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R. Kulikov

Institut für Toxikologie und Genetik

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Regulation des Tumorsuppressor Proteins p53 durch die Glykogen Synthase Kinase 3

ZUSAMMENFASSUNG

Das Mdm2 Onkoprotein reguliert Menge und Aktivität des p53 Protein, eines der wichtigsten Tumorsupressorproteine in höheren eucaryotischen Zellen. Für einen effizienten Abbau des p53 Proteins muss das Mdm2 Protein an mehreren zusammenhängenden Phosphorylierungsstellen innerhalb des zentralen konservierten Bereichs phosphoryliert sein.

Während meiner Doktorarbeit fand ich, dass die Glykogen synthase kinase 3 (GSK 3) das Mdm2 Protein *in vitro* und *in vivo* innerhalb des zentralen Bereichs phosphoryliert. Hemmung von GSK 3 mit synthetischen Inhibitoren oder siRNA verhinderte den Abbau des p53 Proteins in einer Mdm2 abhängigen Weise, obwohl das p53 Protein an das Mdm2 Protein gebunden war und durch das Mdm2 Protein ubiquityliert wurde. Auch die Lokalisierung der p53 und Mdm2 Proteine oder die Interaktion der Mdm2 und MdmX Proteine war durch die Inhibition von GSK 3 nicht betroffen. Die Hemmung von GSK 3 verminderte jedoch die Bindung des Mdm2 Proteins an das Proteasom. Die Mengenzunahme des p53 Protein führte zur Transkription des *p21/waf1* und *mdm2* Gens, nicht aber zur Transkription apoptotischer Gene wie *bax* oder *puma*. Nach ionisierender Strahlung, die zur Mengenzunahme des p53 Proteins führt, wird GSK 3 an Serin 9 phosphoryliert. Diese Phosphorylierung geht der p53 Mengenzunahme voraus und überlappt teilweise mit ihr. Darüberhinaus reduzierte die Expression einer GSK 3 Mutante, die gegen die Phosphorylierung durch ionisierende Strahlung resistent war, die Ansammlung des p53 Proteins.

Ich schließe aus diesen Ergebnissen, dass GSK 3 das p53 Protein reguliert, indem es wichtige Phosphorylierungsstellen phosphoryliert, die im zentralen Bereich des Mdm2 Proteins liegen. Meine Daten weisen außerdem auf einen neuen Mechanismus für die schadensinduzierte Ansammlung des p53 Proteins hin und beschreiben eine post-ubiquityläre Funktion des Mdm2 Proteins, die Interaktion des Mdm2 Proteins mit dem Proteasom.

ABSTRACT

The Mdm2 oncoprotein regulates abundance and activity of the p53 protein, one of the most important tumor suppressor proteins in higher eucaryotic cells. For efficient degradation of p53, the Mdm2 protein needs to be phosphorylated at several contiguous residues within the central conserved domain.

I found that glycogen synthase kinase 3 (GSK 3) phosphorylated the Mdm2 protein *in vitro* and *in vivo* within the central domain. Inhibition of GSK 3 with synthetic compounds or siRNA rescued p53 from degradation in an Mdm2-dependent manner despite its association with the Mdm2 protein and ubiquitylation. Localization of the p53 and Mdm2 proteins or the interaction of the Mdm2 and MdmX proteins were not affected but inhibition of GSK 3 decreased the association of the Mdm2 protein with the proteasome. The accumulated p53 protein induced transcription of the *p21/waf1* and *mdm2* gene but not of pro-apoptotic genes such as *bax* or *puma*. Ionizing irradiation, which leads to p53 accumulation directed phosphorylation of GSK 3 at serine 9, which preceded and overlapped with the increase in p53 levels. Moreover, expression of a GSK 3 mutant refractory to ionizing irradiation-induced phosphorylation reduced the accumulation of p53.

I therefore conclude that GSK 3 regulates p53 levels by phosphorylating key sites in the central domain of the Mdm2 protein. My data also reveal a new mechanism for DNA damage-induced p53 accumulation and describe a post-ubiquitylation function of the Mdm2 protein, the interaction of the Mdm2 protein with the proteasome.

Acknowledgements

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Abbreviations

aa	aminoacid
AAA ATPases	ATPases associated with diverse cellular activities
APS	ammonium persulfate
Arf	alternative reading frame protein
ATM	ataxia telangiectasia mutated kinase
ATP	adenosine triphosphate
Bis I	bisindolylmaleimide I
Bis IX	bisindolylmaleimide IX
bp	base pairs
BSA	bovine serum albumin
cdk	cyclin dependent kinase
cDNA	complementary DNA
Chk2	checkpoint kinase 2
Ci	Curie
CKI	casein kinase I
DCS	donor calf serum
ddNTP	di-deoxynucleotide triphosphate
DEPC	diethyl pyrocarbonate
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DNA-PK	DNA-activated protein kinase
dNTP	deoxynucleotide triphosphate
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EDTA	ethylendiamine-N,N-tetracetate
ER	endoplasmic reticulum
FCS	foetal calf serum
g	gram
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GSK 3	glycogen synthase kinase 3
GST	glutathione S-transferase
h	hour
HA	hemagglutinin
HEPES	2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid
HRP	horseradish peroxidase

Ig	immunoglobulin
IP	immunoprecipitation
IPTG	isopropyl-β-D-thiogalactopyranoside
IR	ionizing irradiation
kb	kilobases
kDa	kilodalton
LB	Luria–Bertani
m	milli
μ	micro
M	molar
Mdm2	mouse double minute 2
min	minute
MOPS	4-morpholinepropanesulfonic acid
mRNA	messenger RNA
n	nano
NES	nuclear export signal
NLS	nuclear localization signal
NoLS	nucleolar localization signal
NP-40	nonidet P-40.
NZF	Npl4 zinc finger
OD	optical density
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PI3K	phosphatidylinositol 3-kinase
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
PMSF	phenylethylsulphonyl fluoride
PNK	polynucleotide kinase
PUMA	p53-upregulated modulator of apoptosis
RING	really interesting new gene
RNA	ribonucleic acid
rpm	rounds per minute
RT	room temperature
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
sec	second

siRNA	small interfering RNA
TBS	tris buffered saline
TEMED	N,N,N',N'+tetramethylethylenediamine
Tris	tris-(hydroxymethyl)-aminomethane
U	units
UV	ultra violet
V	volt
V/v	volume on volume
W/v	weight on volume
WB	western blot
wt	wild type
YY1	ying yang 1

1. INTRODUCTION

The p53 tumour suppressor is a tightly regulated protein that acts by blocking cell cycle progression or promoting apoptosis when cells encounter stress signals such as oncogene activation or DNA damage (for a review, Hofseth et al., 2004). It is mutated in about every second tumor and deletion of both alleles of the *p53* gene results in early onset of cancer (Vogelstein et al., 2000). The p53 protein has a short half-life, and it is normally maintained at low levels in unstressed cells by continuous ubiquitylation and subsequent degradation by the 26S proteasome. Ubiquitylation is mainly due to the interaction of the p53 protein with the RING finger ubiquitin E3 ligase Mdm2 (Honda et al., 1997; Oliner et al., 1993; Mommand et al., 1992). When cells are stressed, p53 degradation is blocked and p53 accumulates in the nucleus where it functions as a transcriptional activator or repressor of distinct target genes that contain p53 sequence-specific DNA binding sites. The mechanisms of p53 regulation are under intense investigation. Despite the significant progress in understanding the degradation pathway of the p53 protein, the exact mechanisms underlying the p53 response to DNA damage are not known. The recent observation that the degradation of the p53 protein is regulated by hypophosphorylation of the central domain of the Mdm2 protein after ionizing irradiation suggests the existence of a signalling cascade that regulates Mdm2 function via phosphorylation of its central domain (Blattner et al., 2002). However, no kinases are known that phosphorylate the central domain of the Mdm2 protein.

In my PhD thesis I have identified glycogen synthase kinase 3 (GSK 3) as a kinase that phosphorylates the central domain of Mdm2 *in vitro* and *in vivo*. I found that inhibition of GSK 3 rescued p53 from degradation in an Mdm2-dependent manner despite its association with Mdm2 and ubiquitylation. Moreover, inhibition of GSK 3 decreased the association of the Mdm2 protein with the proteasome. The accumulated p53 protein induced transcription of *p21/waf1* and *mdm2* but interestingly not of proapoptotic proteins such as *bax* or *puma*. In consistency with the hypophosphorylation of the central domain of the Mdm2 protein after ionizing irradiation, GSK 3 became phosphorylated and inactivated after DNA damage. Correspondingly, expression of a constitutively active mutant of GSK 3 reduced p53 accumulation after ionizing irradiation. These data provide not only a new mechanism for DNA damage-induced p53 accumulation but also shed some light on the post-ubiquitylation function of Mdm2, the interaction of the Mdm2 protein with the proteasome. In the following chapters I will introduce the players of the p53 degradation pathway and their regulation in response to DNA damage.

1.1. The p53 tumor suppressor protein

Since its discovery 25 years ago, p53 has become one of the most studied tumour suppressor genes. Originally it was identified as a cellular protein of 53 kDa that bound to SV40 large T-antigen and accumulated in cancer cells (Lane and Crawford, 1979; Linzer and Levine, 1979). However, 10 years later, researchers discovered that they worked previously with the mutant p53 gene instead of its wild type counterpart. Subsequent research clearly demonstrated that wild type p53 is not an oncogene but in fact a tumor suppressor gene (Baker et al., 1989; Finlay et al., 1989).

In approximately half of all human tumors, the p53 gene was found to be mutated and some of these p53 missense mutations gain oncogenic activity and promote tumorigenesis (Sigal and Rotter, 2000). In the remaining cases, the p53 response pathway may not be functional, due to overexpression of p53 inhibitors, such as Mdm2, or inactivation of p53 activators such as p14Arf (Vogelstein et al., 2000).

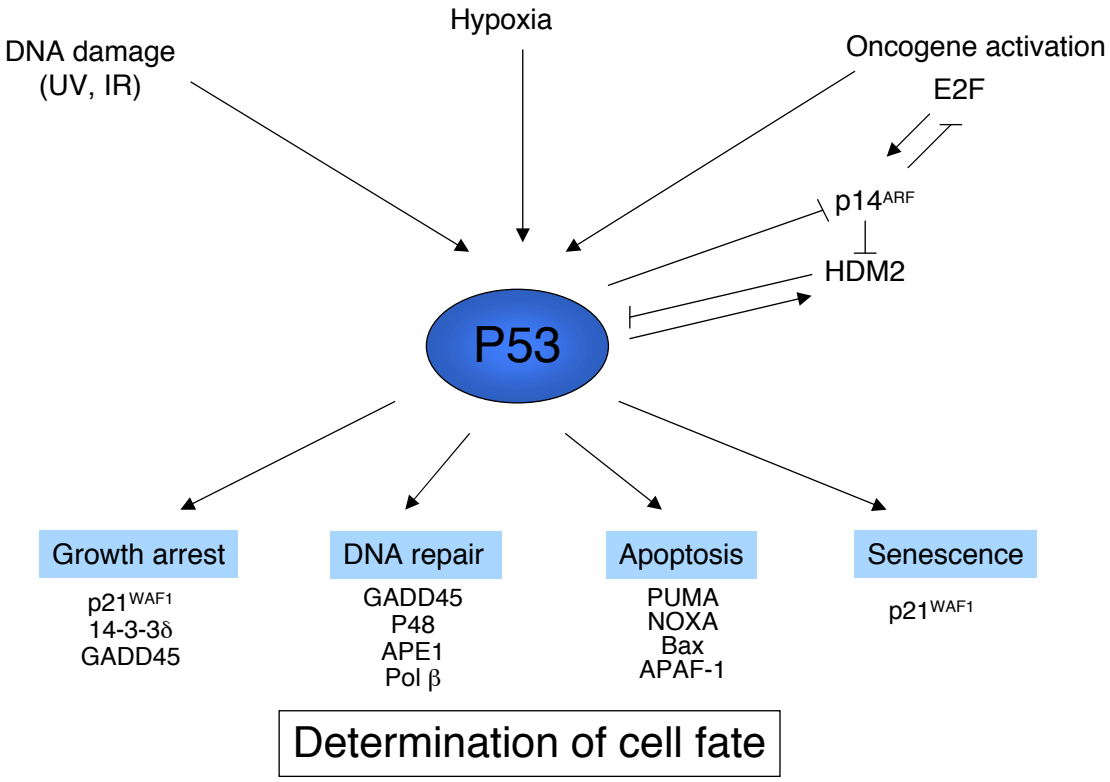


Figure 1.1. The p53 protein is at the crossroads of multiple cellular stress pathways. After stress p53 accumulates and activates transcription of genes that play a role in cell-cycle checkpoints, apoptosis, DNA repair or cellular senescence. (Adopted from Hofseth et al., 2004)

1.1.1 Transcriptional activation function of the p53 protein

The p53 protein has multiple functions in cells, most of them are based on its activity as a transcription factor. In the absence of stress, the p53 protein is transcriptionally inactive. Several signalling pathways stabilize the p53 protein in response to stress and lead to its

activation. Depending on the conditions of cell growth, the type and duration of stress, p53 selectively activates a different subset of target genes which cause either apoptosis or growth arrest (for a recent review, Hofseth et al., 2004; Fig. 1.1). The p53 protein regulates the G1-S checkpoint in response to relatively low doses of DNA damage, heat shock, hypoxia and other forms of stress. The targets for transcriptionally active p53 protein that mediate efficient G_1 arrest are the cyclin-dependent kinase inhibitor $p21^{WAF1}$, GADD45 and 14-3-3σ. Growth arrest in response to mild DNA damage gives the cell time to repair damaged DNA. Under extreme stress and severe DNA damage, the p53 protein triggers the activation of genes implicated in the apoptotic cascade including bax, APAF-1, NOXA, PUMA and many others (Fig. 1.1). The mechanisms that enable p53 to selectively trigger one of the two distinct pathways are not known and are subject of intense investigations (Slee et al., 2004).

1.1.2 P53, direct activator of apoptosis

There is growing evidence that the p53 protein can induce apoptosis independently of its ability to bind DNA and to activate transcription. A number of publications reported recently about the localization of the p53 protein to mitochondria in response to DNA damage or hypoxia. The p53 protein translocates to the mitochondria at the onset of p53-induced apoptosis and contributes directly to apoptotic signalling at the mitochondria. At the mitochondria, the p53 protein interacts directly with anti-apoptotic proteins such as Bcl-2 and Bcl-X_L (Marchenko et al., 2000; Mihara et al., 2003). Interestingly, transcriptionally inactive mutants of the p53 protein, when targeted to mitochondria, induce apoptosis as effectively as wild-type p53 (Mihara et al., 2003).

1.1.3 Structure of the p53 protein

The human p53 protein is a nuclear phosphoprotein that consists of 393 amino acids. The protein is commonly divided into three functional domains. The amino-terminal domain is required for transcriptional activation of target genes. The Mdm2 protein interacts with this N-terminal transactivation domain and thus inhibits p53 transcriptional activity (Chen et al., 1995). Most of the interactions between the p53 protein and its target proteins take place in the central core domain of the p53 protein, which mediates sequence-specific DNA-binding. Towards the carboxyl-terminal end of the p53 protein, there is a tetramerization domain, a nuclear export signal and nuclear localization signals.

The p53 protein is a target for a complex and diverse array of covalent post-translational modifications (reviewed in Bode and Dong, 2004). The most common of these post-translational modifications of the p53 protein include phosphorylation of serines and threonines, and acetylation, ubiquitylation and sumoylation of lysine residues. Post-translational phosphorylation and acetylation is supposed to lead to p53 stabilization,

accumulation and activation in the nucleus (reviewed in Bode and Dong, 2004). More recent reports suggest, however, that the role of phosphorylation might have been overestimated (Thompson et al., 2004: Jackson et al., 2004).

