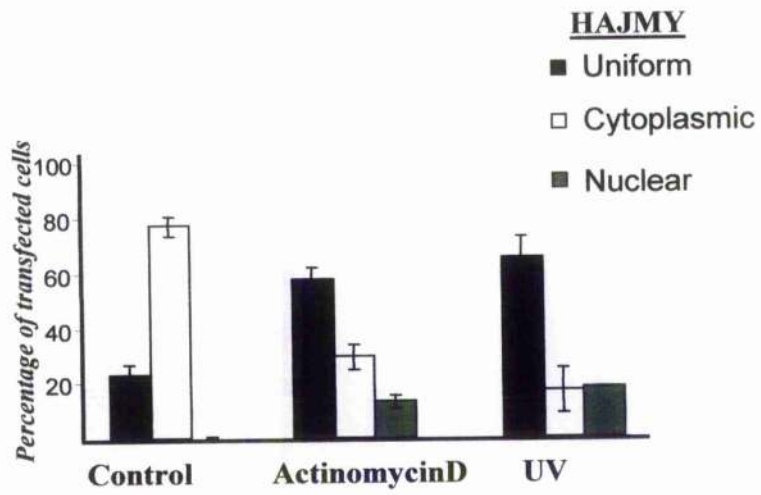
B.



C. DNA damage reduces the relocalisation of JMY by Hdm2

70-100 cells were scored with respect to the subcellular localisation of JMY. The data represent average values from 3 separate experiments. Only cells with nuclear Hdm2 staining (representing the majority of cells) were included in the analysis.

C.



4.4. Hdm2 does not promote the cytoplasmic translocation of

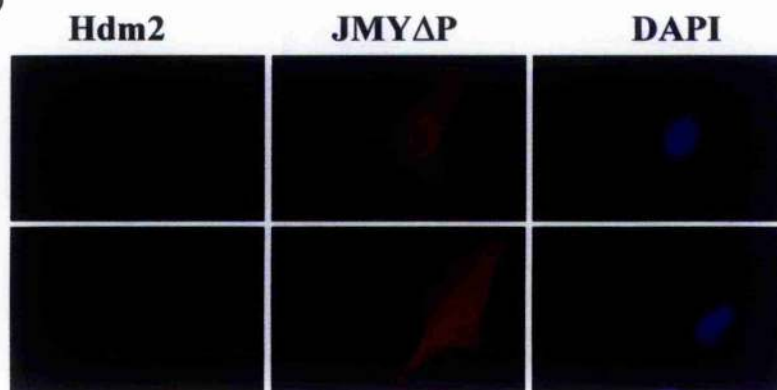
JMY ΔP

A (i). U2OS cells were transfected with 3 μ g of pCMV-HAJMY ΔP and 9 μ g pcDNA3 empty vector. The subcellular localisation of JMY ΔP was determined by indirect immunofluorescence using an anti HA antibody (Y-11) and a red fluorescent secondary antibody. Nuclei were visualised with the nuclear stain DAPI.

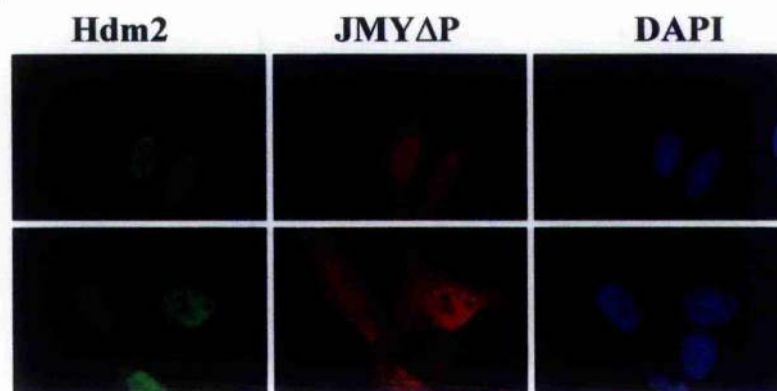
(ii). U2OS cells were transfected with 3 μ g of pCMV-HAJMY ΔP and 9 μ g of the Hdm2 expression vector pCHDM1A. The subcellular localisation of JMY ΔP was visualised by indirect immunofluorescence with the polyclonal anti-HA antibody Y-11 and a red fluorescent secondary antibody. Hdm2 was detected with the monoclonal antibody Ab-1 and a green fluorescent secondary antibody. DAPI staining shows the position of nuclei.

A.

(i)



(ii)



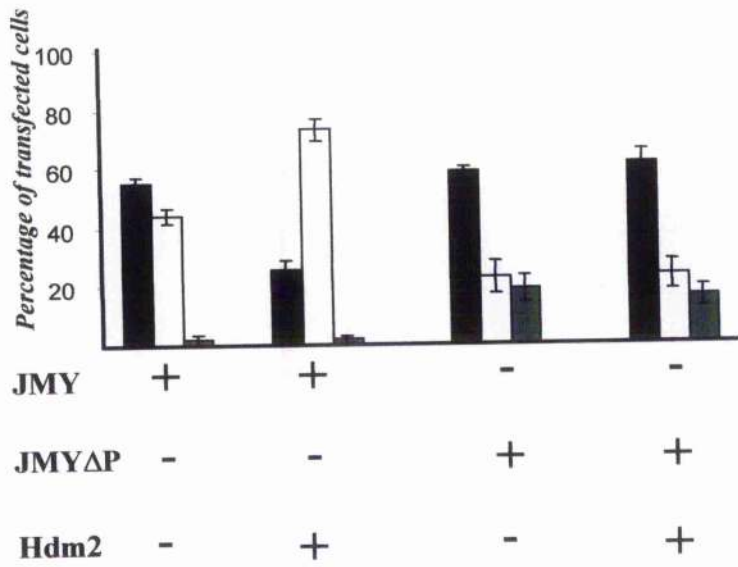
***B. Comparison of the effect of Hdm2 on the subcellular
relocalisation of JMY and JMY ΔP***

100-150 cells were scored with respect to the subcellular localisation of JMY and JMY ΔP in the presence of elevated levels of Hdm2. The data represent average values from 3 separate experiments.

B.

HA-JMY/JMY Δ P

- Uniform
- Cytoplasmic
- Nuclear

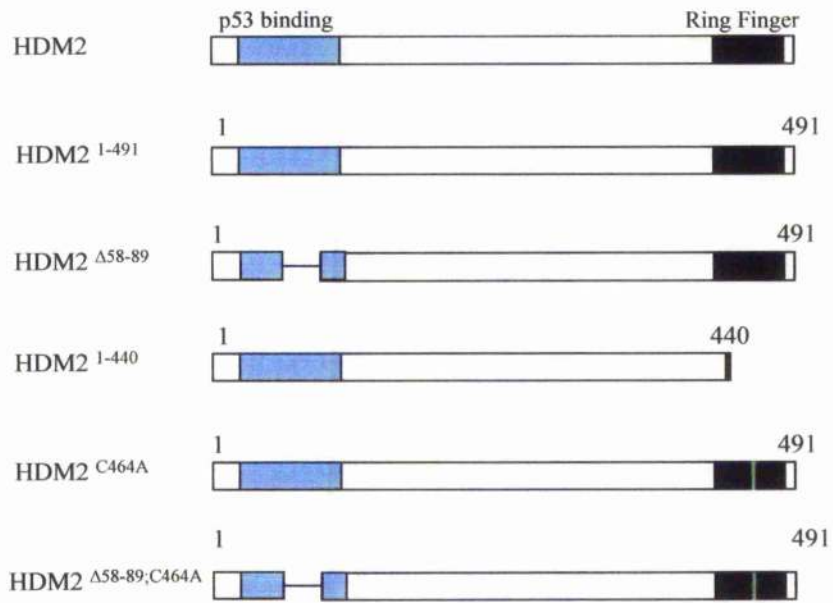


4.5. Mapping of the domains in Hdm2 that are required for JMY export

A. Diagram illustrating the Hdm2 mutants used

The p53 binding domain is shown in blue. JMY Δ 58-89 lacks a region that is essential for binding to p53. The mutants, 1-440, C464A and Δ 58-89;C464A all have deficient ligase activity due to ring finger deletion (1-440) or substitution in a critical, Zinc co-ordinating cysteine (C464A).