1.1.4 P53 is degraded via the ubiquitin-dependent proteasome degradation

In normal, unstressed cells, the p53 protein is very unstable with a half-life ranging from 5 to 30 minutes (Maltzman and Czyzyk, 1984). In consequence, p53 is present at very low cellular levels owing to continuous proteasomal degradation that is largely mediated by the Mdm2 protein (Maki et al., 1996). Recently, three other proteins have been discovered, Pirh2, Cop1 and topors, that also facilitate degradation of the p53 protein via the ubiquitin-proteasome pathway (Leng et al., 2003; Dornan et al., 2004; Rajendra et al., 2004). The physiological significance of p53 degradation by Cop1, Pirh2 and topors is not as yet clear. Therefore, Mdm2-dependent p53 degradation will be discussed in the next chapters.

1.2 General principles of ubiquitylation-dependent proteasomal degradation.

The most common role of ubiquitylation is to render proteins susceptible to degradation by the 26S proteasome (for a review, Fang and Weissman, 2004). It occurs as a consequence of modification of proteins with chains of four or more ubiquitins linked through lysine 48 (K48) of ubiquitin and the specific recognition of these tagged substrates by the 19S cap of the 26S proteasome (Thrower et al., 2000).

In general, ubiquitylation results from the sequential action of three classes of enzymes, E1 or ubiquitin activating enzyme, E2 or ubiquitin conjugating enzyme, and E3 or ubiquitin protein ligase (Fig. 1.2). As a result of this enzyme cascade, an ubiquitin monomer becomes attached to the ε-amino group of an internal lysine residue of the substrate (Passmore and Barford, 2004). In some cases, the ubiquitin molecule is attached to a free alpha-amino group of the substrate rather than to a lysine residue (Breitschopf et al., 1998; Kuo, 2004; Ben-Saadon et al., 2004). The ubiquitylation reaction is repeated until polyubiquitin chains are formed. For several proteins, the activity of an additional protein, called E4 ligase, is required for polyubiquitylation (Koegl et al., 1999).

Substrate specificity is determined largely by the E3 ubiquitin ligase. Ubiquitin ligases can be identified by specific E3 motifs. Most of the E3 ligases fall into two big classes: HECT domain (Homologous to E6-AP Carboxyl Terminus; Huibregtse et al., 1995) or RING domain (Really Interesting New Gene) ubiquitin ligase (Joazeiro and Weissman, 2000). A few other motifs are, however, also associated with ubiquitin ligase activity. These include U-box (Cyr et al., 2002), PHD (Plant Homeo-Domain; Coscoy et al., 2001) or LAP (Leukemia-Associated Protein) finger domains (Boname and Stevenson, 2001).

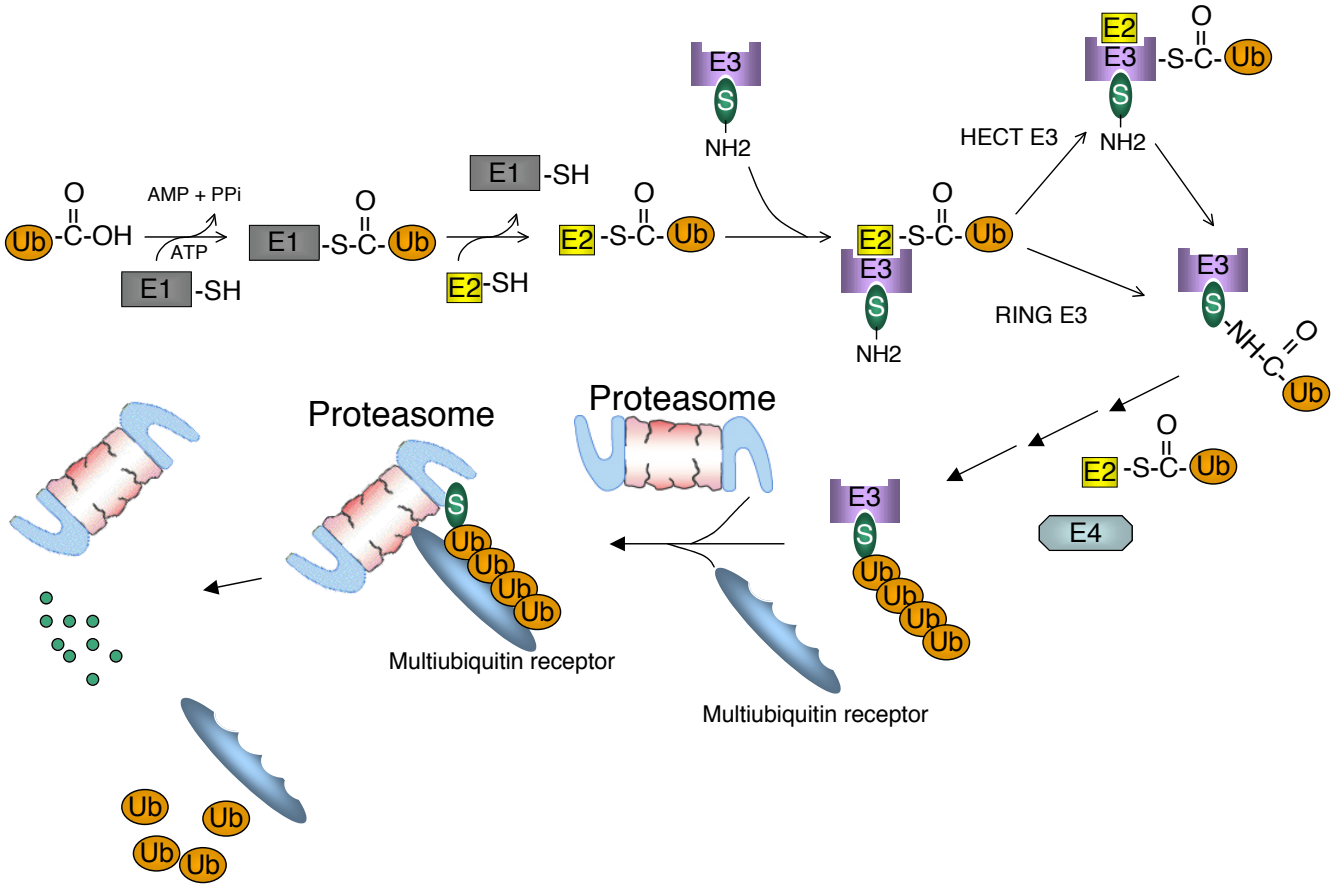


Figure 1.2. The ubiquitylation-dependent proteasomal degradation pathway. Free ubiquitin (Ub) is activated in an ATP-dependent manner with the formation of a thiol-ester linkage between E1 and the carboxyl terminus of ubiquitin. The activated ubiquitin monomer is transferred to one of several E2s. For HECT domain E3s, ubiquitin is transferred from the E2 protein to the active site cysteine of the HECT domain of the ubiquitin ligase, and subsequently transfered to a substrate (S) or to a substrate-bound multi-ubiquitin chain. For RING-finger E3s, E2s associate with E3s, which may have the substrate already bound and the ubiquitin is transferred directly from the E2 enzyme to the substrate. The ubiquitylation reaction is repeated until a multiubiquitin chain of four or more K48-linked ubiquitin moieties is formed. Some substrates require the activity of an additional enzyme, an E4 ubiquitin ligase for polyubiquitylation. The polyubiquitylated substrate is recognized by proteasomal multiubiquitin receptors, unfolded and degraded in the central catalytic chamber of the 26S proteasome. Single ubiquitin molecules are regenerated and can be used again.

Once the chains of at least four ubiquitin proteins are formed, the substrate is targeted for proteasomal degradation (Thrower et al., 2000). The polyubiquitin chains are recognized by multiubiquitin receptors on the regulatory subunit of the proteasome that target proteins to AAA ATPase-dependent unfolding and subsequent degradation in the central catalytic chamber of the 20S proteasome (Fig. 1.2; Sauer et al., 2004). The nature of polyubiquitin receptors was actively investigated recently (Hartmann-Petersen and Gordon, 2004).

A few proteins are able to bind polyubiquitin chains, among them are Dsk2/PLIC, Rad23, Cdc48, S6' AAA ATPase and S5a non-ATPase subunit of the proteasome. Genetic

studies in yeast have demonstrated indirectly that Rad23 and Dsk2/PLIC proteins play a role in polyubiquitin recognition (Hartmann-Petersen and Gordon, 2004). However, the only direct evidence for recognition of polyubiquitin chains by Rad23 was received recently when Deshais and co-workers developed a new cell-free degradation system and were able to show that Rad23 is a receptor for polyubiquitylated forms of the cdk inhibitor Sic1 (Verma et al., 2004). Importantly, in the same system Rad23 was not targeting Cln2 and other proteins for degradation revealing a high level of specificity (Verma et al., 2004). Another hypothesis proposes that specific ubiquitin ligases may contribute to the delivery of their substrates to the proteasome (Xie and Varshavsky, 2000). Indeed, a number of different E3 ubiquitin ligases have been shown to interact with the proteasome. In human cells, the KIAA10 E3 has been shown to bind to the human S2/Rpn1 subunit (You and Pickart, 2001) and the von Hippel-Lindau protein (VHL) interacts with S6a, a component of 19S regulatory complex of proteasome (Tsuchiya et al., 1996) Moreover, its ability to degrade its substrate, HIF1α, depends on the interaction of VHL with the proteasome (Corn et al., 2003). In budding yeast, the Ubr1 and Ufd2 ubiquitin ligases interact directly with the proteasome (Xie and Varshavsky, 2000). Future investigations will reveal whether delivering substrates for proteasomal degradation is a general property of ubiquitin ligases and which role multiubiquitin receptors play in this process.

1.3 The p53 degradation pathway

The p53 protein is degraded via the classical ubiquitin-proteasome pathway. It is polyubiquitylated at several C-terminal lysines and subsequently degraded by the proteasome (for a Review, Yang et al., 2004). Mdm2 acts as an E3 ligase for p53 (Honda et al., 1997) and promotes its ubiquitylation and degradation *in vivo* (Haupt et al., 1997; Kubbutat et al., 1997). The corresponding E2 enzyme was identified recently (Saville et al., 2004). UbcH5B/C supports the degradation of the p53 degradation *in vivo* and is the most likely E2 candidate for Mdm2-dependent ubiquitylation of p53 (Saville et al., 2004), although a direct interaction of the UbcH5B/C proteins with the Mdm2 protein was not shown. In addition to Mdm2, other E3 ligases have been shown to ubiquitylate the p53 protein and promote its proteasome-mediated degradation. Pirh2 interacts with the p53 protein and promotes Mdm2-independent p53 ubiquitylation and degradation (Leng et al., 2003). Similar to Mdm2, Pirh2 is a target gene of the p53 protein and participates in autoregulatory negative feedback loop (Leng et al., 2003). Another E3 ligase, COP1, has also been described recently as a direct ubiquitin ligase for p53 (Dornan et al., 2004). The depletion of COP1 by siRNA enhances p53-mediated growth arrest and sensitizes cells to ionizing irradiation (Dornan et al., 2004). The third and most recently discovered physiological ubiquitin ligase for p53 is the human topoisomerase I- and p53-binding protein topors (Rajendra et al., 2004). It is yet uncertain how these proteins are

specifically regulated and under which situations they may be specifically activated. However, the redundancy of four ubiquitin ligases for p53 emphasizes the importance of keeping this tumor suppressor protein under tight control.

1.3.1 The Mdm2 protein is a major negative regulator of p53.

Mdm2-dependent ubiquitylation of the p53 protein is believed to be the most important pathway for p53 degradation and it was actively investigated over the last years. The *mdm2* (mouse double minute 2) gene was originally identified as one of three genes (*mdm1*, *2*, and *3*) which were amplified in a spontaneously transformed mouse BALB/c cell line (3T3-DM; Cahilly-Snyder et al., 1987). The gene product of *mdm2* was subsequently shown to be responsible for the transformation of cells when it is overexpressed (Fakharzadeh et al., 1991). Particularly, genetic deletion of the *mdm2* gene in the mouse demonstrated a critical role of the Mdm2 protein for the regulation of the p53 protein. Mdm2-null mouse embryos died early after implantation (before E6.5), but they were fully rescued when p53 was absent (Jones et al., 1995; Montes de Oca Luna et al., 1995). This result provides genetic evidence that the most important role of the Mdm2 protein is the physiological regulation of p53 protein function, at least in early development (Jones et al., 1995; Montes de Oca Luna et al., 1995).

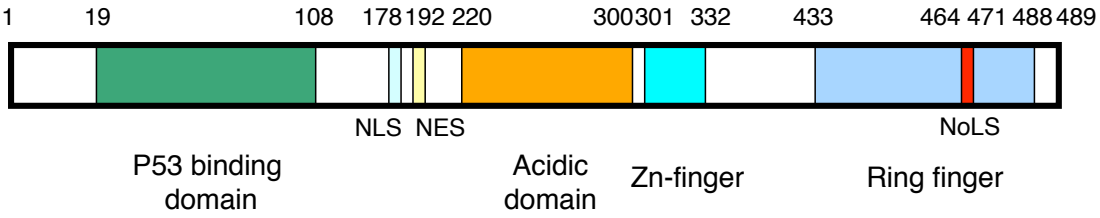


Figure 1.3. Functional domains of the Mdm2 protein. *NLS*, nuclear localization signal; *NES*, nuclear export signal; *Zn-finger*, zinc-finger domain; *NoLS*, nucleolar localization signal; *RING-finger*, RING-finger domain. The numbers above denote amino acid numbers.

The Mdm2 protein is a nuclear phosphoprotein that contains several conserved functional domains (Fig. 1.3). The amino terminus contains p53-interacting domain (Chen et al., 1993; Kussie et al., 1996). Within the central domain, there are binding sites for many Mdm2 regulators, e.g. p300, Arf, L5, L11, L23 and YY1 (Grossman et al., 1998; Llanos et al., 2001; Marechal et al., 1994; Lohrum et al., 2003; Dai et al., 2004; Sui et al., 2004). The central region also contains nuclear localization and nuclear export signals, that mediate nucleo-cytoplasmic shuttling, and a Npl4 zinc finger (NZF) domain. NZF domains are known to bind ubiquitin but the NZF domain of Mdm2 has a very weak ubiquitin-binding activity and its function is largely unknown (Meyer et al., 2002). The carboxyl-terminus of the Mdm2 protein contains a C3H4 RING finger domain that is required for

Mdm2-mediated ubiquitin transfer (Honda and Yasuda, 2000). The C-terminus of Mdm2 also contains a nucleolar localization sequence and binds RNA (Elenbaas et al., 1996).

The Mdm2 protein controls the activity of the p53 protein in two ways. It inhibits the transcriptional activity of p53 by binding to its transactivation domain and concealing it from coactivators and the basic transcriptional machinery (Momand et al., 1992; Kussie et al., 1996). This inhibition has been attributed to the intrinsic transcriptional repressive activity of the N-terminal region of Mdm2 and to the recruitment of a transcriptional corepressor CtBP2 by Mdm2 (Mirnezami et al., 2003). The Mdm2 protein also promotes ubiquitylation and degradation of the p53 protein. Overexpression of the Mdm2 protein decreases p53 protein levels (Haupt et al., 1997; Kubbutat et al., 1997). Further studies demonstrated that Mdm2 is a ubiquitin ligase (Honda et al., 1997). The activity of Mdm2 towards p53 both *in vitro* (Fang et al., 2000; Honda and Yasuda, 2000) and in cells (Fang et al., 2000) is mediated by its C-terminal RING finger. Mdm2 itself is induced by p53 (Barak et al., 1993). Thus, p53 and Mdm2 are linked through an autoregulatory negative feedback loop that maintains low cellular levels of p53. Multiple stimuli including DNA damage, oncogene activation disrupt the p53-Mdm2 negative feedback loop, leading to p53 accumulation and transcriptional activation of p53 target genes (reviewed in Alarcon-Vargas and Ronai, 2002).