A.



B. Deletion of the ring finger in Hdm2 1-440 abolishes its effect on the localisation of JMY

U2OS cells were transfected with 3 μ g of pCMV-HAJMY and 6 μ g of pCHDM 1-440. The subcellular localisation of JMY was visualised by indirect immunofluorescence with the anti-HA antibody Y-11 and a red fluorescent secondary antibody.

Hdm2 1-440 was detected with the monoclonal antibody Ab-1 and a green fluorescent secondary antibody. The DAPI staining shows the position of nuclei.

C. Hdm2 C464A is unable to promote the nuclear export of JMY

The localisation of JMY was examined in U2OS cells transfected with the expression vectors pCMV-HAJMY (3 μ g) and pCHDM 464 Ala (6 μ g). Y-11 and a red fluorescent secondary antibody were used to detect HAJMY while Hdm2 C464A detection was with Ab-1 and a green fluorescent.

B.



C.



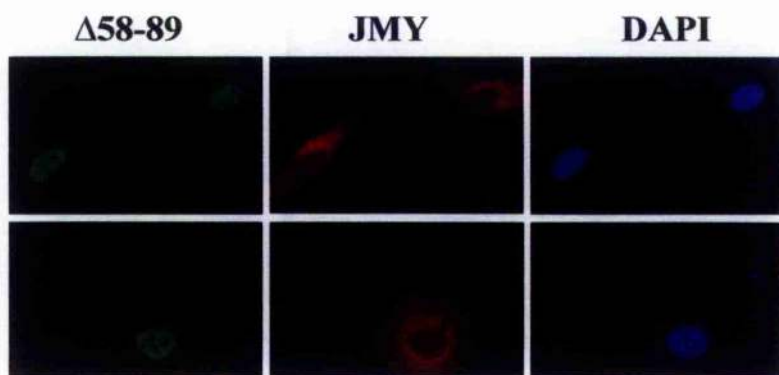
D. Hdm2 Δ 58-89 enhances the cytoplasmic localisation of JMY

U2OS cells were transfected with 3 μ g of pCMV-HAJMY and 9 μ g of pCHDM Δ 58-89. Thirty six hours following transfection, JMY was detected with Y-11 and a red fluorescent secondary antibody; and Hdm2 Δ 58-89 was detected with the monoclonal antibody Ab-1 and a green fluorescent secondary antibody.

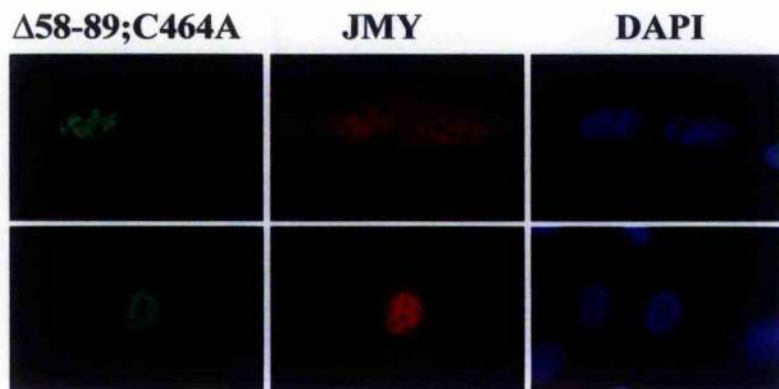
E. Mutation of the ring finger in Hdm2 Δ 58-89 abolishes the relocalisation of JMY into the cytoplasm

U2OS cells were transfected with 3 μ g of pCMV-HAJMY and 6 μ g of an expression vector for the double mutant Hdm2 Δ 58-89;C464A (pCHDM Δ 58-89;C464A). Thirty six hours following transfection, JMY was detected with Y-11 and a red fluorescent secondary antibody; and Hdm2 Δ 58-89;C464A was detected with the monoclonal antibody Ab-1 and a green fluorescent secondary antibody.

D.



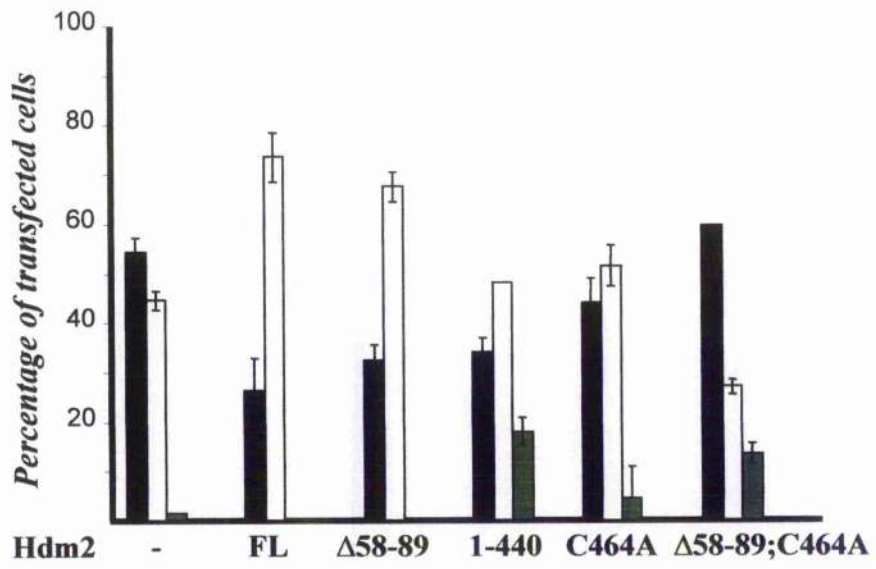
E.



F. Quantification of the effect of Hdm2 mutation on the subcellular localisation of JMY

100-150 cells were scored with respect to the subcellular localisation of JMY. The data represent average values from at least 4 separate experiments. Only cells with nuclear Hdm2 staining (which represent the majority of cells) were included.

F.



Chapter 5. Interaction of JMY and Hdm2 in cells

5-1. Introduction

The oncogenic potential of Mdm2 is largely attributed to its ability to tightly regulate the activity of the p53 tumour suppressor (Momand *et al.*, 2000). However, increasing evidence for p53-independent functions of Hdm2 suggests that alternative roles may also contribute to the oncogenic properties of Hdm2 (Daujat *et al.*, 2001).

The list of Mdm2 interacting partners includes cell cycle regulators such as Rb and E2F1 (Martin *et al.*, 1995; Xiao *et al.*, 1995, Hsich *et al.*, 1999), the tumour suppressor PML (Wu *et al.*, 2003), ribosomal proteins (Marechal *et al.*, 1994), and tissue /or cell type factors such as Numb (Juven-Gershon *et al.*, 1998; Yogosawa *et al.*, 2003) and Myo D (Fiddler *et al.*, 1996). Nevertheless, the physiological significance of the majority of these interactions is yet to be elucidated, and may point to the involvement of Mdm2 in the regulation of cell proliferation and /or differentiation.

I have shown above that Hdm2 can affect the intracellular distribution of JMY in the absence of stress but not under conditions of DNA damage. This implied the existence of a functional interaction between the two proteins, and prompted the analysis of the physical interaction between JMY and Hdm2 and its potential regulation by cellular conditions.

5-2. JMY and Hdm2 interact in cells

To investigate whether JMY and Hdm2 can form a complex, HA-tagged JMY and Hdm2 were overexpressed in U2OS cells, and the cell extracts were immunoprecipitated using a monoclonal anti- HA antibody (Figure 5.2.A lane 2), or a monoclonal antibody against Mdm2 (Ab-1) (Figure 5.1.A. lane 3). Western blot analysis demonstrated the presence of Hdm2 in JMY immunoprecipitates. Endogenous p53 also co-immunoprecipitated with HAJMY, confirming the previous results that JMY associates with the p53 complex (Shikama *et al.*, 1999).