1.3.1.1 The interaction of Mdm2 and p53 is a prerequisite for p53 ubiquitylation and degradation.

The interaction of the p53 and Mdm2 proteins is a necessary but insufficient requirement for p53 degradation (Fig. 1.4). The Mdm2 and p53 proteins form a complex via their N-terminal domains (Chen et al., 1993). The minimal Mdm2-binding site on the p53 protein comprises residues 18-26 of which Phe19, Trp23 and Leu26 are the most critical (Chen et al., 1993; Bottger et al., 1997). Accordingly, p53 mutants, in which the Mdm2 binding site has been mutated are resistant to Mdm2-mediated degradation (Haupt et al., 1997; Kubbutat et al., 1997). In comparison to p53, p53-binding region of Mdm2 is relatively large, the direct interaction with the p53 protein has been localized to a hydrophobic pocket (19-108 aa) at the amino terminus of the Mdm2 protein that consists of two structurally similar parts, which form a deep cleft into which the p53 protein inserts as an amphipathic α-helix (Kussie et al., 1996). Nevertheless, sequences far away from the hydrophobic cleft have been shown to affect p53-Mdm2 binding. For instance, phosphorylation of the central domain of the Mdm2 protein at Thr216 by cyclin A/cdk2 weakens its interaction with p53 (Zhang and Prives, 2001). Interestingly, although the N-terminal part of p53 contains the primary docking site for Mdm2, a p53 protein lacking its N-terminus is still able to interact with Mdm2 in the presence of RNA (Burch et al., 2000).

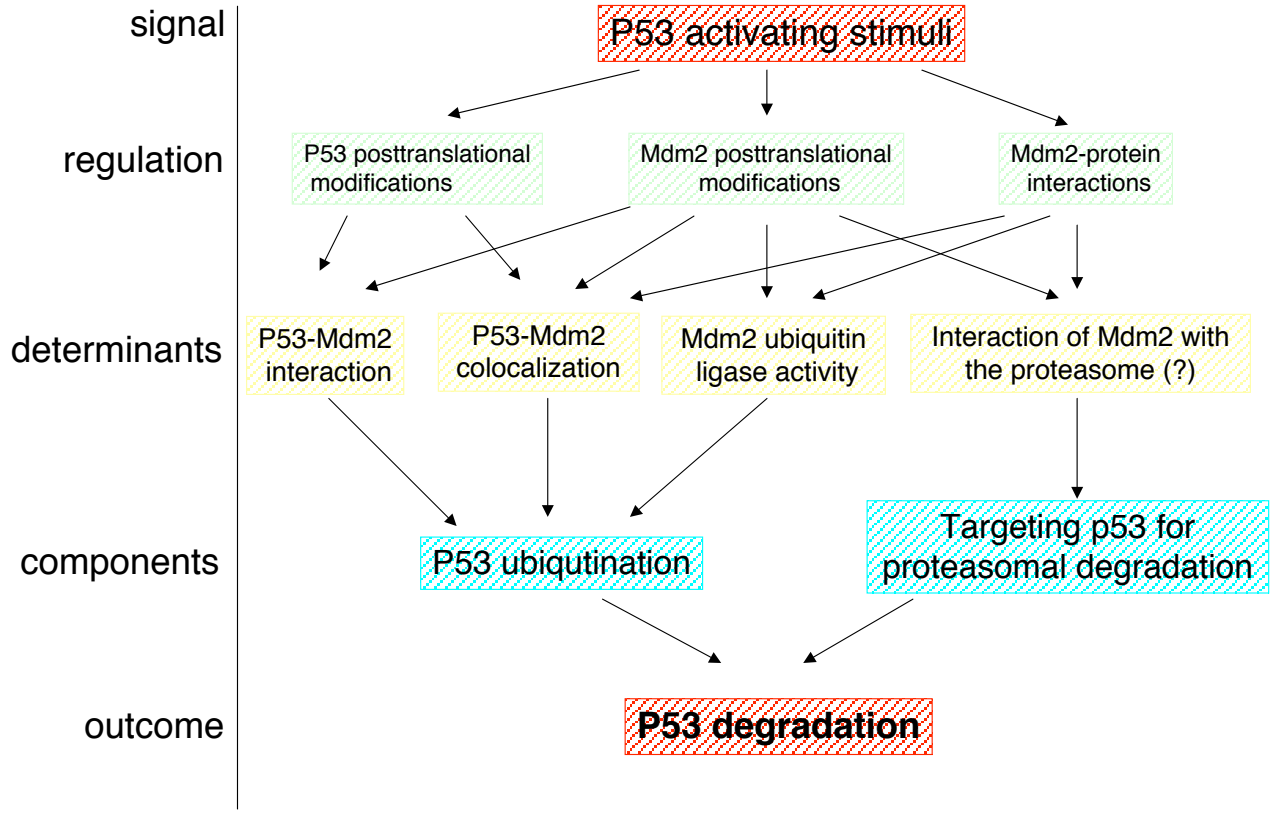


Figure 1.4. Schematic representation of p53 degradation pathway.

This additional interaction site has been mapped to the core domain of the p53 protein where it regulates p53 ubiquitylation and it regulates p53 ubiquitylation as mutation of the critical for the interaction Ser-261 and Leu-264 leads to increased p53 ubiquitylation (Shimuzu et al., 2002). Importantly, nuclear magnetic resonance studies have revealed global conformational changes of the Mdm2 protein after binding of a N-terminal peptide derived from the p53 protein (Schon et al., 2002). It is possible, that the binding of the Mdm2 and p53 proteins via their N-termini exposes a secondary binding motif in the p53 protein that inhibits p53 ubiquitylation by binding to a so far unrecognized domain in the Mdm2 protein. Additional requirement for the interaction of the p53 and Mdm2 proteins includes the p53 homo-tetramerization domain. Monomeric mutants of the p53 protein are not degraded by the Mdm2 protein (Kubbutat et al., 1998). The tetramerization domain of the p53 protein enhances p53 degradation, possibly by improving the Mdm2 binding.

The Mdm2-p53 interaction is thought to be regulated by a number of posttranslational modifications. The N-terminus of the p53 protein can, for instance, be phosphorylated by many kinases (for a review, Bode and Dong, 2004). On the basis of the interaction of the p53 and Mdm2 proteins, it has been suggested that phosphorylation of the p53 protein at

serine residues within the N-terminus is a critical event for the accumulation of the p53 protein in response to DNA damage or oncogene activation since it releases the p53 protein from the inhibitory action of the Mdm2 protein (Shieh et al., 1997; Chehab et al., 1999). However, the mutation of N-terminal serines into alanines doesn't significantly change the ability of the p53 protein to interact with Mdm2 *in vivo* (Lai, et al., 2000) and mutant p53 proteins, were the critical residues have been mutated still accumulate in response to DNA damage (Wu et al., 2002; Blattner et al., 1999b; Ashcroft et al., 1999). Therefore, phosphorylation of N-terminal serines is dispensable for p53 accumulation which is further supported by the fact that several stimuli, e.g. induction of p14ARF, lead to p53 accumulation without N-terminal phosphorylation of the p53 protein (Jackson et al., 2004). Nevertheless, phosphorylation of Thr18 may interfere with the interaction of the p53 and Mdm2 protein and weaken it several fold (Schon et al., 2002).

Not only the p53 protein but also the Mdm2 protein is phosphorylated in response to DNA damage (Khosravi et al., 1999; de Toledo et al., 2000). Phosphorylation of the Mdm2 protein at Ser-17 by DNA-PK is supposed to disrupt the interaction of the p53 and Mdm2 protein (Mayo et al., 1997). Another kinase, ATM, phosphorylates both, the Mdm2 and the p53 protein in response to DNA damage (de Toledo et al., 2000). Although there are no data available regarding the phosphorylation of the N-terminus of the Mdm2 protein by the ATM kinase *in vivo,* ATM phosphorylates Mdm2 *in vitro* at multiple sites, including one site between residues 1 and 115 (Khosravi et al., 1999). In this region only Ser-17 fits with the SQ-motif that is required for DNA-PK or ATM activity. NMR studies have shown that Mdm2 residues 16-24 form a lid over the p53-binding site of Mdm2 (McCoy et al., 2003). When Ser-17 is replaced with an aspartic acid, thus mimicking a phosphorylated serine, the lid has a higher affinity for the p53-binding site of the Mdm2 protein than wild type and in consequence, it prevents the interaction of the Mdm2 protein with the p53 protein (McCoy et al., 2003). Thus, allosteric regulation of Mdm2 may play a role in DNA-damage induced p53 activation.

1.3.1.2 Co-localization of p53 and Mdm2 is a prerequisite for Mdm2-mediated degradation of the p53 protein

Localization of the Mdm2 and p53 proteins is a very important issue for the regulation of p53. Thus, factors that influence their localization will also affect p53 degradation (Fig. 1.4). Several proteins regulate the localization of the Mdm2 protein by inducing nucleoplasmic shuttling (reviewed in O'Brate and Giannakakou, 2003). For example, Akt-dependent phosphorylation of Mdm2 on serines 166 and 168 is necessary for the translocation of the Mdm2 protein from the cytoplasm into the nucleus where it ubiquitylates p53 (Mayo and Donner, 2001; Ogawara et al., 2002; Zhou et al., 2001). In contrast, p14ARF sequesters the Mdm2 protein in nucleoli thus preventing the Mdm2

protein from physical interaction and ubiquitylation of p53 (Tao and Levine, 1999; Weber et al., 1999). The localization of the p53 protein is also regulated by post-translational modifications and protein-protein interactions. In some tumors, e.g. breast adenocarcinoma, undifferentiated neuroblastoma, retinoblastoma or colorectal carcinoma, the p53 protein is located in the cytoplasma and therefore functionally inactive (O'Brate and Giannakakou, 2003).

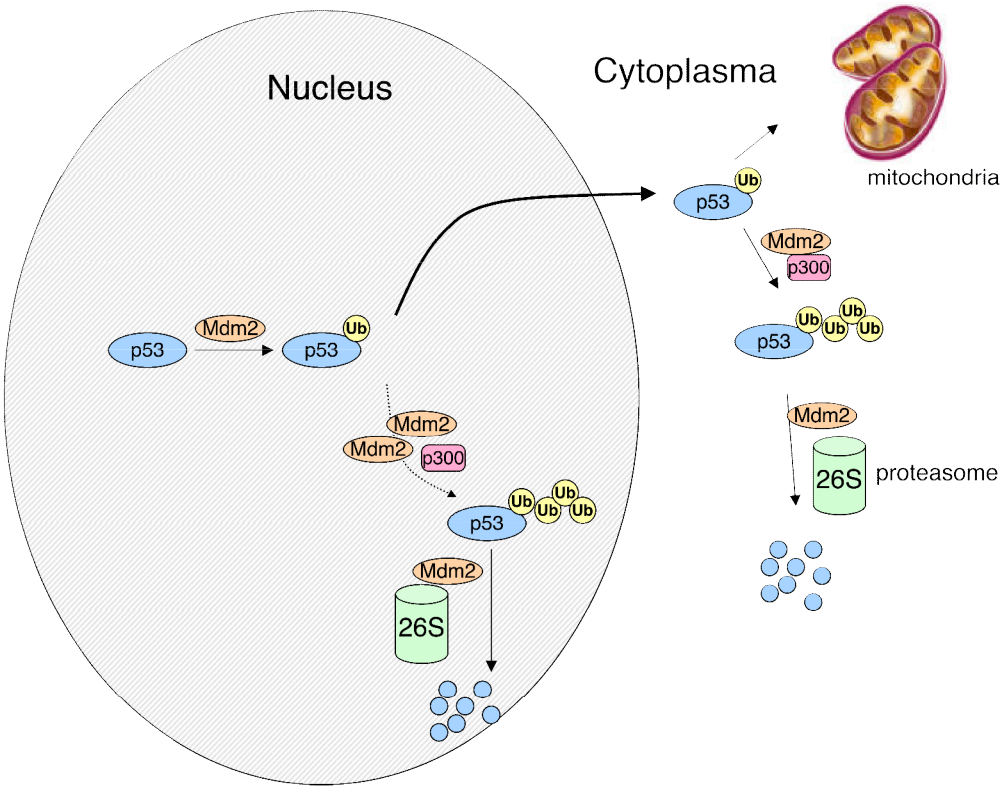


Figure 1.5. Topology of p53 ubiquitylation. Mdm2 monoubiquitylates p53 in the nucleus. Monoubiquitylated p53 is exported out of the nucleus and degraded by cytoplasmic proteasomes. It also mediates apoptosis through its interaction with mitochondria. When Mdm2 levels are high, p53 is polyubiquitylated in the nucleus and degraded by nuclear proteasomes.

In the past, it has been thought that the p53 protein needs to be translocated to the cytoplasm for efficient degradation (Zhang and Xiong, 2001). More recently, however, it was shown that p53 can be degraded in both nucleus and cytoplasm (Xirodimas et al., 2001a). Interestingly, recent evidence suggests monoubiquitylation as a critical signal for regulating nuclear export, and Mdm2 is capable of inducing both monoubiquitylation and polyubiquitylation of p53 (Li et al., 2003). When Mdm2 protein levels are low, it catalyzes monoubiquitylation of p53 that is then effectively exported out of the nucleus and subjected to further modifications in the cytoplasm (Fig. 1.5). When Mdm2 protein levels are high, the p53 protein is quickly polyubiquitylated within the nucleus and degraded by nuclear proteasomes. The reasons for moving monoubiquitylated p53 out of the nucleus are not clear. It is possible that monoubiquitylated p53 has functions in the cytoplasm

other than transcriptional activation. Indeed, cytoplasmic p53 has recently been shown to play an important role in apoptosis through interactions with the mitochondria (Murphy et al., 2004).

1.3.1.3 Ubiquitylation of the p53 protein is tightly regulated

When the p53 and Mdm2 proteins colocalize in the same cellular compartment and interact, Mdm2 transfers ubiquitin moiety to the C-terminus of p53. At the same time, p53 can become deubiquitylated by the ubiquitin protease HAUSP (Li et al., 2002; Li et al., 2004). In response to stresses, ubiquitylation of p53 is inhibited through diverse pathways, depending on the nature of stimulus and cell type.

Significant progress has been made in recent years in understanding the molecular mechanisms of p53 ubiquitylation. The Mdm2 ubiquitin ligase activity is a subject to multiple regulatory events involving Mdm2 posttranslational modifications and protein-protein interactions (Fig. 1.4) and emerging evidence suggests that inhibition of the E3 activity of the Mdm2 protein or of its post-ubiquitylation function are common mechanisms for rescuing the p53 protein from degradation.

1.3.1.3.1 The Mdm2 ubiquitin ligase activity is regulated by protein-protein interactions

Several proteins have recently been shown to play an important role in p53 regulation through their interaction with the Mdm2 protein. By interacting with Mdm2, they regulate the abundance of the p53 protein in an indirect way, namely by activating or inhibiting the Mdm2 ubiquitin ligase activity, or the Mdm2 post-ubiquitylation function (Fig. 1.6; Fig. 1.7). From the other hand, many Mdm2-interacting proteins represent the p53-independent substrates of Mdm2 (Fig. 1.7).