The complementary analysis of immunoprecipitation with an Hdm2 antibody showed that, in contrast to endogenous p53, which was efficiently co-immunoprecipitated with Hdm2, JMY was not detectable in these immunoprecipitates (Figure 5.1.A lane 3, and Figure 5.1.B lane 2). A possible explanation is that the Hdm2 antibody Ab-1 interferes with the JMY-Hdm2 interaction. To test this possibility, the experiment was repeated using the SMP14 anti- Mdm2 antibody, which recognises a different epitope from Ab-1 (Ab-1 recognises an epitope between residues 26-150, and the epitope for SMP14 is between amino acids 154-167). Both JMY and endogenous p53 were detected in the SMP14 Hdm2 immunoprecipitation products (Figure 5.1.B. lane 3), but not in immunoprecipitates of an irrelevant antibody (Figure 5.1.B. lane 4) confirming the interaction of JMY and Hdm2 in cells (Figure 5.1.B. lane 3).

Further, the interaction of Hdm2 and the JMY isoform JMY Δ P was investigated to determine whether the inability of Hdm2 to translocate JMY Δ P to the cytoplasm reflects lack of interaction between the two proteins.

HAJMY Δ P and Hdm2 were overexpressed in U2OS cells and the cell extracts were immunoprecipitated with a monoclonal anti-HA antibody, or an irrelevant, anti-Gal4 antibody (Figure 5.1.C). Examination of the HAJMY Δ P immunoprecipitates showed the presence of Hdm2 as well as endogenous p53 (lane 2) whereas the two proteins were not detected in the control immunoprecipitates (lane 3). Therefore, the proline-rich region of JMY is not essential for its ability to interact with Hdm2.

Mutations in the ring finger of Hdm2 abrogated its ability to promote the nuclear export of JMY whereas deletion of the p53 binding domains had no effect. To test whether these phenotypes are the result of differential binding to JMY, HAJMY was coexpressed with wild type Hdm2, the ring finger mutant C464A or the p53 binding mutant Δ 58-89. The interaction was tested by immunoprecipitation using the anti-Mdm2 antibody SMP14 and the presence of endogenous p53 in the immunoprecipitates was examined as a positive control (Figure 5.1.D).

p53 efficiently co-immunoprecipitated with both wild type Hdm2 (lane 2) and Hdm2 C464A (lane 4) but not with Hdm2 Δ 58-89 (lane 6). A small level of p53 is detectable in the Hdm2 Δ 58-89 immunoprecipitates which reflects the interaction of p53 with endogenous Hdm2 in U2OS cells (lane 6).

Examination of the immunoprecipitation products revealed the ability of JMY to interact with Hdm2 Δ 58-89 and Hdm2 C464A as well as with wild type Hdm2. However, the interaction of JMY with Hdm2 C464A is reduced relative to that with wild type Hdm2 and Hdm2 Δ 58-89. This observation may provide a potential explanation for the inability of Hdm2 C464A to promote the cytoplasmic relocalisation of JMY.

5-3. The interaction of JMY and Hdm2 is regulated by cellular conditions

The main known function of Mdm2 is the maintenance of low levels and activity of p53 under normal conditions. A considerable body of evidence supports a role of DNA damage in attenuating the inhibition of p53 by Mdm2, which allows the activation of the tumour suppressor. Several mechanisms have been described that interrupt the p53-Mdm2 regulatory loop, including phosphorylation events that may interrupt or reduce the interaction between the two proteins, or that reduce the ability of Mdm2 to downregulate p53 without affecting their interaction (Inoue *et al.*, 2001; Maya *et al.*, 2001; Alcaron-Vargas and Ronai, 2002).

Since DNA damage also attenuated Hdm2-mediated redistribution of JMY, it was of interest to determine the effect of DNA damage on the JMY-Hdm2 interaction. To this end, Hdm2 and HAJMY were co-expressed in U2OS cells, which were grown under normal conditions (Figure 5.2.A), or in the presence of the DNA damaging drug actinomycin D for 16 hours (Figure 5.2.B). The interaction of

HMJMY and Hdm2 was assessed by immunoprecipitation with a monoclonal anti-HA antibody (HA11) and western blot analysis with the relevant antibodies.

Consistent with the previous results, both Hdm2 and endogenous p53 were co-immunoprecipitated with JMYY under normal conditions. Actinomycin D caused the stabilisation of p53, which was reflected in its increased levels in the JMYY immunoprecipitates. However, although Hdm2 was still detected in the immunoprecipitates, its levels were reduced by approximately two-fold (reduced by 56%) relative to those observed in unstressed cells (compare 5.2.A lane 2 and 5.2.B lane 2). This suggests that the JMYY-Hdm2 interaction is attenuated by actinomycin D treatment, and is consistent with the reduced ability of Hdm2 to stimulate the translocation of HMJMY into the cytoplasm of actinomycin D treated cells.

Next, the effect of serum starvation on the JMYY-Hdm2 interaction was examined. HMJMY and Hdm2-programmed U2OS cells were incubated in low serum media (0.2%) for 16 hours and the cell extracts were immunoprecipitated as above, using a monoclonal anti-HA antibody (Figure 5.2.C). Under these conditions, neither Hdm2 nor endogenous p53 were detected in HMJMY immunoprecipitates. Since low serum conditions cause the nuclear exclusion of JMYY, it is conceivable that under these conditions, JMYY dissociates completely from the p53-Hdm2 complex.

5-4. Conclusions

The data presented in this chapter indicate the ability of JMY and Hdm2 to interact in cells. The N-terminal domain of Hdm2 might be involved in its ability to interact with JMY as suggested by the absence of interaction in immunoprecipitation assays targeting the N-terminus of Hdm2. However, the ability of the p53-binding deficient mutant ($\Delta 58-89$) of Hdm2 to interact with JMY indicates that this region is not necessary for the interaction. Interestingly, mutation of the ubiquitin ligase domain reduces the JMY-Hdm2 interaction, which might explain the inability of this mutant to enhance the cytoplasmic localisation of JMY.

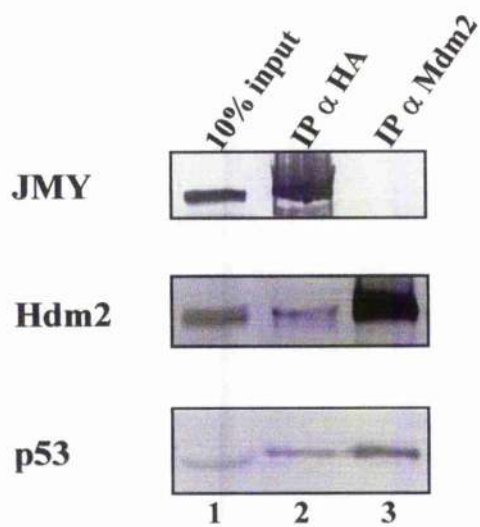
Deletion of the proline rich region in JMY did not affect its ability to interact with Hdm2, indicating that the failure of JMY Δ P to undergo Mdm2-mediated export is not the result of lack of interaction between the proteins. This suggests that the interaction with Hdm2 is not sufficient for cytoplasmic relocalisation of JMY and that additional properties of JMY and/ or additional factors may be involved.

5.1. JMY and Hdm2 interact in cells

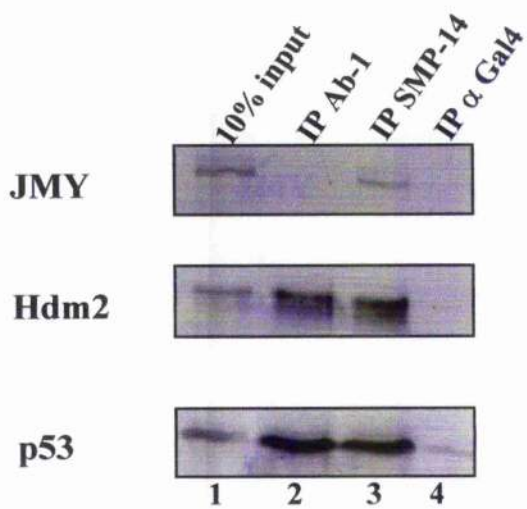
LW
A. U2OS cells were transfected with pCMV-HAJMY(10 μ g) and pCHDMIA (10 μ g), and were harvested 36 hours post-transfection. JMY was immunoprecipitated from cell extracts with the mouse anti-HA antibody HA11 (lane 2); and Hdm2 was immunoprecipitated with the monoclonal Ab-1 antibody (lane 3). JMY was detected with a rabbit anti-HA antibody (Y-11), Hdm2 with the polyclonal antibody H-221 and p53 with sheep anti-p53 serum. The input lane (lane 1) represents 10% of the cellular extract.