Positive regulators of p53 ubiquitylation

Although the Mdm2 protein has ubiquitin ligase activity towards the p53 protein *in vitro*, in a cellular context it requires several cofactors that support polyubiquitylation of the p53 protein. One of most important cofactors of the Mdm2 protein is Mdmx, an Mdm2 family member that also contains a RING domain (for a review, Marine and Jochemsen, 2004). Nevertheless, despite its appearance as an E3 ligase, the MdmX protein does not seem to have any ubiquitin ligase activity towards p53. Moreover, when it is overexpressed, it can stabilize both, the Mdm2 and the p53 protein (Jackson and Berberich, 2000; Stad et al., 2001). Surprisingly, genetic deletion of both *mdmX* alleles revealed that the MdmX protein does function as a negative regulator of the p53 protein, resulting in embryonic lethality, in a similar way to deletion of the *mdm2* gene (Migliorini et al., 2002). The mechanism for MdmX-mediated p53 stabilization is not clear.

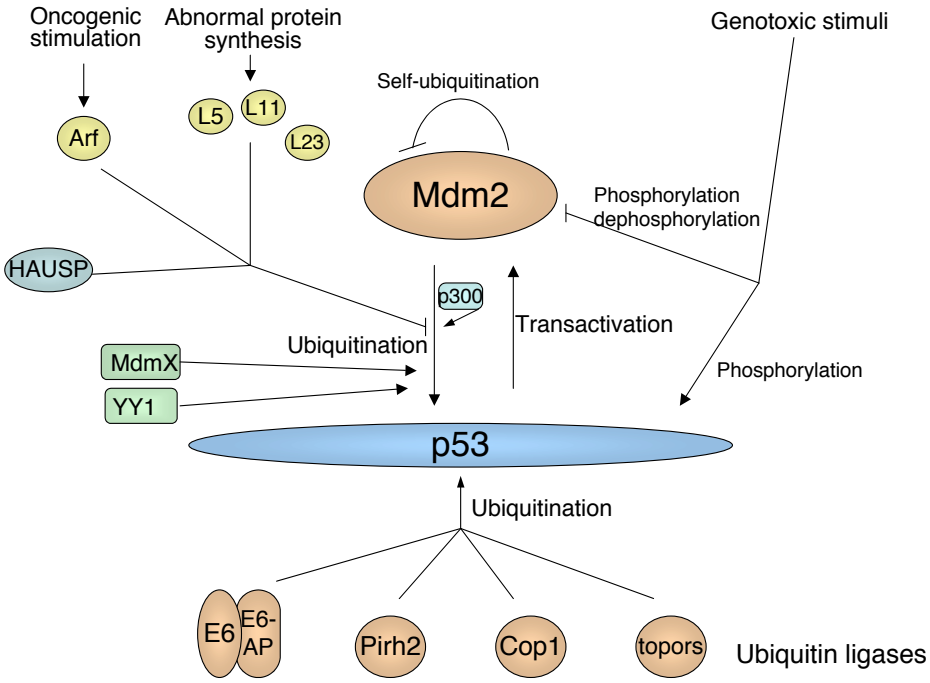


Figure 1.6. Regulation of p53 ubiquitylation. P53 is ubiquitylated mainly by the Mdm2 protein, although other endogenous E3 ligases are capable of ubiquitylating p53. p53 ubiquitylation is stimulated by MdmX, YY1 and p300. Different stress stimuli lead to inactivation of the Mdm2 protein via protein-protein interactions or posttranslational modifications.

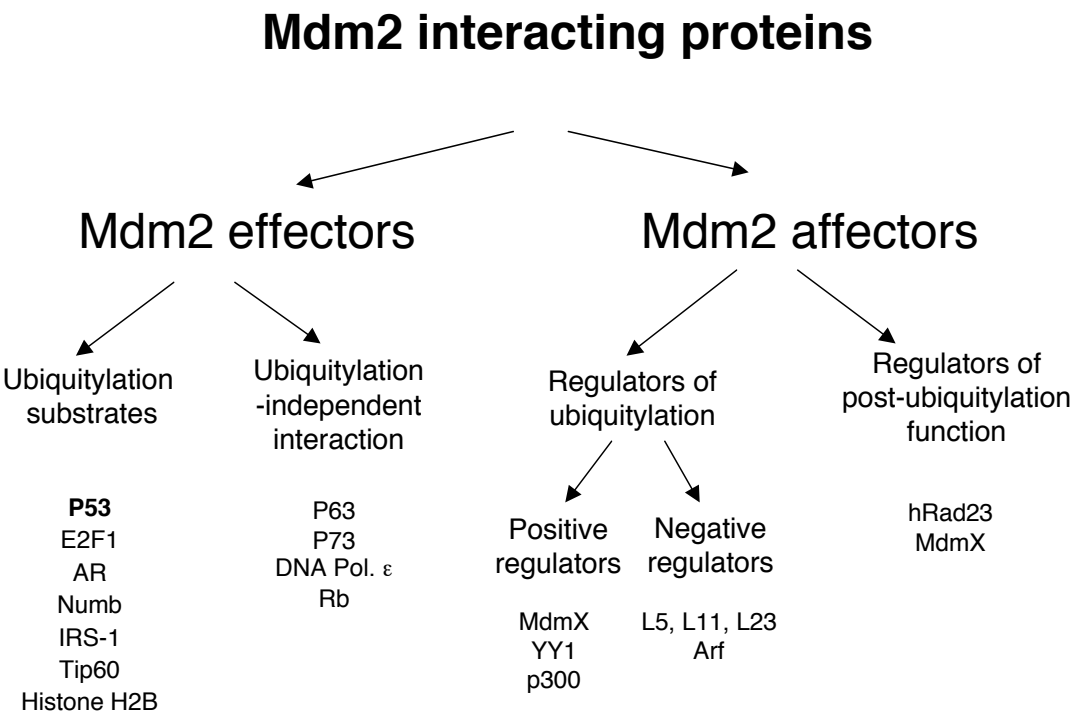


Figure 1.7. Mdm2-interacting proteins.

MdmX seems to block the degradation of p53 rather than its ubiquitylation since polyubiquitylated forms of p53 readily accumulate within the nucleus after MdmX overexpression (Jackson and Berberich, 2000; Stad et al., 2001). However, when the ratio of the MdmX:Mdm2 proteins is low, these proteins cooperatively decrease p53 levels and therefore, mutual dependence model was recently proposed (Gu et al., 2002). According to this model, MdmX stimulates Mdm2 activity towards p53 degradation. The precise mechanism of MdmX function is, however, not as yet clear.

The Mdm2 protein also interacts with the transcriptional co-activator p300 (Grossman et al., 1998). When the levels of the Mdm2 protein are low, it does not polyubiquitylate p53 *in vitro* (Lai et al., 2001). A role for the transcriptional coactivator p300 in p53 polyubiquitylation has been suggested by the observation that adenovirus E1A protein stabilizes the p53 protein, possibly by affecting its ubiquitylation. This effect may depend partly on the interaction of the E1A protein with p300 (Chiou and White, 1997). More recently, p300 has been shown to possess ubiquitin ligase activity and to act as a E4 ubiquitin ligase for p53 *in vivo* (Grossman et al., 2003).

Another transcription factor, Ying Yang 1 (YY1) interacts with both the Mdm2 and p53 protein (Sui et al., 2004). It is a negative regulator of the p53 protein and stimulates Mdm2-dependent polyubiquitylation of p53, possibly by stabilizing the association of the p53 and Mdm2 proteins (Sui et al., 2004; Gronroos et al., 2004). Inactivation of endogenous YY1 enhances the accumulation of the p53 protein as well as the expression of p53 target genes in response to DNA damage, and it sensitizes cells to DNA-damage-induced apoptosis (Gronroos et al., 2004).

Negative regulators of p53 ubiquitylation

Recent work from Rubbi and Milner puts forward a very interesting hypothesis that all p53-inducing signals such as low oxygen levels, heat shock, nucleotide depletion, abnormalities in cell growth and proliferation and DNA damage are actually transmitted through the nucleolus via one and the same mechanism – by disrupting nucleoli (Rubbi and Milner, 2003). When the authors irradiated small sections of the nucleus with ultraviolet light in a way that damaged the DNA but did not compromise nucleolar function, the p53 protein was not stabilized. Conversely, when they injected an antibody that disrupted the nucleolar organisation but did not damage the DNA, the p53 protein was accumulated (Rubbi and Milner, 2003). Obviously, DNA damage *per se* is not enough to stabilize the p53 protein. Stabilization rather depends on nucleolar disruption. One of the hypotheses how the nucleolar disruption leads to p53 accumulation is the release of nucleolar proteins that bind to Mdm2 and inactivate its ubiquitin ligase function.

P14^{ARF} or ARF protein was the first nucleolar protein that was discovered to inhibit p53 ubiquitylation (Ruas and Peters, 1998). p14^{ARF} functions upstream of the Mdm2 protein and blocks its activity. This model is strengthened by the tumorigenicity of ARF knockout mice (Lowe and Sherr, 2003). Still, the exact mechanistic function of p14^{ARF} is not completely understood. The protein is capable of sequestering the Mdm2 protein in the nucleolus, though it can also stabilize the p53 protein independently of Mdm2 relocalization (Llanos et al., 2001). p14^{ARF} directly inhibits the enzymatic function of Mdm2 *in vitro*, however, *in vivo* it only reduces the amount of polyubiquitylated forms of the p53 protein and shows no effect on Mdm2 self-ubiquitylation (Honda and Yasuda, 1999; Midgley et al., 2000; Xirodimas et al., 2001b).

Al least three other nucleolar proteins bind the Mdm2 protein *in vivo*: L5, L11 and L23. A recent report shows that all these proteins associate with the Mdm2 protein in one complex (Dai and Lu, 2004), although Mdm2-L5, Mdm2-L11 and Mdm2-L23 interactions were individually reported previously (Marechal et al., 1994; Lohrum et al., 2003; Dai et al., 2004). Overexpression of any of these proteins activates the p53 protein by preventing Mdm2-mediated ubiquitylation and degradation. It is possible that these ribosomal proteins sense nucleolar stress and subsequently inhibit the Mdm2 protein so that the p53 protein can be efficiently stabilized.

1.3.1.3.2 Non-protein ligands change Mdm2 binding specificity

The Mdm2 protein is a conformationally flexible protein. NMR studies revealed global conformational changes of the overall structure of the Mdm2 protein upon binding of p53-derived peptides (Schon et al., 2002). Interestingly, non-protein ligands alter the conformation of the Mdm2 protein as well (Burch et al., 2000; Burch et al., 2004). The C-terminal domain of Mdm2 has been shown to be a functional zinc-binding domain and RNA-binding domain (Lai et al., 1998; Elenbaas et al., 1996) that can alter the association of the Mdm2 protein with the p53 protein (Burch et al., 2000). Depending on whether the Mdm2 protein is ligand-free, zinc-bound or RNA bound, the conformation of the Mdm2 protein changes and it interacts with different proteins as determined by phage peptide display (Burch et al., 2004).

1.3.1.3.3 The Mdm2 protein interacts with many proteins that regulate multiple pathways

The p53 protein represents a classical protein that is ubiquitylated by the Mdm2 protein and as a consequence, degraded by the proteasome. But it is not the only protein that is regulated by the Mdm2 protein. Mdm2 interacts with multiple factors that regulate transformation, cell cycle control, differentiation, DNA synthesis, RNA biosynthesis, transcription and cell surface turnover (reviewed in Iwakuma and Lozano, 2003).

Recently, several proteins were identified as substrates for Mdm2-mediated ubiquitylation (Fig. 1.7). Among them are Numb (Yogosawa et al., 2003), androgen receptor (Lin et al., 2002), insulin receptor substrate IRS-1 (Usui et al., 2004), histone acetyltransferase Tip60 (Legube et al., 2002), histone H2B (Minsky and Oren, 2004) and, possibly, E2F1 (Blattner et al., 1999a). All these proteins associate with the N-terminus of the Mdm2 protein. Importantly, the same proteins may modulate p53 ubiquitylation possibly via competing for binding the N-terminal substrate-recognition domain of Mdm2 (Legube et al., 2004). The Mdm2 protein also regulates many pathways via ubiquitylation-independent pathways (for review see: Iwakuma and Lozano, 2003), such as the interaction of the E2F1 protein with Rb (Sdek et al., 2004), the suppression of p73 function (Zeng et al., 1999) and the stimulation of DNA polymerase ε (Asahara et al., 2003).

1.3.1.3.4 Mdm2 functions are regulated by post-translational modifications

The ability of the Mdm2 protein to target the p53 protein for proteasomal degradation is regulated by multiple posttranslational modifications of the Mdm2 protein such as phosphorylation, acetylation, ubiquitylation and sumoylation (Fig. 1.4; Fig. 1.8). Depending on the site of modification, one of the different functions of the Mdm2 protein may be affected. The modifications are regulated in response to different types of cellular stresses and most of them are critical for DNA-damage induced p53 activation.

Almost 20% of the amino acids of the Mdm2 protein are serine or threonine residues, and the Mdm2 protein is phosphorylated at multiple sites *in vivo* (Hay and Meek, 2000). The consequences of these phosphorylations are multiple and complex, and every aspect of Mdm2 function seems to be regulated by phosphorylation (for review, Meek and Knippschild, 2003). The interaction of the p53 protein with the Mdm2 protein is, for instance, regulated by phosphorylation of the amino-terminus of the Mdm2 protein by a DNA-PK-like kinase (Mayo et al., 1997). Mdm2 ubiquitin ligase activity and Mdm2 nucleoplasmic shuttling is affected by Akt phosphorylation (Ashcroft et al., 2002; Ogawara et al., 2002) and the post-ubiquitylation function of the Mdm2 protein is regulated by GSK 3-dependent phosphorylation as I demonstrate in my work (Fig. 1.8 and Results). Two clusters of phosphorylation sites are located at the amino terminal (amino acids 1-193) and central (amino acids 200-300) domains of the Mdm2 protein (Hay and Meek, 2000). Mapping of these clusters fits well with more recent studies which have identified a number of sites that are phosphorylated in a cellular context, including (with the modifying enzymes, where known, given in parentheses) Ser17 (DNA-PK; Mayo et al., 1997), Ser166 (Akt; Zhou et al., 2001), Ser186 (Akt; Zhou et al., 2001), Ser188 (Akt; Milne et al., 2004), Thr216 (cyclin A-cdk1/2; Zhang und Prives, 2001), Ser229, Ser232, Ser240, Ser242, Ser246 (CKIδ; Winter et al., in press), Ser253, Ser256, Ser260, Ser262, Ser269

(CK2; Gotz et al., 1999), Tyr394 (c-Abl; Goldberg et al., 2002), and Ser395 (ATM; Maya et al., 2001). Although, many sites were shown to be phosphorylated by specific kinases *in vitro*, for most of them there is very little evidence that they are phosphorylating the Mdm2 protein *in vivo*.

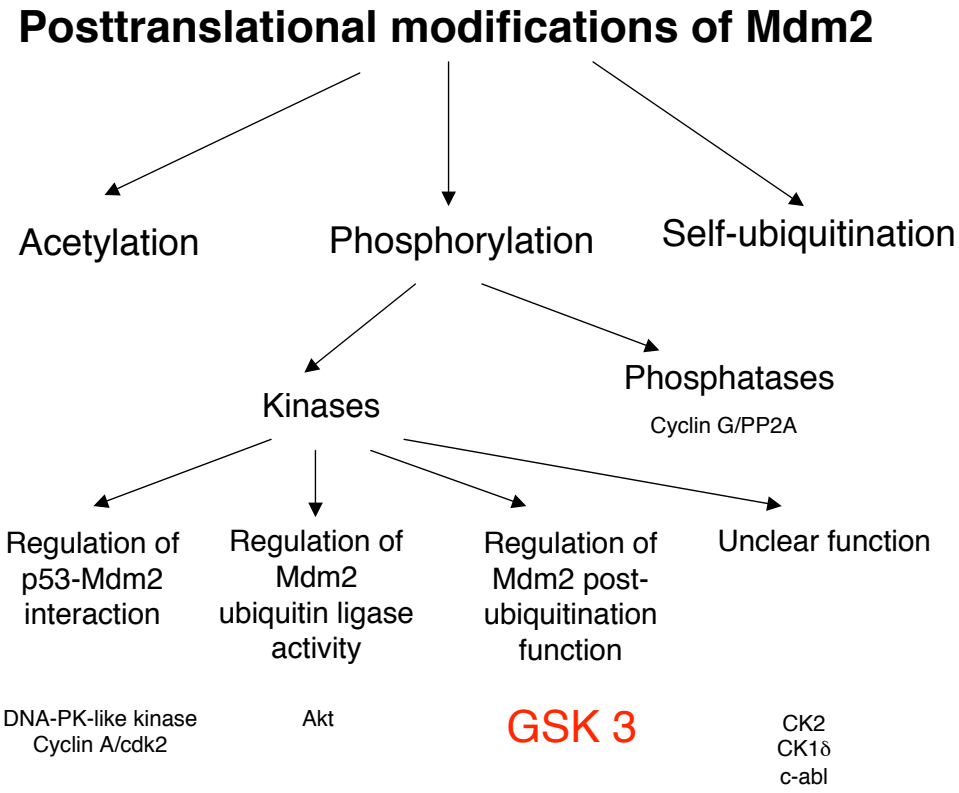


Figure 1.8. Posttranslational modifications of Mdm2.

Apart from several kinases, phosphatase PP2A is also implicated in the regulation of Mdm2 phosphorylation. Two more recent studies have shown that cyclin G1 interacts directly with the Mdm2 protein and with the protein phosphatase 2A (PP2A) holoenzyme encompassing the B' subunit. In this complex, cyclin G1 can act both as a targeting subunit and as a selectivity factor that stimulates PP2A phosphatase activity towards the Mdm2 protein (Kimura and Nojima, 2002; Okamoto et al., 2002). The cyclin G1-PP2A complex dephosphorylates the Mdm2 protein at residues Thr216 and Ser166 (Okamoto et al., 2002). Phosphorylation of these sites has opposing effects on Mdm2 activity (Zhou et al., 2001; Zhang and Prives, 2001), and the biological consequence of cyclin G1-PP2A mediated dephosphorylation is not clear.

The Mdm2 protein is also sumoylated (SUMO-1, Small Ubiquitin-like Modifier 1) *in vivo* by the SUMO E3 ligases Ubc9, PIAS1, and PIASxβ (Buschmann et al., 2001; Miyauchiet al.,

2002). Current evidence suggests that the site(s) of SUMO modification lies within aminoacids 134-212, a region which contains four lysines (Xirodimas et al., 2002). It is proposed that the Mdm2 protein becomes sumoylated as it enters the nucleus, by RanBP2 and further sumoylated by PIAS within the nucleus itself (Miyauchi et al., 2002).

The CBP protein (CREB-binding protein) acetylates the Mdm2 protein *in vitro* and *in vivo* at lysines 466/467 located within the RING finger domain of Mdm2 (Wang et al., 2004), The Mdm2 mutant K466/467Q, which mimicks the Mdm2 protein with an acetylated lysine is severely reduced in its activity regarding p53 ubiquitylation and degradation, as well as in Mdm2 autoubiquitylation (Wang et al., 2004).

The Mdm2 protein is an E3 ubiquitin ligase, which mediates ubiquitylation of the p53 protein and of other substrates as well as the self-ubiquitylation (Iwakuma and Lozano, 2003). Specificity in the transfer of ubiquitin to p53 and Mdm2 itself resides, in part, in the RING domain. Substitution of this domain with a heterologous RING finger permits autoubiquitylation but abolishes ubiquitylation of the p53 protein (Fang et al., 2000). Certain deletion mutants in the central domain of Mdm2 allow to uncouple the p53 ubiqutination and self-ubiquitylation function of Mdm2.
The balance between auto- and substrate-ubiquitylation of Mdm2 is modulated physiologically by posttranslational modifications and by protein-protein interactions. Upon SUMO conjugation, the E3 ligase activity of the Mdm2 protein is shifted towards the p53 protein, while self-ubiquitylation is minimized (Buschmann et al., 2001). Phosphorylation of Mdm2 by Akt induces stabilization of the Mdm2 protein due to a reduction in self-ubiquitylation while p53 ubiquitylation is enhanced (Feng et al., 2004). Also the interaction of the Mdm2 protein with MdmX inhibits the self-ubiquitylation of the Mdm2 protein but does not interfere with p53 degradation (Stad et al., 2001). Obviously, the regulation of p53 ubiquitylation and Mdm2 self-ubiquitylation is different and the underlying mechanisms of these two processes need to be uncovered.

1.3.1.4 hRad23 proteins regulate the post-ubiquitylation function of the Mdm2 protein

hRad23A and hRad23B proteins contain UBL (ubiquitin-like) domains that bind to the S5a subunit of proteasomes and two UBA (ubiquitin associating domains) domains that interact with polyubiquitin chains. Because of these properties the hRad23 proteins were proposed to be the receptors for proteasome substrate recognition (Verma et al., 2004). hRad23 proteins are involved in the regulation of p53 turnover. The overexpression of hRad23B leads to the accumulation of ubiquitylated p53 in Mdm2-dependent manner suggesting that it blocks a post-ubiquitylation function of Mdm2 (Glockzin et al., 2003;

Brignone et al., 2004). The current model states that hRad23 proteins represent a link between the Mdm2 protein and proteasome, which is required for efficient degradation of polyubiquitilated p53 proteins (Glockzin et al, 2003; Brignone et al., 2004). However, several facts contradict to this hypothesis as the silencing of hRad23 with siRNA leads to the increased rates of p53 degradation rather then one would expect from the described model (Brignone et al., 2004). Second, degradation-defective mutant of Mdm2 (Δ211-240) displays an increased binding to hRad23 but it is not able to target p53 for degradation (Brignone et al., 2004). It seems that a role of hRad23 proteins is more complex than it was thought before, and the further investigations should clarify this question.

1.3.2 The central domain of the Mdm2 protein: at the crossroads of p53 ubiquitylation and degradation.

The central domain of the Mdm2 protein encompasses approximately 90 aminoacids, from aminoacid 210 to 300. It is characterized by a very high number of negatively charged aminoacids. The function of this domain is not as yet known but it is clear that it is crucial for p53 ubiquitylation and degradation. Initially, the central domain was only implicated in post-ubiquitylation processing of the p53 protein. Neither the deletion of 222-272 nor 217-246 aminoacids influenced the Mdm2 E3 ligase activity or nucleocytoplasmic shuttling of the Mdm2 protein. However, mutant Mdm2 was not able to target the p53 protein for proteasomal degradation (Argentini et al., 2001; Zhu et al., 2001). Two recent reports demonstrate that the acidic domain is also critical for p53 ubiquitylation (Kawai et al., 2003; Meulmeester et al., 2003). The central domain also contains the determinants of substrate specificity of the Mdm2 protein; for instance, the mutation of 247-252 aminoacids blocks the degradation of p53 but not MdmX (Uldrijan and Vousden, 2004), and the replacement of the whole central domain of the Mdm2 protein with the equivalent domain of the MdmX protein blocks the ability of the Mdm2 protein to ubiquitylate the p53 protein but doesn't block Mdm2 self-ubiquitylation activity (Kawai et al., 2003; Meulmeester et al., 2003).

The regulation of Mdm2 ubiquitin ligase activity by protein-protein interactions is also mediated by the central domain. So, the central domain represents a binding platform for Arf, L5, L11, L23, YY1, p300, hRad23, for all the proteins regulating p53 degradation pathway with possible exception of MdmX (Grossman et al., 1998; Llanos et al., 2001; Marechal et al., 1994; Lohrum et al., 2003; Dai et al., 2004; Sui et al., 2004). Of note, while the interaction of MdmX with Mdm2 stimulates Mdm2 ubiquitylation activity towards p53 and the heterooligomerization of Mdm/MdmX was mapped to the Ring finger domains of both proteins (Tanimura et al., 1999), the central domain of Mdm2 might directly contribute to the Mdm2/MdmX heterooligomerization as the RING finger domain directly interacts with the central domain of Mdm2 *in vitro* (Dang et al., 2002).

A cluster of phosphorylation sites in the central domain of the Mdm2 protein is constitutively phosphorylated in cells (Blattner et al., 2002). This cluster includes serines 238, 240, 244, 251, 254, 258 and 260 (Hay and Meek, 2000; Blattner et al., 2002). Modification of each of these residues may contribute significantly to Mdm2-mediated turnover of the p53 protein. This conclusion is based on the use of a series of mutants of the Mdm2 protein where serines were replaced with an alanine, either as a single mutation or in pairs, block the degradation of the p53 protein to various degrees, (Blattner et al., 2002). Similar to the deletion mutants of the acidic domain, the ability of these mutant proteins to ubiquitylate p53 is not affected indicating that phosphorylation of these serines is critical for a post-ubiquitylation function of the Mdm2 protein. In response to ionizing irradiation, key residues in this domain including serines 238, 240, 244, 251, 254, 258 and 260 become rapidly hypophosphorylated and this hypophosphorylation clearly precedes p53 accumulation (Blattner et al., 2002). Hypophosphorylation could occur through stress-dependent inhibition of kinase(s) or stimulation of a phosphatase(s) or both. Up to now, enzymes that modify this region in response to DNA damage were not known.

1.4 Glycogen Synthase Kinase 3 (GSK 3)

GSK 3 was discovered over 20 years ago as one of several protein kinases that phosphorylated and inactivated glycogen synthase (Embi et al., 1980). It was subsequently purified to homogeneity from skeletal muscle (Woodgett and Cohen, 1984), and molecular cloning revealed that there were two closely related isoforms, GSK 3α and GSK 3β, which are expressed ubiquitously in mammalian tissues (Woodgett, 1990; Woodgett, 1991). The proteins share 97% sequence similarity within their kinase catalytic domains, but differ significantly from one another outside this region, with GSK 3α possessing an extended N-terminal glycine-rich tail.

For many years, GSK 3 was linked exclusively to glycogen metabolism. However, during the last years, GSK 3 was recognized as a multifunctional kinase that regulates many cellular functions as diverse as glucose metabolism, protein synthesis, cell proliferation, microtubule dynamics, cell motility and Wnt-signalling (for a review see: Jope and Johnson, 2004). At present at least 50 protein substrates have been reported for GSK 3, ranging from metabolic enzymes over structural proteins, to transcription factors. Although most of these proteins have not as yet met all of the criteria for being regarded as a physiological GSK 3 substrate (Frame and Cohen, 2001), this large number of substrates illustrates the great potential of GSK 3 to affect many cellular functions. Moreover, it suggests that the activity of GSK 3 must be carefully regulated to avoid indiscriminate phosphorylation of its many substrates. Although the mechanisms regulating GSK 3 are not fully understood, precise control appears to be achieved by a combination of phosphorylation, alteration of localization, and regulation of interactions with GSK 3-binding proteins.

1.4.1 Regulation of GSK 3

1.4.1.1 Regulation of GSK 3 by phosphorylation

GSK 3 is one of the few protein kinases that are constitutively active. Its activity is, however, significantly reduced by phosphorylation of an N-terminal serine, Ser9 in GSK 3β and Ser21 in GSK 3α (Sutherland et al., 1993; Sutherland and Cohen, 1994). Several kinases can phosphorylate these serines (Fig. 1.9), including PKB, protein kinase A (PKA), protein kinase C (PKC), p90Rsk and p70S6K (Goode et al., 1992; Cross et al., 1995; Sutherland and Cohen, 1994; Li et al., 2000). Accordingly, many signaling pathways control GSK 3 activity. It is possible that each kinase affects only a specific pool of the GSK 3 present in cells because of the subcellular distribution of both GSK 3 and each regulatory kinase (Frame and Cohen, 2001). Scaffolding proteins provide one mechanism to restrict interactions between the kinases. For example, PKA-anchoring protein 220 binds GSK 3 to facilitate its phosphorylation by PKA (Tanji et al., 2002). In contrast to the inhibitory regulation by phosphorylation of serines in the N-terminal domain, GSK 3 activity is enhanced by

phosphorylation of Tyr216 in GSK 3β and Tyr279 in GSK 3α (Hughes et al., 1993). This might occur by autophosphorylation or by other tyrosine kinases but little is known about regulation of the processes that modulate tyrosine phosphorylation of GSK 3 (Frame and Cohen, 2001).

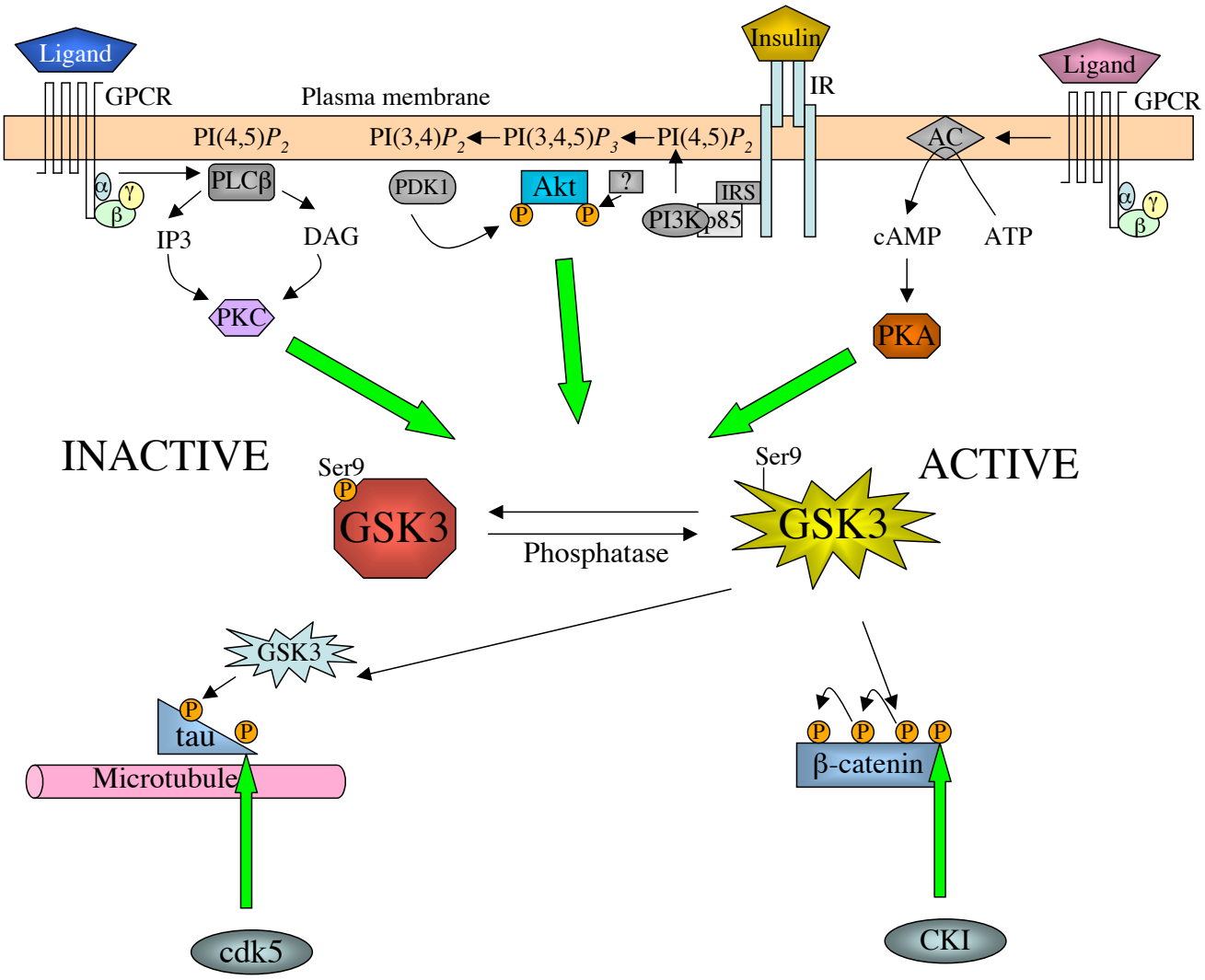


Figure 1.9. Regulation of glycogen synthase kinase 3 (GSK 3) by phosphorylation. Several signaling cascades activate kinases that directly inhibit GSK 3 by phosphorylating a regulatory N-terminal serine of GSK 3 (shown as Ser9 of GSK 3β). Reactivation of GSK 3 is mediated by specific protein phosphatases. Many substrates of GSK 3 must be primed, which means they need to be pre-phosphorylated at a serine or threonine, four residues away from the serine or threonine that will be phosphorylated by GSK 3. Thus, the consensus site for phosphorylation of primed substrates by GSK 3 is [S/T]xxx[S/T](*P*). Priming provides an additional regulatory mechanism to control phosphorylation of substrates by GSK 3 because signalling pathways that prime its substrates must be active before GSK 3 can phosphorylate the substrates. Two examples are shown, pre-phosphorylation of the microtubule-associated protein tau by cdk5 facilitates phosphorylation of the tau protein by GSK 3 (Cho and Johnson, 2003). β-catenin must be pre-phosphorylated by casein kinase I (CKI) at Ser45 for GSK 3 to phosphorylate β-catenin at sites Ser41, Ser37, Ser33 and to target it for proteasomal degradation (Amit et al., 2002).