B. U2OS cells were transfected as described in (A) and the cell extracts were subject to immunoprecipitation with the anti-Mdm2 antibodies Ab-1 (lane 2), or SMP14 (lane 3). Monoclonal anti-Gal4 antibody was used as a control for specificity (lane 4). JMY was detected with a rabbit anti-HA antibody (Y-11), Hdm2 with the polyclonal antibody H-221 and p53 with sheep anti-p53 serum. 10% of the extract was loaded as input (lane 1).

A.



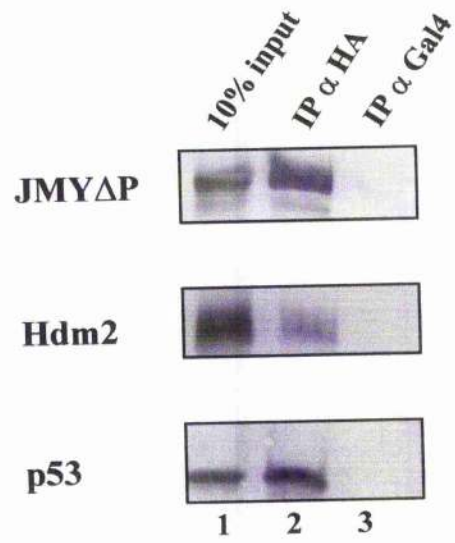
B.



C. JMY Δ P interacts with Hdm2

U2OS cells were transfected with 10 μ g of pCMV-HAJMY Δ P and 10 μ g pCHDM1A, and harvested 36 hours after transfection. Cell extracts were immunoprecipitated with monoclonal anti-HA (lane 2), or with monoclonal anti-Gal4 antibody (lane 3). The immunoprecipitates were analysed for Hdm2 and p53 binding. JMY Δ P was detected with a rabbit anti-HA antibody (Y-11), Hdm2 with the polyclonal antibody H-221 and p53 with sheep anti-p53 serum. The input lane (lane 1) represents 10% of the cellular extract.

C.



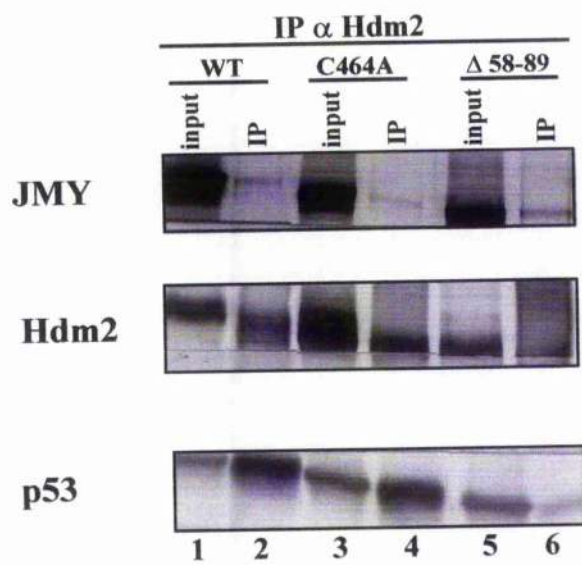
D. JMY interacts with p53 binding and ring finger mutants of

Hdm2

U2OS cells were transfected with 10 µg pCMV-HAJMY and 10 µg of pCHDM1A (lanes 1 and 2), pCHDM^{464A} (lanes 3 and 4) and pCHDM Δ58-89 (lanes 5 and 6). Thirty six hours post-transfection, cell extracts were immunoprecipitated with the anti-Mdm2 SMP14 antibody (lanes 2, 4, 6). The immunoprecipitates were analysed for the presence of p53 and HAJMY with shecp anti-p53, and rabbit anti JMY (1289) sera, respectively. Hdm2 was detected with the polyclonal H-221 antibody.

L² up

D.



5.2. The JMY-Hdm2 interaction is regulated by cellular conditions

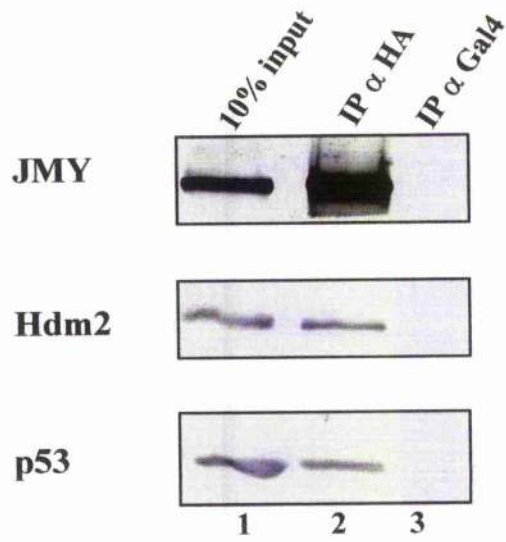
A. In unstressed cells

U2OS cells were transfected with pCMV-HAJMY(10 μ g) and pCHDMIA (10 μ g). The cell extracts were immunoprecipitated with mouse anti-HA antibody (lane2); or with mouse anti-Gal4 antibody as a control (lane 3). The immunoprecipitates were analysed for Hdm2 and p53 binding. JMY was detected with a rabbit anti-HA antibody (Y-11), Hdm2 with the rabbit H-221 antibody, and p53 with sheep anti-p53 serum. Lane 1 shows the input (10% of cell extract).

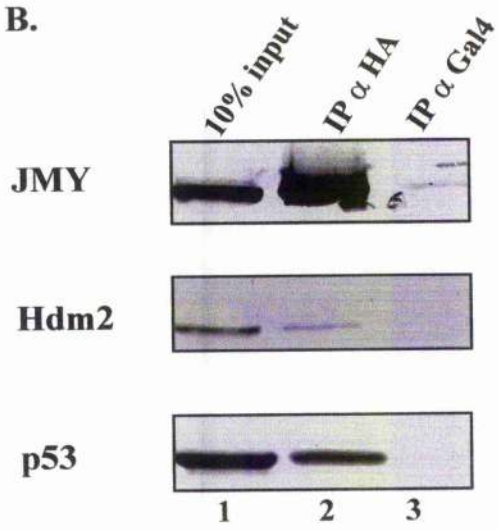
B. Following ActinomycinD treatment

U2OS cells were transfected with the pCMV-HAJMY and pCHDMIA expression vectors (10 μ g of each). Twenty four hours after transfection, cells were incubated in media containing 20 nM actinomycin D and were harvested 16 hours later. The lysates were immunoprecipitated with an anti-HA antibody (lane 2), or anti-Gal4 antibody (lane 3). JMY, p53 and Hdm2 were detected by western blot analysis as described in (A).

A.



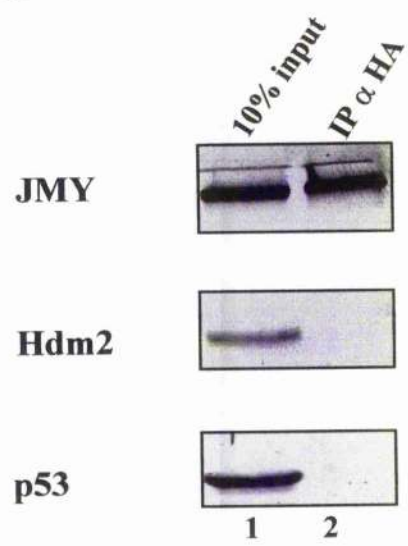
B.



C. Serum starvation

Twenty four hours following transfection with pCMV- HAJMY (10 μ g) and pCHDMIA (10 μ g), U2OS cells were incubated with media supplemented with 0.2% foetal calf serum, and harvested 16 hours later. The presence of Hdm2 and p53 in HAJMY immunoprecipitates (lane 2) was examined with the polyclonal antibody H-221 for Hdm2 and a sheep serum for p53. HAJMY was immunoprecipitated with a mouse anti-HA antibody, and detected with the rabbit anti-HA antibody Y-11.

C.



Chapter 6. Discussion

A multitude of cellular strategies ensure the tight regulation of cellular proliferation. Defects in the pathways that control the cell's response to DNA damage, cell cycle checkpoints or growth stimuli can contribute to inappropriate cell division and the initiation, or progression of cancer.

In addition to control of protein stability and post-translational modification, regulated nuclear transport is proving to be a fundamental and critical mechanism in the regulation of protein function. Regulation of both nuclear import and nuclear export has emerged as an efficient mechanism of translating signals related to the cellular environment into patterns of gene expression. This control mechanism has the advantage of allowing a rapid response to a particular signal, and of restricting the access of transcriptional regulators to their target genes in the absence of the appropriate stimulus.

Intensive research in the field of transcriptional regulation yielded multiple examples of the control of transcription factors by regulated nuclear transport. Examples include the cytokine -induced nuclear translocation of STATs, hormone-induced translocation of ligand-dependent transcription factors, and the nuclear export of the p53 tumour suppressor by its negative regulator Mdm2. Moreover, the activity of another class of transcriptional regulators, coactivator proteins, is increasingly viewed as another layer of transcriptional control and recent studies have highlighted the importance of nuclear transport in the regulation of some transcriptional cofactors (Muller *et al.*, 2002).

For example, stimulation of Rho signalling induces the translocation of the transcriptional coactivator FHL2, which results in the activation of androgen receptor responsive genes (Muller *et al.*, 2002). Moreover, the steroid receptor coactivator SRC-2, which is involved in skeletal muscle differentiation, translocates into the nucleus during the differentiation process (Chen *et al.*, 2001).

The data presented here relate to the regulation of the p53 cofactor, JMY. JMY is regulated at the level of subcellular localisation. While conditions of DNA damage enhanced the nuclear localisation of JMY, serum deprivation as well as the Mdm2 oncoprotein had the opposite effect, enhancing the relocalisation of JMY to the cytoplasm.

6-1. DNA damage enhances the nuclear localisation of JMY

JMY cooperates with p300 to stimulate the p53 response. Conditions of DNA damage enhance the association of JMY with the p53 complex, indicating that stress-dependent mechanisms allow the recruitment of JMY to the p53 complex (Shikama *et al.*, 1999). The data presented in this thesis suggest that enhancement of the nuclear localisation of JMY is one such mechanism.

The DNA-damage induced nuclear translocation of JMY would be predicted to facilitate its ability to act as a transcriptional cofactor for p53 and to augment p53-dependent apoptosis. Examination of the effect of UV treatment on the cell cycle effect of JMY revealed that JMY does not augment apoptosis under these conditions. Considering that the transcriptional targets of p53 include genes

involved in DNA repair and angiogenesis as well as cell cycle arrest and apoptosis, it is conceivable that in response to UV, JMY is involved in responses other than apoptosis.

The nuclear translocation of JMY was not triggered by all types of DNA damaging treatments. Whilst UV and actinomycin D increased the nuclear localisation of JMY, etoposide failed to produce such an effect. A possible explanation relates to the type of damage resulting from each treatment.

Actinomycin D interacts with DNA, forming obstructive structures for the movement of RNA polymerase, whereas UV promotes the formation of bulky adducts within the DNA structure, which would also affect the processivity of the enzyme. Etoposide, in contrast, favours the formation of double and single strand DNA breaks due to its interference with Topoisomerase II (Friedberg *et al.*, 1995). Therefore, it is conceivable that JMY responds to particular signal transduction pathways which are activated upon the formation of bulky DNA structures, or stalled transcription.

Two major components of the DNA damage checkpoint, ATM and ATR, are thought to contribute differentially to the DNA damage checkpoint. ATM has been mostly implicated in responses to DNA double strand breaks, whereas ATR is thought to regulate responses to various types of damage, including double strand breaks, stalled replication and pyrimidine dimers (Zhou and Elledge, 2000). Therefore, it is an attractive, yet unproven speculation that the ATR pathway may be involved in enhancing the nuclear localisation of JMY. This hypothesis can be

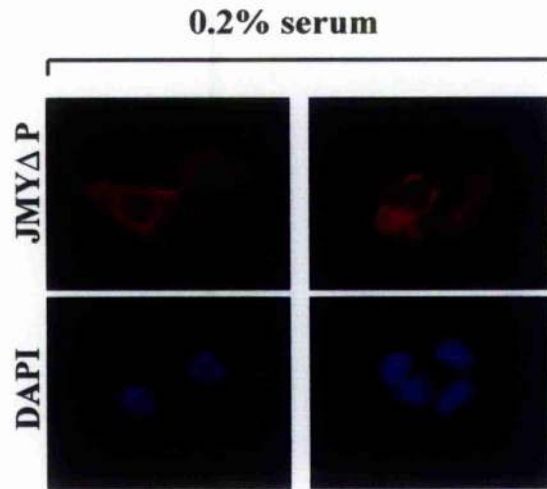
assessed by overexpression of a kinase-defective mutant of ATR, which would be predicted to attenuate or abolish the nuclear translocation of JMY in response to UV and actinomycin D.

6-2. Serum deprivation promotes the nuclear export of JMY

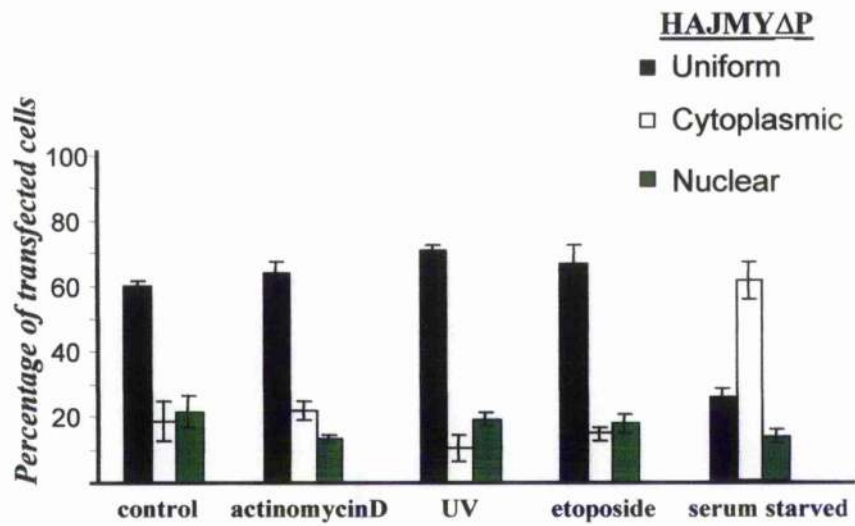
In contrast to DNA damage, low serum conditions stimulate the translocation of JMY into the cytoplasm. The effect on subcellular localisation is rapid (observed after 1 hour of serum withdrawal) and is reversed by the reintroduction of serum. The rapid kinetics of JMY localisation in response to serum withdrawal, and its ability to re-enter the nucleus following serum stimulation suggest a role for growth factor signalling in the regulation of JMY.

Cellular signalling pathways commonly involve signal-regulated translocation of proteins between cellular compartments. Phosphorylation is the most frequent post-translational modification known to regulate nuclear transport, and a considerable number of examples illustrate the link between phosphorylation and the subcellular localisation of signal transduction molecules as well as transcription regulators. For example, phosphorylation of the transcription factor NF-AT in the presence of moderate calcium levels exposes its NES, thus enhancing its nuclear export (Turpin *et al.*, 1999). In contrast, phosphorylation of p53 has been proposed to block its nuclear export and allow its nuclear accumulation in response to DNA damage (Zhang and Xiong, 2001).

D.



E.



The appearance of a phosphatase-sensitive, electrophoretically faster migrating band of JMY following serum deprivation suggests that such conditions mediate the phosphorylation of JMY. Although a more common manifestation of a phosphorylation event is a shift to a slower migrating form, examples of faster electrophoretic mobility have been reported, and include Mdm2 following phosphorylation by ATM (Khosravi *et al.*, 1999).