The activity of GSK 3 is often regulated by the phosphorylation state of its substrates. Most substrates must be pre-phosphorylated (primed) to allow phosphorylation by GSK 3 (Fig. 1.9). This type of regulation allows to inhibit GSK 3 phosphorylation indirectly, via inhibiting the activity of the priming kinase. Thus, two sets of phosphorylation-based signaling systems are integrated to regulate the actions of GSK 3. Upstream of GSK 3, signaling systems transmit regulatory information to GSK 3 via kinases that directly phosphorylate GSK 3 to control its activity. Downstream of GSK 3, another set of signaling systems transmit information by pre-phosphorylating substrates of GSK 3. The timing and location of these signals individually control the activity of GSK 3 towards each substrate.

1.4.1.2 Regulation of the intracellular localization of GSK 3

In addition to regulation by phosphorylation, mechanisms that regulate the intracellular localization of GSK 3 control its access to substrates. GSK 3 is predominantly localized in the cytoplasm, but to some extent also in nuclei and mitochondria, where it is highly active in comparison with cytosolic GSK 3 (Bijur and Jope, 2003). Nuclear levels of GSK 3 change dynamically in response to stimuli. They fluctuate during the cell cycle, being highest in the S-phase, which allows phosphorylation of nuclear cyclin D1 by GSK 3 (Diehl et al., 1998). Nuclear levels of GSK 3 rapidly increase early in apoptosis, thus enabling GSK 3 to modulate gene expression by regulating transcription factors (Bijur and Jope, 2001). During replicative senescence in human fibroblasts, GSK 3 accumulates in the nucleus and contributes to senescence-associated p53 accumulation (Zmijewski and Jope, 2004).

1.4.1.3 Regulation of GSK 3 by binding proteins

GSK 3 activity is also regulated by protein-protein interactions. The best characterized example is the canonical Wnt signaling pathway where GSK 3 binding proteins control access to its substrate, β-catenin (for a recent review, Patel et al., 2004). In the absence of a stimulus, the scaffold protein axin binds GSK 3, casein kinase I, β-catenin and other proteins. This enables casein kinase I to phosphorylate Ser45 on β-catenin thus creating a priming site for GSK 3. In turn, GSK 3 phosphorylates Thr41 and, subsequently, Ser37 and Ser33 (Fig. 1.9). These modifications promote β-catenin degradation (Amit et al., 2002). Wnt stimulation activates disheveled (dvl), which, together with the GSK 3-binding protein Frat, facilitates disruption of the axin-based complex. This decreases the phosphorylation of β-catenin, which results in β-catenin accumulation and activation (Patel et al., 2004).

AIM

The aim of this thesis is to identify kinases that phosphorylate the central domain of the Mdm2 protein. The Mdm2 protein is constitutively phosphorylated *in vivo* at several sites that are critical for p53 degradation. Until recently, no kinases were known that phosphorylate the Mdm2 protein at these sites. Since glycogen synthase kinase 3 (GSK 3) has two consensus phosphorylation sites within the central domain of Mdm2, my first goal was to test whether the Mdm2 protein is phosphorylated by GSK 3 *in vitro* and *in vivo*. Because GSK 3 phosphorylated the Mdm2 protein at sites that are critical for p53 degradation, I characterized the effect of GSK 3 inhibition on p53 ubiquitylation and degradation. Since the central domain of the Mdm2 protein is hypophosphorylated upon ionizing irradiation, I tested the role of GSK 3 for the DNA damage induced p53 accumulation.

2. MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Chemicals

All chemicals were of the highest quality and were purchased from:

Agarose	*Peqlab*, Erlangen, Germany
Alsterpaullone	*Calbiochem*, Bad Soden, Germany
Ampicillin/streptomycin, 100 U/ml	*Gibco-BRL*, Karlsruhe, Germany
Bacto-agar	*Otto-Nordwald KG*, Hamburg, Germany
Bisindolylmaleimide I	*Calbiochem*, Bad Soden, Germany
Bisindolylmaleimide IX	*Calbiochem*, Bad Soden, Germany
BSA, Fraction V	*PAA Laboratories GmbH*, Pasching, Austria
Diethyl pyrocarbonate	*Fluka*, Buchs, Germany
DMSO	*Fluka*, Buchs, Germany
Guanidinium chloride	*BDH Laboratory Supplies*, Poole, England
Hydrogen peroxide 30%	*Fluka*, Buchs, Germany
Hydromount	*National Diagnostics*, Hessle, England
IPTG	*Calbiochem*, Bad Soden, Germany
Jet-Pei	*PolyPlus*, Illkirch, France
β-Mercaptoethanol	*Serva*, Heidelberg, Germany
MG132 Proteasome inhibitor	*Calbiochem*, Bad Soden, Germany
Phosphate-buffered saline (PBS)	*Gibco-BRL*, Karlsruhe, Germany
Puromycin dihydrochloride	*Calbiochem*, Bad Soden, Germany
Skimmed milk powder	Saliter, Obergünzburg, Germany
Trypsin	*Difco*, Detroit, USA

All other chemicals were, unless otherwise stated, purchased from *Carl Roth GmbH* & Co. (Karlsruhe, Germany), *Merck* (Darmstadt, Germany) or *Sigma* (Deisenhofen, Germany).

Radiochemicals:

[γ-^{32}P]-dATP	5 mCi/mmol, 10 mCi/ml	*Perkin-Elmer*, Rodgau-Jügesheim, Germany
[α-^{32}P]-dCTP	1 tip, 50 µCi, 3000 Ci/mmol	*Amersham*, Freiburg, Germany
[α-^{33}P]-ddNTP	1500 Ci/mmol, 450 mCi/ml	*Amersham*, Freiburg, Germany
[^{32}P]-orthophosphate	10 mCi/ml	*Perkin-Elmer*, Rodgau-Jügesheim, Germany

2.1.2 Kits

Easy Pure DNA Purification Kit	*Biozym Diagnostik GmbH*, Oldendorf, Germany
MACSelect™ Transfected Cells Selection Kit	*Miltenyi Biotech*, Bergisch Gladbach, Germany
Prime-a-gene Labelling Kit	*Promega*, Mannheim, Germany
Qiagen Plasmid Maxi Kit	*Qiagen*, Hilden, Germany
QuikChange® site-directed mutagenesis kit	*Stratagene*, Amsterdam, the Netherlands
Zero® Blunt® TOPO PCR cloning kit	*Invitrogen*, Karlsruhe, Germany

2.1.3 Binding matrices

Glutathione Sepharose 4B	*Amersham*, Freiburg, Germany
Ni-NTA-Agarose	*Qiagen*, Hilden, Germany
Protein A Sepharose	*Pierce*, Bonn, Germany
Sephadex G-50	*Amersham*, Freiburg, Germany
Oligo(dT)-cellulose	*Amersham*, Freiburg, Germany

2.1.4 Oligonucleotides

All the oligonucleotides were purchased from *Biomers.net* (Ulm, Germany) or *MWG Biotech* (Ebersberg bei München, Germany).

Oligonucleotides for site-directed mutagenesis:

GSK 3β Ser9→Ala:

S9A GSK3_fw:	5'-CGGCCCAGAACCACCGCCTTTGCGGAGAGCTGC-3'
S9A GSK3_rev:	5'-GCAGCTCTCCGCAAAGGCGGTGGTTCTGGGCCG-3'

Mdm2 point mutations:

S227,230A Mdm2_fw:	5'-GATGGCGTAGCTGAGCATGCTGGTGATTGCCTG-3'
S227,230A Mdm2_rev:	5'-CAGGCAATCACCAGCATGCTCAGCTACGCCATC-3'
S238,240A Mdm2_fw:	5'-GGATCAGGATGCAGTTGCTGATCAGTTTAGCG-3'
S238,240A Mdm2_rev:	5'-CGCTAAACTGATCAGCAACTGCATCCTGATCC-3'
S244A Mdm2_fw:	5'-GTTTCTGATCAGTTTGCCGTGGAATTTGAAG-3'
S244A Mdm2_rev:	5'-CTTCAAATTCCACGGCAAACTGATCAGAAAC-3'
S251,254A Mdm2_fw:	5'-GAAGTTGAGGCTCTGGACGCGGAAGATTAC-3'
S251,254A Mdm2_rev:	5'-GTAATCTTCCGCGTCCAGAGCCTCAACTTC-3'
S258,260A Mdm2_fw:	5'-CTCGGAAGATTACGCCCTGGCTGACGAAGGGCAC-3'

S258,260A Mdm2_rev:	5'-GTGCCCTTCGTCAGCCAGGGCGTAATCTTCCGAG-3'
S267A Mdm2_fw:	5'-GGGCACGAGCTCGCAGATGAGGATGAT-3'
S267A Mdm2_rev:	5'-ATCATCCTCATCTGCGAGCTCGTGCCC-3'
S284,288A Mdm2_fw:	5'-GACAGGAGAAGCCGATACAGACGCTTTTGAAGGAG-3'
S284,288A Mdm2_rev:	5'-CTCCTTCAAAAGCGTCTGTATCGGCTTCTCCTGTC-3'
S296A Mdm2_fw:	5'-GATCCTGAGATTGCCTTAGCTGAC-3'
S296A Mdm2_rev:	5'-GTCAGCTAAGGCAATCTCAGGATC-3'

Mdm2 deletion mutants:

Delta201-300_fw:	5'CTGAGGGAGATGTGCAGCTGGAAGTGTACCTCATG-3'
Delta201-300_rev:	5'-CATGAGGTACACTTCCAGCTGCACAATCTCCCTCAG-3'
Delta201-235_fw:	5'-CTGAGGGAGATGTGCAGCCAGGATTCAGTTTCTGA-3'
Delta201-235_rev:	5'-TCAGAAACTGAATCCTGGCTGCACATCTCCCTCAG-3'
Delta236-260_fw:	5'-TCTGGTGATTGCCTGGATGACGAAGGGCACGAGCT-3'
Delta236-260_rev:	5'-AGCTCGTGCCCTTCGTCATCCAGGCAATCACCAGA-3'
Delta261-300_fw:	5'-GAAGATTACAGCCTGAGTTGGAAGTGTACCTCATG-3'
Delta261-300_rev:	5'-CATGAGGTACACTTCCAACTCAGGCTGTAATCTTC-3'

Oligonucleotides for cloning the N-terminus (1-206 aa), central domain (220-305 aa) and C-terminus (296-491 aa) of Mdm2 into the pGEX4T-2 vector:

Central domain_fw:	5'-GTCGACGCCCTCGCATCAGGATCTTGACG-3'
Central domain_rev:	5'-TCAATAGTCAGCTAAGGAAATCTCAGGATC-3'
N-terminus_fw:	5'-CCAGGAATTCCCATGTGCAATACCAACATGTCTGTACC-3'
N-terminus_rev:	5'-GGGCGGCCGCTAACATATCTCCCTTATTACACACAG-3'
C-terminus_fw:	5'-CCAGGAATTCCCGAAATTTCCTTAGCTGACTATTGG-3'
C-terminus_rev:	5'-GGGCGGCCGCTAGGGGAAATAAGTTAGCACAATCAT TTG-3'

Oligonucleotides for cloning into pSUPER or pSUPER.neo-gfp vectors:

SiRNA_mdm2_1260_fw:	5'-GATCCCCGACAAAGAAGAGAGTGTGGTTCAAGAGACC ACACTCTCTTCTTTGTCTTTTTGGAAA-3'
SiRNA_mdm2_1260_rev:	5'-AGCTTTTCCAAAAAGACAAAGAAGAGAGTGTGGTCTC TTGAACCACACTCTCTTCTTTGTCGGG-3'
SiRNA_GSK3β_fw:	5'-GATCCCCCATAGTCCGATTGCGTTATTTCAAGAGAATA ACGCAATCGGACTATGTTTTTGGAAA-3'

SiRNA_GSK3β_rev:	5'-AGCTTTTCCAAAAACATAGTCCGATTGCGTTATTCTCT TGAAATAACGCAATCGGACTATGGGG-3'

Oligonucleotides for cloning p21 from cDNA:

P21_fw:	5'-CCGGAATTCTTGAAAATGTCAGAACCGGCTGGG GATG-3'
p21_rev:	5'-GGGCGGCCGCCTAGGGCTTCCTCTTGGAGAAG-3'

Oligonucleotides for adding a C-terminal Flag-tag (DYKDDDDK) to GSK 3β:

GSK3_fw:	5'-CCGGAATTCTTGAAAATGTCAGGGCGGCCCAGA ACCACC-3'
GSK3-Flag_rev:	5'-GGGCGGCCGCTCATTTGTCATCGTCGTCCTTGT AGTCGGTGGAGTTGGAAGCTGATGC-3'

2.1.5. Plasmids

pcDNA3.1, a vector for eucaryotic expression under the control of the CMV promotor, was purchased from *Invitrogen* (Karlsruhe, Germany).

pSUPER, pSUPER.neo-gfp, vectors for eucaryotic expression of siRNA-like transcripts under the control of the promoter of the polymerase-III H1-RNA gene. pSuper.neo-gfp vector also encodes a neomycin resistance gene that is fused with the enhanced green fluorescent protein gene (EGFP). Both vectors were purchased from *Oligoengine.*

pGEX4T-2, a vector for inducible bacterial expression of (Glutathione S-Transferase) GST-fusion proteins under the control of the tac promotor, purchased from *Amersham* (Freiburg, Germany).

pMACS K^k.II, a vector for eucaryotic expression of the cell surface marker H-$2K^k$, purchased from *Miltenyi Biotech* (Bergisch Gladbach, Germany).

pcDNA3 His-ubiquitin was a gift from S. Mittnacht.

pcDNA3-wtGSK3β, pcDNA3-R96A-GSK3β were a gift from T. Hagen.

pcDNA3-Mdm2, pcDNA3-mdm2 S238/240A, pcDNA3-Mdm2 S251A/S254A, pcDNA3-p53 and **pDWM 659 N-Myc-Mdm2** have been described previously (Blattner et al., 2002).

pcDNA3-S9A-GSK3β was created by site-directed mutagenesis of pcDNA3-wtGSK3 using the QuikChange® site-directed mutagenesis kit.