It is conceivable that phosphorylation of JMY triggers its nuclear export or impairs its nuclear import, resulting in a predominantly cytoplasmic protein. Alternatively, nuclear transport may facilitate the phosphorylation of JMY in the cytoplasm. Identification of the phosphorylation sites and the kinases involved will provide further clues with as to the role of JMY phosphorylation, and its connection with the observed nuclear export.

The relocalisation of JMY to the cytoplasm occurs via a leptomycin B-, and sodium azide-sensitive mechanism. Leptomycin B is an inhibitor of NES-dependent export by CRM1 (Fornerod *et al.*, 1997; Wolff *et al.*, 1997) whilst sodium azide acts by depleting the levels of RanGTP, thus disturbing the RanGTP/RanGDP gradient across the nuclear membrane. Given that the RanGTP/GDP gradient is required for multiple rounds of nuclear transport (Schwoebel *et al.*, 2002), and that sodium azide reduces the nuclear export of JMY, these data support the involvement of the CRM1-dependent pathway in JMY export, and indicate that it constantly shuttles between the nucleus and the cytoplasm.

Considering that CRM1-dependent nuclear export requires the interaction of CRM1 with leucine-rich nuclear export sequences (NES), it is likely that JMY contains an NES signal. Examination of the primary protein sequence of JMY reveals the presence of a leucine-rich sequence which fits the criteria established for an NES (Bogerd *et al.*, 1996) (figure 6.1). The importance of this sequence in the nuclear export of JMY can be assessed by mutation analysis. Moreover, fusion of the minimal sequence to a heterologous, non-shuttling protein would establish whether it is sufficient to mediate nuclear export without the assistance of an NES-containing binding partner. Of note, the data presented in this thesis do not exclude the possibility that JMY export is mediated by another protein which would provide the NES required for nuclear export.

The physiological significance of the cytoplasmic translocation of JMY in response to serum withdrawal is unclear. It is possible that nuclear export of JMY acts to inhibit its activity as a transcriptional cofactor for p53 and that JMY is maintained in an inactive state in the cytoplasm. This hypothesis is supported by the observation that the ability of JMY to augment apoptosis is abolished under low serum conditions. An alternative and attractive possibility is that JMY possesses an additional function in the cytoplasm which is related to serum/growth factor signalling.

Serum signalling also affects actin dynamics, and the absence of serum stimulation is associated with increased levels of unpolymerised actin (G actin) (Miralles *et al.*, 2003). In this respect, it is intriguing that the C-terminus of JMY

(residues 916-933) contains a region with high homology to the Wiscott Aldrich homology domain (WH2) which has been implicated in actin polymerisation. Considering the transcriptional coactivation properties of JMY and its ability to shuttle between the nucleus and the cytoplasm, it would be interesting to examine the functional importance of this domain to assess any potential links between transcription and the organisation of actin. Such a link has been demonstrated for another transcriptional cofactor, MAL, which acts as a transcriptional coactivator for the serum response factor SRF (Miralles *et al.*, 2003).

The subcellular localisation of MAL displays similar dynamics to that of JMY. In serum starved cells, MAL localises predominantly to the cytoplasm due to its association with actin monomers. Serum stimulation causes a decrease in the levels of actin monomers, thus allowing the relocalisation of MAL into the nucleus, where it associates with SRF and activates SRE (serum response element) mediated gene expression (Miralles *et al.*, 2003).

6-3. Differential regulation of the subcellular localisation of JMY and JMY Δ P

The JMY isoform JMY Δ P possesses distinct properties from the wild type protein (Shikama *et al.*, 1999). JMY Δ P has been shown to have negligible effects on p53-dependent apoptosis, and to augment G1 arrest whereas wild type JMY has a more marked effect on apoptosis. At the level of subcellular localisation, JMY Δ P displays stronger nuclear staining than JMY and, in contrast to JMY, its nuclear localisation is not enhanced by DNA damage. A possible explanation for the

observed differences is that nuclear-cytoplasmic shuttling of JMY may provide an additional control mechanism to prevent inappropriate induction of apoptosis.

Proline-rich domains induce bends in protein structure and have been shown to create binding sites for SH3 domains (Yu *et al.*, 1994). Therefore, it is possible that structural differences between JMY and the ΔP isoform, and/or potential interactions with SH3 containing proteins may influence their association with the nuclear transport machinery, and with components of the damage response.

In contrast, serum withdrawal promotes the relocalisation of JMY ΔP to the cytoplasm in a similar fashion to the wild type protein, indicating that the proline-rich region is not essential for the serum response.

Thus, in addition to having different effects on the p53 response, JMY and JMY ΔP are differentially regulated by DNA damaging conditions whereas they appear to be regulated in a similar fashion in response to serum withdrawal. Taken together, these data imply the possibility that different mechanisms regulate JMY in response to DNA damage and to serum withdrawal.

Considering that JMY can constantly shuttle between the nucleus and the cytoplasm, and the observed changes in its intracellular distribution under different cellular conditions, it is proposed that the steady state distribution of JMY is determined by a balance between import and export mechanisms (figure 6.2). Depending on the cellular conditions, this balance is shifted by changes in

the rate of import, export or both. Thus, DNA damage facilitates nuclear accumulation by reducing the rate of export relative to import. DNA damage has been shown to enhance complex formation between JMY, p300 and p53 (Shikama *et al.*, 1999), which could potentially reduce its ability to shuttle to the cytoplasm. In contrast, serum starvation increases nuclear export relative to import, thus resulting in predominantly cytoplasmic localisation of JMY.

6-4. Mdm2 affects JMY localisation

The oncoprotein Mdm2 is a major negative regulator of the p53 tumour suppressor, and mediates its inhibitory function partly through enhancing the nuclear export of p53.

The data presented here indicate that Mdm2 can also affect the subcellular distribution of the p53 cofactor JMY in a leptomycin B-sensitive, p53-independent manner.

The ability of Mdm2 to relocalise JMY to the cytoplasm was abrogated following treatment with actinomycin D and UV, which are inducers of DNA damage and activators of p53. This may explain the enhanced nuclear localisation of JMY under these conditions and may be accounted for by modifications affecting JMY, Mdm2, or both. Another potential explanation would be that DNA damage affects the molecular composition of the JMY-Mdm2 complex in a way that renders JMY resistant to Mdm2-mediated cytoplasmic relocalisation.

Mdm2-dependent inhibition of p53 is similarly attenuated under DNA damage conditions. For example, ionising radiation-induced Mdm2 phosphorylation appears to reduce its ability to promote p53 degradation and export (Maya *et al.*, 2001), whereas UV and certain alkylating agents reduce the mRNA and protein levels of Mdm2, allowing the accumulation of p53 (Inoue *et al.*, 2001).

6-5. Mdm2-mediated export of JMY is independent of p53 binding but requires an intact ring finger of Mdm2

Mutations that abolish the binding of Mdm2 to p53 do not reduce its effect on JMY sub-cellular distribution. This indicates that Mdm2-mediated relocalisation of JMY is independent of its ability to bind p53 and is consistent with the fact that Mdm2 can promote the redistribution of JMY into the cytoplasm in the p53-null SAOS2 cells.

In contrast, ring finger domain mutants of Mdm2 fail to promote the cytoplasmic localisation of JMY, suggesting that the ubiquitin ligase activity of Mdm2 might be involved. Alternatively, mutations in this region might affect the interaction between JMY and Mdm2. Interestingly, in a small proportion of cells, the nuclear localisation of JMY increases in the presence of these mutants relative to cells overexpressing JMY alone. This was also observed in two studies assessing the role of the ring finger domain in Mdm2-dependent export of p53 (Boyd *et al.*, 2000; Geyer *et al.*, 2000). These studies used cells that express endogenous Mdm2 (NIH 3T3 and U2OS, respectively), and showed the inability of ring finger

mutants to relocalise p53 to the cytoplasm. In both studies, the proportion of cells with nuclear p53 was higher in the presence of the ring finger mutants relative to that observed in cells expressing p53 alone. Since U2OS/NIH3T3 cells already contain endogenous Mdm2, it is conceivable that the ring finger mutants act in a dominant negative fashion and inhibit the effect of endogenous Mdm2 on JMY, and p53. Figure 6.3. summarises the effect of the described Mdm2 mutations on p53 and JMY binding and nuclear export.

Mdm2-mediated export of JMY might account for the variations in its distribution in U2OS cells, which express endogenous Hdm2, and SAOS2 cells, which express almost undetectable levels of Hdm2 due to their *p53* null status. The proportion of cells displaying nuclear exclusion of JMY is almost twofold higher in U2OS than SAOS2 cells, which might reflect a degree of nuclear export by endogenous Hdm2 in U2OS cells.

However, a significant percentage of cells display cytoplasmic JMY staining even in SAOS2 cells and in U2OS cells in the absence of overexpressed Mdm2. This suggests that additional factors might affect the distribution of JMY, and that Mdm2 enhances the cytoplasmic localisation of JMY as opposed to being the single main regulator of JMY localisation. Similarly, although Mdm2 can significantly enhance p53 export, p53 can shuttle between the nucleus and the cytoplasm independently of Mdm2. For example, an Mdm2-binding defective mutant of p53 has been shown to retain shuttling activity and p53 localisation can be regulated by DNA damage independently of Mdm2 (Stommel *et al.*, 1999,

Zhang and Xiong, 2001). The situation where the subcellular localisation of a protein is regulated by an interacting protein as well as independently of the binding partner is not uncommon. For example, the subcellular localisation of the oncogenic transcription factor β -catenin is determined by an intrinsic nucleocytoplasmic shuttling activity in growth factor stimulated cells. However, in the absence of stimulus, the tumour suppressor APC actively exports β -catenin into the cytoplasm (Henderson and Fagotto, 2002).

JMYAP is resistant to Mdm2-mediated nuclear export despite maintaining the ability to undergo nuclear export in response to serum deprivation. This suggests that one or more features associated with the polyproline region of JMY are essential for Mdm2-mediated export. Further, these data indicate that elevated levels of Mdm2 and serum deprivation may utilise different properties of JMY to promote its cytoplasmic relocalisation.

6-6. Mdm2 does not target JMY for degradation

The data presented above suggest that Mdm2 can regulate the subcellular localisation of JMY and p53 by a similar mechanism. However, this similarity does not extend to the regulation of protein levels. In contrast to its ability to promote the degradation of p53, Mdm2 fails to downregulate the protein levels of JMY. This might be a reflection of the fact that JMY is a relatively stable protein and does not seem to be regulated at the level of its stability.

6-7. JMY and Mdm2 interact in cells

Consistent with the ability of Mdm2 to promote the redistribution of JMY to the cytoplasm, the two proteins can interact in cells. However, the ability of JMYΔP to interact with Mdm2 indicates that the presence of JMY and Mdm2 in the same complex is not sufficient for Mdm2 to promote the cytoplasmic translocation of JMY, and that an additional property of JMY that is associated with the proline rich region is required.

Accumulating evidence shows that Mdm2 does not always promote both nuclear export and degradation of associated proteins, and complex formation with Mdm2 does not necessarily translate into reduced levels and nuclear export. For example, although it can inhibit p73 function, Mdm2 does not target it for degradation nor nuclear export. The inability of p73 to undergo Mdm2-directed nuclear export is attributed to its non-functional nuclear export sequence (Gu *et al.*, 2001).

Another example relates to the regulation of the E2F/ DP heterodimer (Loughran and La Thangue, 2000). Mdm2 downregulates the levels of E2F-1 only in the presence of its heterodimeric partner DP-1, whereas it can promote the degradation of the latter independently of E2F-1. Moreover, the interaction of Mdm2 with DP-1 promotes its nuclear accumulation as opposed to export (Loughran and LaThangue, 2000).

Thus it appears that properties intrinsic to Mdm2-binding proteins determine, at least in part, the outcome of the association with Mdm2. This may explain the inability of Mdm2 to promote the degradation of JMY, and may also account for its inability to enhance the nuclear export of JMY Δ P.

6-8. Regulation of the JMY-Mdm2 complex by cellular conditions

Examination of JMY-containing complexes in cells reveals its ability to associate with both Mdm2 and p53. The p53-Mdm2-JMY complex seems to possess a dynamic nature as it is affected by cellular conditions. Whereas the three proteins coexist in a complex under normal conditions, actinomycin D-induced DNA damage reduces the ability of Mdm2 but not p53 to co-immunoprecipitate with JMY. This is consistent with the observation that actinomycin D compromises Mdm2-mediated nuclear export of JMY, and might contribute to the nuclear accumulation of JMY in response to actinomycin D.

In contrast, serum starvation conditions, which stimulate the nuclear export of JMY, not only block the interaction between JMY and Mdm2, but also prevent the association of JMY and p53. A possible explanation is that under these conditions, JMY dissociates from the p53 and Mdm2 complex, and is exported into the cytoplasm via an Mdm2-independent mechanism.

A model for the regulation of the subcellular localisation of JMY is proposed (figure 6.4). Under normal conditions, JMY constantly shuttles between the nucleus and the cytoplasm and can interact with p53 and Mdm2. Mdm2

enhances the export of JMY, which presumably attenuates its function as an apoptotic cofactor for p53. DNA damage stimulates the nuclear translocation of JMY and causes a reduction in its association with Mdm2, thus reducing the rate of JMY export and allowing its accumulation in the nucleus. In the nucleus, JMY cooperates with p300 to enhance p53-dependent transactivation.

Under conditions of growth factor/serum deprivation, an Mdm2-independent export mechanism is proposed to stimulate the relocalisation of JMY to the cytoplasm and, as a result, JMY dissociates from the p53-Mdm2 complex.

6-9. Concluding comments:

The results presented in this thesis relate to the regulation of the p53 cofactor JMY. JMY is demonstrated to shuttle between the nucleus and the cytoplasm in a regulated manner, and cellular conditions are shown to shift the intracellular distribution of JMY towards a more nuclear or more cytoplasmic localisation. The present study focused on the subcellular localisation of ectopially expressed JMY due to difficulty in generating antibodies that are suitable for the analysis of the endogenous protein by immunofluorescence.

In the absence of serum, a nuclear export mechanism results in a predominantly cytoplasmic localisation of JMY. The opposite effect is produced by certain conditions of DNA damage, which facilitate the recruitment of JMY to the nucleus, the site of p53 action. Furthermore, the interaction of JMY and p53 is regulated by these conditions. Whereas JMY can associate with p53 in the presence of DNA damage, this interaction is abrogated in the absence of serum. The purification and comparison of JMY-containing complexes from DNA damaged or serum starved cells would provide further insight into the regulation of JMY and could potentially reveal other cooperating molecules or targets of JMY function.

The finding that the JMY and JMY Δ P do not respond to DNA damage treatment in the same manner suggests the existence of divergent regulatory mechanisms for the two isoforms. An intriguing question relates to whether different stress signals result in the preferential use of a particular JMY isoform in the p53

response. Given the ability of JMY and JMY Δ P to modulate the outcome of the p53 activation, further understanding of their mechanisms of regulation could provide valuable clues regarding the choice of the p53 response.

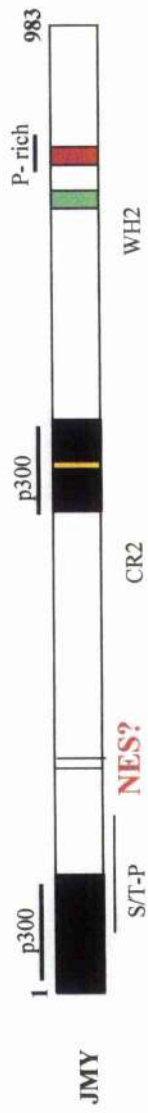
Interestingly, JMY can associate with the p53 inhibitor Mdm2, an interaction regulated by cellular conditions. Furthermore, elevated levels of Mdm2 result in enhanced cytoplasmic localisation of JMY and this effect is attenuated under DNA damage conditions.