pcDNA3-Flag-wt-GSK3β and **pcDNA3-Flag-S9A-GSK3β** encoding wt GSK3β and S9A GSK3β with C-terminal Flag-tag were created by PCR using GSK3-Flag_rev and GSK3_fw primers and pcDNA3-wtGSK3β or pcDNA3-S9A-GSK3β as templates. The PCR products were ligated into pCR®-Blunt II-TOPO® vector. pCR®-Blunt II-TOPO-wt GSK3-

Flag and pCR®-Blunt II-TOPO-S9A-GSK3-Flag were digested with *EcoRI* and *NotI*. The resulting inserts were isolated and ligated into pcDNA3.1.

pDWM659 Myc-Mdm2-Δ201-300, pDWM659 Myc-Mdm2-Δ201-235, pDWM659 Myc-Mdm2-Δ236-260, pDWM659 Myc-Mdm2-Δ261-300, pDWM659 N-Myc-Mdm2 S227,230A, pDWM659 N-Myc-Mdm2 S238,240A, pDWM659 N-Myc-Mdm2 S244A, pDWM659 N-Myc-Mdm2 S251,254A, pDWM659 N-Myc-Mdm2 S258,260A, pDWM659 N-Myc-Mdm2 S267A, pDWM659 N-Myc-Mdm2 S284,288A Mdm2 and **pDWM659 N-Myc-Mdm2 S296A** were generated by mutating pDWM659 N-Myc-Mdm2 using the QuikChange® site-directed mutagenesis kit and Delta201-300_fw, Delta201-300_rev, Delta201-235_fw, Delta201-235_rev, Delta236-260_fw, Delta236-260_rev, Delta261-300_fw, Delta261-300_rev, S227,230A Mdm2_fw, S227,230A Mdm2_rev, S238,240A Mdm2_fw, S238,240A Mdm2_rev, S244A Mdm2_fw, S244A Mdm2_rev, S251,254A Mdm2_fw, S251,254A Mdm2_rev, S258,260A Mdm2_fw, S258,260A Mdm2_rev, S267A Mdm2_fw, S267A Mdm2_rev, S284,288A Mdm2_fw, S284,288A Mdm2_rev, S296A Mdm2_fw and S296A Mdm2_rev primers.

pcB6-Bax and **pCMV-PUMA** were a gift from K. Vousden.

pSuper-GSK3β and **pSuper.neo.gfp-Mdm2** encoding siRNA against human GSK 3β (region 323-342) and human Mdm2 (region 1232-1241) were created by cloning annealed SiRNA_GSK3β_fw and SiRNA_GSK3β_rev or SiRNA_mdm2_1260_fw and SiRNA_mdm2_1260_rev oligonucleotides into the HindIII and BglII site of pSuper and pSuper-neo.gfp.

pGEX-4T-2 wt mdm2 (220-305), pGEX-4T-2 S238/240A mdm2 (220-305) and **pGEX-4T-2 S251/254A mdm2 (220-305)** encoding wild type or phosphorylation site mutants of the central domain of Mdm2 were generated by amplifying the central domain of Mdm2 with Central domain_fw and Central domain_rev primers using pcDNA3-Mdm2, pcDNA3-mdm2 S238/240A or pcDNA3-Mdm2 S251A/S254A as a template. The PCR products were ligated into pCR®-Blunt II-TOPO® vector. The resulting pCR®-Blunt II-TOPO-mdm2 (220-305), pCR®-Blunt II-TOPO- S238/240A mdm2 (220-305) and pCR®-Blunt II-TOPO-S251/254A mdm2 (220-305) plasmids were digested with EcoRI-NotI and the inserts were ligated into the pGEX4T-2 vector.

pGEX-4T-2 wt mdm2 (1-206) and **pGEX-4T-2 wt mdm2 (296-491)** encoding N-terminus and C-terminus of Mdm2, were obtained from Markus Winter.

2.1.6. Antibodies

Primary antibodies:

p53:	DO-1, monoclonal (Vojtesek et al., 1995)
	AB-2, monoclonal (*Oncogene*, Bad Soden, Germany)
	CM-1, rabbit polyclonal (Midgley et al., 1992)

Mdm2:	4B2, monoclonal (Chen et al., 1993)
PCNA:	PC10, monoclonal (Waseem and Lane, 1990)
GST/GST-fusion proteins:	anti-GST, goat polyclonal (*Rockland*, Gilbertsville, USA)
myc-tagged Mdm2:	9E10, monoclonal (*Oncogene*, Bad Soden, Germany)
GSK 3:	anti-GSK-3β, monoclonal (*BD Biosciences*, Heidelberg, Germany)
	anti-phospho-Ser9-GSK-3β, rabbit polyclonal (*Cell Signaling*, Beverly, USA)
E2F-1:	sc-193, rabbit polyclonal (*Santa Cruz*, Santa Cruz, USA)
S6b /Rpt3:	TPB7-27, monoclonal (*Biomol*, Hamburg, Germany)
β-catenin:	anti-β-catenin, monoclonal (*BD Biosciences*, Heidelberg, Germany)

Secondary antibodies:

anti-mouse:	P0161, HRP-conjugated (*DakoCytomation GmbH*, Hamburg) Germany)
	Cy-2 conjugated (*Dianova*, Hamburg, Germany)
anti-rabbit:	P0448, HRP-conjugated (*DakoCytomation GmbH*, Hamburg) Germany)
	Cy-3 conjugated (*Dianova*, Hamburg, Germany)
anti-goat:	PO160, HRP conjugated (*DakoCytomation GmbH*, Hamburg) Germany)

2.1.7. Enzymes

Casein Kinase I δ	*New England Biolabs*, Beverly, USA
Chymotrypsin, sequencing grade	*Sigma*, Deisenhofen, Germany
DNA Polymerase I, Klenow Fragmen	*Promega*, Mannheim, Germany
Glygogen Synthase Kinase 3 β (GSK 3β)	*Upstate Biotechnology*, Charlottesville, USA
Lysozyme	*Sigma*, Deisenhofen, Germany
M-MLV Reverse Transcriptase	*Promega*, Mannheim, Germany
Pfu polymerase	*Promega*, Mannheim, Germany
PfuTurbo DNA polymerase	*Stratagene*, Amsterdam, the Netherlands
Proteinase K	*Sigma*, Deisenhofen, Germany
T4 DNA ligase	*Promega*, Mannheim, Germany
T4 Polynucleotide kinase	*Promega*, Mannheim, Germany
Thermo Sequenase	*USB*, Cleveland, USA

All *restriction endonucleases* were purchased from *Promega*, Mannheim, Germany,

2.1.8 Bacteria

E. coli BL21: F$^-$, *omp*T, *hsd*S (rB$^-$, mB$^-$), *gal*, *dcm*, bacterial strain for expression of the GST-fusion proteins, were purchased from *Amersham*, Freiburg, Germany.

E. coli DH5α: *sup*E44Δ*lac*U169(φ80*lac*ZΔM15)*hsd*R17*rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1, were obtained from Christine Blattner.

E. coli TOP10: F$^-$ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *deo*R *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G, were included in the Zero® Blunt® TOPO PCR cloning Kit.

E. coli XL-1 Blue: *rec*A1 *end*A1 *gyr*A96 *thi*-1 *hsd*R17 *sup*E44 *rel*A1 *lac* [F′ *pro*AB *lac*I^{q}ZΔM15 Tn10 (Tet^r)] were included in the QuikChange® site-directed mutagenesis kit.

2.1.9 Cell lines and media

NIH 3T3 cells were grown in Dulbecco's modified Eagle medium (DMEM; *Gibco-BRL*, Karlsruhe) supplemented with 10% donor calf serum (*Gibco-BRL*, Karlsruhe, Germany) and 100 U/ml streptomycin and penicillin (*Gibco-BRL*, Karlsruhe, Germany) in a humidified atmosphere with 5% CO_2 at 37°C. All other adherent cell lines were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum (*PAA Laboratories GmbH*, Pasching, Austria) and 100 U/ml streptomycin and penicillin.

U2OS, human, osteosarcoma, epithelial, ATCC number HTB-96, obtained from Christoph Englert.

H1299, human, lung carcinoma, epithelial, ATCC number CRL-5803, obtained from David Lane.

COS-7, African green monkey, kidney, SV40 transformed fibroblasts, ATCC number CRL-1651, obtained from David Meek.

NIH/3T3 mouse embryonic immortalized fibroblasts, obtained from Margarethe Litfin.

2.1.10 Other materials

20 × 20 cm cellulose chromatography plates	*Merck*, Darmstadt, Germany
ECL Hyperfilm	*Amersham*, Freiburg, Germany
Filter paper 3MM	*Bender&Hobein*, Karlsruhe, Germany
Hybond N+ membrane	*Amersham*, Freiburg, Germany
Immobilon-P (PVDF membrane)	*Millipore*, Bedford, USA
MP Hyperfilm	*Amersham*, Freiburg, Germany
P81 ion-exchange paper	*VWR*, Darmstadt, Germany
Petri dishes	*Greiner Labortechnik*, Nürtingen, Germany

2.2 METHODS

The composition of commonly used buffers and routine methods were taken from Molecular Cloning (Maniatis et al., 1989) unless otherwise stated.

2.2.1 CELL CULTURE AND TRANSFECTION METHODS

2.2.1.1 Cell culture

All cell lines were maintained at 37°C in a Steri-Cult 200 incubator (*Forma Scientific*, Marietta, USA) in 5% CO_2 and 95% air humidity. Adherent cells were grown until they reached 80-90% confluence. For trypsinization, the medium was aspirated and cells were washed once with PBS (137 mM NaCl, 2.7 mM KCl, 6.5 mM Na_2HPO_4, 1.5 mM K_2HPO_4). 1 ml of 0.25% trypsin was added per 50 cm^2 and the cells were incubated at 37°C until they detached from the culture dish. The trypsin solution was diluted 1:5 with fresh culture medium, and the cell suspension was transferred to a centrifugation tube. Cells were collected by centrifugation for 2 min at 1200 rpm and re-plated.

2.2.1.2 Freezing and thawing of cells

For freezing, logarithmically growing cells were trypsinized as described in section 2.2.1.1 and collected by centrifugation. The cells were resuspended in freezing medium (DMEM, 20% FCS, 10% DMSO) and transferred to cryovials. After incubation on ice for 30 min, the cells were stored at -80°C for 16-24 h before they were transferred into liquid nitrogen. For re-propagation, cells were thawed quickly at 37°C and transferred to fresh medium. The next day, the medium was replaced with fresh culture medium.

2.2.1.3 Transfection of cells with jet-Pei reagent

Cells were plated the day before transfection. For a 75 cm^2 culture dish, 20 μl of 1× Jet-Pei™ reagent was diluted in 150 mM NaCl to a total volume of 500 μl. The diluted Jet-Pei™ solution was added to 15 μg of DNA in 500 μl of 150 mM NaCl and incubated at RT for 30 min. The mixture was added dropwise to the cells and distributed evenly by gentle swirling. 24 hours after transfection, the medium was replaced with fresh culture medium.

2.2.1.4 Transfection of cells with calcium phosphate (Chen and Okayama, 1987)

The day before transfection, cells were trypsinized and 1×10^5 cells were plated onto 30 cm^2 culture dishes. 5 μg DNA was diluted in 250 mM $CaCl_2$ and mixed dropwise with an equal volume of 2× HBS buffer (280 mM NaCl, 1.5 mM Na_2HPO_4, 50 mM HEPES, pH 7.05). The calcium phosphate-DNA precipitate was added to 10 volumes of culture medium and mixed by gentle swirling. 12 hours after transfection, the culture medium was removed. The cells were incubated with 15% glycerol in PBS for 2 min, washed once with PBS and grown in fresh culture medium for at least 24 hours.

2.2.1.5 Magnetic separation of transfected cells

1×10^6 U2OS cells were transfected with 1 μg pMACS K^k.II vector, which expresses the truncated mouse MHC class I molecule H-$2K^k$ as a selection marker, and 9 μg pSuper.neo-gfp vector expressing siRNA against human Mdm2 or empty vector. 24 hours after transfection, cells were treated with the GSK 3 inhibitor alsterpaullone or left untreated for control. Two hours after treatment, cells were washed twice with PBS and 500 μl of 0.25% trypsin solution was added. When the cells were dissociated from the culture dish and from each other, trypsinization was stopped by adding 100 μl 100% FCS. 80 μl MACSelect K^k MicroBeads were added and incubated on ice for 15 minutes with gentle rocking. 1.32 ml of pre-cooled degassed PBE buffer (PBS, 2 mM EDTA, 0.5% BSA) were added and the cells were pipetted up and down to ensure a single-cell suspension. The MS separation column was placed into a MiniMACS Separation Unit and washed with 500 μl of degassed PBE. The resuspended cells were applied to the column in 4 portions of 500 μl each. The column was washed 3 times with 500 μl of degassed PBE buffer and the flow-through which contained the untransfected fraction of the cells was collected. The column was removed from the Separation Unit and placed into a fresh tube. 500 μl of PBE were added to the column and the transfected cells were flushed out using a plunger.

2.2.1.6 Treatment of cell lines

Ionizing irradiation: Cells were irradiated in culture medium with 7.5 Gray using a ^{60}Cobalt γ-source with a dose rate of 2 Gray/min.

UV irradiation: the medium was removed from the cells. Cells were irradiated with 30 Joules/m^2 UV-C light and the initial culture medium was added back to the cells.

GSK 3 inhibition: alsterpaullone was used at a final concentration of 10 μM, bisindolylmaleimide I at a final concentration of 4 μM, bisindolylmaleimide IX at a final concentration of 2 μM and LiCl at a final concentration of 50 mM.

Transfected cells were selected with 300 μg/ml neomycin or 1.5 μg/ml puromycin.

Cycloheximide was used at a final concentration of 30 μg/ml and MG132 at a final concentration of 10 μg/ml.

2.2.2 NUCLEIC ACIDS METHODS

2.2.2.1 Determination of nucleic acid concentration

The concentration of nucleic acids was determined by measuring their optical density (OD) at 260 and 280 nm. An OD_{260} = 1 is equivalent to 50 μg/ml double stranded DNA or 40 μg/ml RNA. A ratio of OD_{260}/OD_{280} ranging from 1.8 to 2.0 indicates an acceptable purity of the nucleic acid.

2.2.2.2 Plasmid DNA preparation

2.2.2.2.1 Large scale plasmid preparation

Plasmid DNA was prepared on a large scale using the Qiagen Plasmid Maxi Kit. A single bacterial colony was inoculated into 200 ml of LB-medium (10 g tryptone; 5 g yeast extract, 10 g NaCl ad 1 l) supplemented with the appropriate antibiotic and incubated overnight with shaking (220 rpm) at 37°C. The bacteria were collected by centrifugation in a fixed angle rotor at 6000 rpm for 10 min and the pellet was resuspended in 10 ml solution P1 (10 mM EDTA, 50 mM Tris-HCl pH 8.0, 400 μg/ml RNAse A) and incubated for 5-10 min at RT. Cells were lysed by addition of 10 ml solution P2 (200 mM NaOH, 1% SDS) and the mixture was neutralised by addition of 10 ml of solution P3 (3 M Na acetate pH 4.8). After 20 min incubation on ice, the lysate was centrifuged at 4000 rpm for 20 min at 4°C in a fixed angle rotor and the supernatant was applied onto a pre-equilibrated Qiagen-tip 500 column. The column was washed twice with QC buffer and the plasmid DNA was eluted with 15 ml QF buffer. The purified DNA was precipitated by adding 10 ml of isopropanol and collected by centrifugation in a swing-out rotor at 10000 rpm for 20 min at 4°C. The DNA pellet was washed with 70% ethanol, resuspended to a final concentration of 1-3 mg/ml in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and stored at -20°C.