The remaining crucial question relates to the functional significance of the JMY-Mdm2 interaction, and the relocalisation of JMY by Mdm2. Mdm2-dependent inhibition of p53 occurs at multiple levels, including inhibition of its interaction with transcriptional coactivators (Wadgaonkar and Collins, 1999), and recruitment of co-repressors (Mirnezami *et al.*, 2003). It is speculated that Mdm2-mediated export of JMY might represent an additional mechanism of modulating the transcriptional activity of p53. Since JMY preferentially stimulates p53-dependent apoptosis (Shikama *et al.*, 1999), its cytoplasmic relocalisation in the presence of Mdm2 might serve to attenuate the apoptotic function of p53. In this respect, it would be interesting to compare the effect of the p53 binding mutant (Hdm2 Δ 58-89) and the combined double mutant (Hdm2 Δ 58-89;C464A) on the coactivation and pro-apoptotic function of JMY. The above hypothesis predicts that Hdm2 Δ 58-89 but not Hdm2 Δ 58-89;C464A (which is deficient in JMY export) can reduce or block the effect of JMY on p53 transcriptional activity and apoptosis. Finally, if the above hypothesis is correct, and if Mdm2-directed

export of JMY attenuates the p53 response, blocking the JMY-Mdm2 interaction might stimulate the p53 response, providing potential use in therapy development.

6.1. Potential NES sequences of JMY

Sequences in the primary protein sequence of JMY that fit the criteria for an NES are shown. The consensus proposed for an NES (Bogerd *et al.*, 1996) sequence is shown



JMY sequences

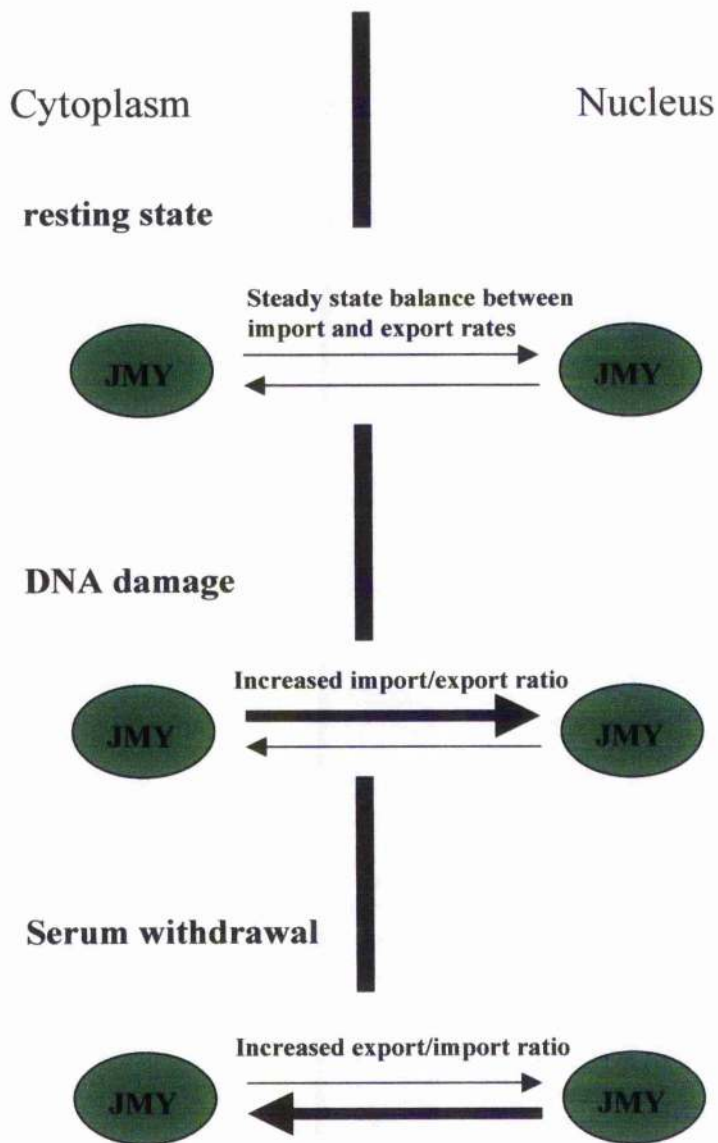
229	L	R	S	Q	L	E	P	C	L	P	V	-	F	241
276	L	C	Y	Q	L	Q	V	Y	L	G	H	G	L	288

Consensus NES

L	X(1-3)	L	X(2-4)	L	-	X-
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**6.2 Model for the regulation of the net subcellular localisation
of JMY**

The distribution of JMY is determined by a balance between nuclear import and nuclear export processes. This distribution can be altered by shifting the balance towards nuclear import or towards export and depends on cellular conditions



Hdm2/mutant	Ubiquitin ligase	p53 binding	p53 nuclear export	JMY binding	JMY nuclear export
Hdm2	+	+	+	+	+
Hdm2 1-440	-	+	-	Nt	-
Hdm2 C464A	-	+	-	Reduced	-
Hdm2 Δ 58-89	+	-	-	+	+
Hdm2 Δ 58-89; C464A	-	-	-	Nt	-

Figure 6.3. Summary of the effect of Hdm2 mutants on p53 and JMY Nt; not tested.

6.4. Model for the regulation of the subcellular localisation of JMY by Mdm2

A. Normal conditions

Under normal conditions, JMY constantly shuttles between the nucleus and the cytoplasm and can interact with p53 and Mdm2. Mdm2 augments the cytoplasmic localisation of JMY in an NES- dependent, leptomycin B-sensitive fashion. This effect of Mdm2 is independent of binding to p53 as the p53-binding mutant Hdm2 Δ 58-89 retains the ability to associate with and promote the nuclear exclusion of JMY. In contrast, mutation of the ring finger domain abolishes Mdm2-mediated relocalisation of JMY, potentially by reducing the ability of the two proteins to interact.

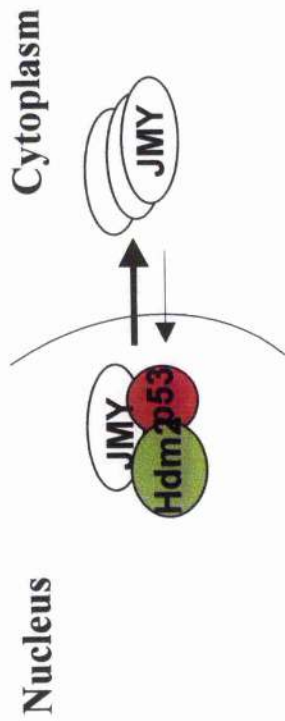
B. DNA damage

The association of Mdm2 with the JMY complex is weakened, thus reducing the rate of the nuclear export of JMY and allowing its accumulation in the nucleus, and enhanced interaction with p53.

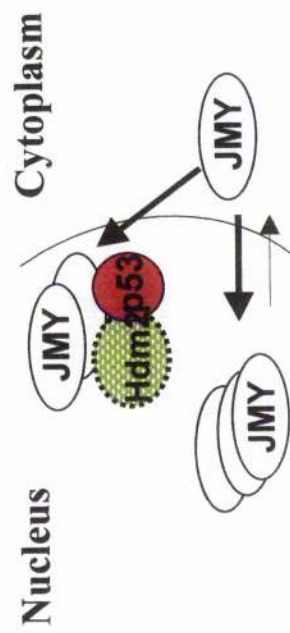
C. Serum deprivation

Under conditions of growth factor/serum deprivation, JMY dissociates from the p53-Mdm2 complex and is shuttled to the cytoplasm via an Mdm2-independent mechanism.

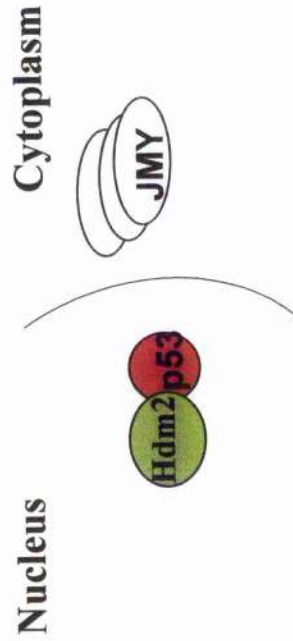
A. Unstressed cells



B. DNA damage



C. Serum withdrawal



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