2.2.2.2.2 Small scale plasmid preparation

A single bacterial colony was inoculated into 2 ml of LB medium supplemented the appropriate antibiotic and grown overnight under constant shaking (220 rpm) at 37°C. The next day, 1.5 ml were transferred to a fresh vial and the bacteria were collected by centrifugation at 5000 rpm for 5 min. The pellet was resuspended in 200 μl of TELT buffer (50 mM Tris-HCl, pH 8.0, 62.5 mM EDTA, 2.5 M LiCl, 0.4% Triton X-100) and the cells were lysed in the presence of lysozyme at a final concentration of 1 mg/ml for 3 min at 96°C. After 5 min on ice, lysate was centrifuged at 13000 rpm for 8 min. The pellet was removed with a yellow pipette-tip, and the DNA was precipitated by adding 100 μl isopropanol. The DNA was collected by centrifugation, washed with 70% ethanol and resuspended in 50 μl water.

2.2.2.3 Restriction endonuclease digestion of DNA

DNA was digested at a concentration of 1 μg/μl in the buffer recommended by the supplier and 2-3 units of a restriction enzyme per μg DNA. The reaction was incubated for 1 hour at 37°C. The quality of the digest was controlled by agarose gel electrophoresis.

2.2.2.4 Agarose gel electrophoresis

1 to 1.5% agarose were boiled in TAE buffer (40 mM Tris, pH 7.2, 20 mM sodium acetate, 1 mM EDTA). Ethidium bromide was added to a final concentration of 0.3 µg/ml and the solution was poured into a horizontal electrophoresis chamber, which was fitted with a comb. After polymerization, the comb was removed, the DNA was mixed with loading buffer (0.5 M EDTA, 50% glycerol, 0.01% bromophenole blue) and loaded into the slots. Electrophoresis was carried out at 50-100 V at RT until the bromophenole blue front had moved 5 cm. The DNA was visualized by transillumination with 320 nm UV light and photographed with an Eagle Eye photocamera system (*Stratagene*).

2.2.2.5 Isolation/purification of DNA from agarose gels

DNA fragment was isolated with the Easy Pure DNA Purification Kit. The DNA was excised from the agarose gel with a scalpel. The gel piece containing the DNA was melted at 55°C in Salt buffer. 5 µl + 1 µl per 1 µg DNA of the DNA-binding resin was added and incubated at RT for 5 min. The resin with the bound DNA was washed twice, dried and the DNA was eluted with water.

2.2.2.6 DNA ligation

Except for TOPO cloning (see 2.2.2.9), DNAs were incubated with 3 units T4 ligase in 1× T4 buffer supplied by the manufacturer (*Promega*) for 1-3 h at RT. Insert: vector ratio was usually 3:1.

2.2.2.7 Sub-cloning

The fragments of DNA was released from the vectors using appropriate restriction endonucleases, resolved by agarose gel electrophoresis, isolated from the gel and ligated into a second vector by using compatible sites or through blunt end ligation.

2.2.2.8 Polymerase Chain Reaction (PCR)

All PCR reactions were carried out in a thermal cycler (GeneAmp PCR System 2400, *Perkin Elmer*). PCR was performed in a total volume of 50 µl containing 100 ng of template, 250 µM dNTP, 250 ng of primers, 3 U of Pfu polymerase and 1× Pfu polymerase buffer. In some cases, $MgCl_2$ was added to a final concentration of 1 mM. PCR was usually carried out with the following cycles: 1 cycle (95°C, 3 min), 30 cycles (95°C, 30 sec; 55°C, 30 sec; 72°C, 2 min/1 kb DNA template) and 1 cycle (72°C, 2 min). The quality of the PCR product was analyzed by agarose gel electrophoresis.

2.2.2.9 Cloning into pCR®-Blunt II-TOPO® vector

PCR products were cloned into the p pCR-Blunt II-TOPO vector by using the Zero® Blunt® TOPO PCR Cloning Kit. The plasmid vector was supplied linearized with blunt ends and covalently bound topoisomerase I. *Vaccinia virus* topoisomerase I binds to duplex DNA at specific sites, forms a covalent bond with the phosphate group of the 3' thymidine and cleaves one DNA strand, thus enabling the DNA to unwind. The enzyme then re-ligates the ends of the cleaved strand and dissociates from the DNA.

For the TOPO-ligation, 4 µl of fresh PCR product, 1 µl of the pCR-Blunt II-TOPO vector and 1 µl sterile water was mixed gently and incubated for 5 min at RT. 2 µl of the reaction were used for transformation of chemically competent *E. coli* TOP10.

2.2.2.10 Transformation of chemically competent bacteria

100 µl chemically competent bacteria were thawed on ice. DNA was added and the mixture was incubated on ice for 30 min. The bacteria were heated to 42°C for 45 s, immediately transferred on ice and diluted to 1 ml with LB medium. The bacteria were incubated for 1 hour at 37°C, spread onto pre-warmed selective agar plates (supplemented with 50 µg/ml ampicillin or kanamycin) and grown overnight at 37°C.

2.2.2.11 Construction of siRNA expressing plasmids

Two complementary oligos were designed according to the graph in Figure 2.1 using the RNAi Design Tool from *Oligoengine* (http://www.oligoengine.com). The oligonucleotides were dissolved in water to a final concentration of 3 mg/ml. 1 µl of a forward and reverse oligonucleotide was added to 48 µl annealing buffer (50 mM HEPES, pH 7.4, 100 mM K-acetate, 2 mM Mg-acetate), heated to 90°C for 4 min, incubated at 70°C for 10 min and slowly cooled down to 10°C. After annealing, 2 µl of oligonucleotides were phosphorylated with 10 units T4 polynucleotide kinase in PNK buffer (70 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 5 mM DTT) and 1 mM ATP for 30 min. PNK was heat-inactivated at 65°C for 20 min and the annealed and phosphorylated oligonucleotides were ligated into pSUPER or pSUPER.neo-gfp vector, linearized with *Bgl*II and *Hind*III. Positive clones were screened by *Hind*III/*Eco*RI digestion.

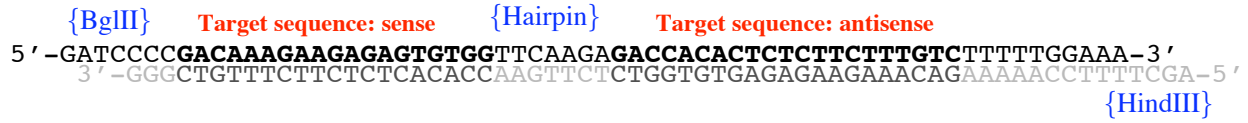


Figure 2.1. Design of oligonucleotides for generating siRNA expressing plasmids.

2.2.2.12 Site-directed mutagenesis

Point mutations and deletions were introduced into wild type plasmids using the QuikChange® site-directed mutagenesis kit. PCR was carried out in total volume of 50 µl containing 5 µl of 5× reaction buffer, 250 ng of each primer, 1 µl of dNTPs, 100 ng of template and 2.5 units PfuTurbo DNA polymerase. Cycling parameters were: 1 cycle (95°C, 30 sec), 30 cycles (95°C, 30 sec; 55°C, 1 min; 68°C, 20 min) and 1 cycle (68°C, 20 min). To create the deletion mutant Δ235-260 of Mdm2, the method was modified according to Wang and Malcolm, 2002. Two PCR reactions each containing only one of the two primers were set up and 5 cycles of PCR were performed. The two PCR reactions were combined and another 30 PCR cycles were performed as described in 2.2.2.8. The DNA templates were digested for 1 h at 37°C with *Dpn*I*, a* restriction endonuclease specific for methylated DNA. 2 µl of the PCR product were used to transform chemically competent *E. coli*. Positive clones were identified by sequencing (see 2.2.2.13).

2.2.2.13 Manual (radioactive) DNA sequencing

DNAs were sequenced using the Thermo Sequenase Radiolabelled Terminator Cycle Sequencing Kit. 1 µg of plasmid DNA was mixed with 3 pmol of a sequencing primer and 8 U of Thermo Sequenase in a total volume of 20 µl. From this mixture, 4.5 µl were transferred into a PCR vial containing 2 µl dNTPs and 0.5 µl of one of four [^{33}P]-ddNTPs. PCR was performed using the following parameters: 95°C, 30 s; 55°C, 30 s and 72°C, 1 min for a total of 50 cycles. The reactions were stopped by addition of 4 µl of the stop solution. After denaturation (95°C, 5 min), 5 µl of the sequencing reaction were loaded onto a 6% polyacrylamide 6M urea/TBE (90 mM Tris-base, 90 mM boric acid, 2.5 mM EDTA, pH 8.3) gel and separated by electrophoresis. When the bromophenole blue front reached the bottom of the plate, the electrophoresis chamber was disassembled. The gel was dried on Whatman 3MM paper at 80°C for 2 hours under vacuum and exposed to MP Hyperfilm. Films were developed 24-48 hours after exposure in a Kodak M35 X-OMAT Processor.

2.2.2.14 Isolation of polyA RNA from cultured cells

All solutions (except for Tris-containing buffers) were pretreated with diethyl pyrocarbonate (DEPC) for at least 1 hour at 37°C or overnight at RT to remove RNases and autoclaved. Cells were grown to 80-90% confluency in 175 cm^2 cell culture dishes. The medium was aspirated and the cells were washed twice with PBS and scraped into 10 ml STE buffer (100 mM NaCl, 20 mM Tris-HCl, pH 7.5, 10 mM EDTA, 0.5% SDS). Genomic DNA was chopped up by a 10 s pulse with an “Ultra-turrax T25” (*Janke & Kunkel IKA Labortechnik*, Staufen, Germany) and proteins were digested with 300 µg/ml proteinase K for 45 min at 37°C. 0.1 g oligo(dT)cellulose and 1 ml 5 M NaCl were added

and the suspension was incubated with end-over-end rotation overnight at room temperature. The cellulose was pelleted by centrifugation and washed 3 times with HSB buffer (300 mM NaCl, 10 mM Tris-HCl, pH 7.5, 5 mM EDTA, 0.1% SDS). Bound RNA was eluted with 3 ml of distilled water and concentrated by ethanol precipitation. After 2 days at -20°C, the RNA mixture was centrifuged at 10000 rpm for 25 min. The RNA-pellet was washed with 70% ethanol to remove the salt, centrifuged for another 10 min and dried. The RNA was resuspended in 50 µl water and used for Northern blotting or reverse transcription.

2.2.2.15 Northern blotting

Agarose to a final concentration of 1.4% was boiled in 1× MOPS buffer (20 mM MOPS, pH 7.0; 5 mM Na-acetate; 1mM EDTA). The solution was cooled down to 37°C and formaldehyde was added to a final concentration of 6%. The agarose solution was poured into a horizontal electrophoresis chamber that was fitted with a comb and allowed to polymerize. 5 µg of polyA RNA were mixed with an equal volume of RNA sample buffer (17.5% formaldehyde, 50% deionized formamide, 1× MOPS buffer, 0.5% ethidium bromide, 0.01% bromophenole blue), denatured for 10 min at 65°C and loaded onto the gel. Electrophoresis was performed in 1× MOPS buffer at 130 V until the bromophenole blue front had moved for 6 cm. The RNA was blotted overnight onto Hybond N+ membrane in 10× SSC buffer (1.5 M NaCl, 150 mM Na-citrate, pH 7.0) and crosslinked by UV-irradiation and heating for 2 hours at 80°C. The membrane was pre-hybridized for 2 hours at 65° C in 4× SSC buffer (600 mM NaCl, 60 mM Na-citrate, pH 7.0), 1× Dennhardt's solution (0.02% Ficoll 400, 0.02% Polyvinylpyrolidin 360, 0.02% BSA), 0.1% SDS, 16 mM Na-phosphate pH 7.3 and 0.05% sodium pyrophosphate and hybridized overnight at 65°C in 4× SSC buffer, 10 mM EDTA and 0.1% SDS in the presence of the radiolabeled cDNA. The membrane was washed at 65°C for 30 min each in buffer A (300 mM NaCl, 30 mM Na-citrate, 16 mM Na-phosphate pH 7.3, 0.01% sodium pyrophosphate), buffer B (150 mM NaCl, 15 mM Na-citrate, 16 mM Na-phosphate pH 7.3, 0.01% sodium pyrophosphate), buffer C (150 mM NaCl, 15 mM Na-citrate, 16 mM Na-phosphate pH 7.3, 0.01% sodium pyrophosphate) and buffer D (75 mM NaCl, 7.5 mM Na-citrate, 0.1% SDS, 16 mM Na-phosphate pH 7.3, 0.01% Na-pyrophosphate) and exposed to MP Hyperfilm. Films were developed in a Kodak M35 X-OMAT Processor.

2.2.2.16 Radioactive labelling of cDNAs for Northern hybridization

Radio-labeling of cDNAs was performed using the prime-a-gene labelling system (*Promega*). cDNAs comprised the open reading frame of human *p53*, *p21/waf1*, *bax*, *PUMA*, *mdm2* and a 1200 bp *PstI* fragment of rat *GAPDH*. 25 – 50 ng of gel purified DNA probes were adjusted with water to a final volume of 35 µl and denatured for 2 min at

95°C. 2 µl unlabelled dNTPs (dATP, dGTP, dTTP, 500 µM each), 2 µl nuclease-free BSA, 10 µl 5× labelling buffer (250 mM Tris-HCl, pH 8.0, 25 mM $MgCl_2$, 10 mM DTT, 1 M HEPES, pH 6.6, 26 A_{260} u/ml random hexadeoxyribonucleotides), 50 µCi [α-^{32}P]-dCTP and 5 units of the Klenow fragment of DNA Polymerase I were added and incubated for 60 minutes at RT. The labelling mix was applied onto a Sephadex G-50 column and spun at 2000 rpm for 2 minutes. The eluate containing the labelled DNA probes was collected in a reaction tube and stored at - 20°C for a maximum of one week. Immediately before use, labelled cDNAs were denatured at 95°C for 2 minutes.

2.2.2.17 Preparation of cDNA

1 µg polyA RNA from the human osteosarcoma U2OS cell line and 1 µg Oligo$(dT)_{12-18}$ (*Invitrogen*) were diluted with water to a final volume of 14 µl, denatured for 5 min at 70°C and cooled down on ice. 5 µl 5× M-MLV RT buffer (*Promega*), 1.25 µl dNTP mix (10 mM each), 25 units Rnasin® Ribonuclease Inhibitor (*Promega*) and 200 units M-MLV Reverse Transcriptase were added and the volume was adjusted to 25 µl with RNAse-free H_2O. After an initial incubation at 40°C for 10 min, the temperature was raised to 55°C. The reaction was incubated for another 50 min and stopped by heat-inactivation (15 min at 70°C). The cDNA was used to amplify *p21/waf1* by standard PCR (see 2.2.2.8).

2.2.3 PROTEIN METHODS

2.2.3.1 Determination of protein concentration

Protein concentrations were determined according to Bradford. The Bradford solution was prepared by dissolving 100 mg Coomassie Brillian Blue G-250 in 50 ml of ethanol. 100 ml 85% phosphoric acid were added and the total volume was adjusted to 1 l with water. The Bradford solution was filtered and stored at 4°C.

To determine the protein concentration in samples, 2 µl of the sample were added to 1 ml of Bradford reagent and measured immediately in an ELISA plate reader at 600 nm. For the calibration curve, 0, 2, 4 and 6 µl of 1 mg/ml BSA were added to 1 ml Bradford reagent and measured in parallel. The concentration of protein samples was determined as OD/2α, where α is OD of 1 µl BSA in 1 ml Bradford reagent.

2.2.3.2 Preparation of cell lysate

Cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed in NP-40 buffer (150 mM NaCl, 50 mM Tris pH 8.0, 5 mM EDTA, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride). The protein extract was cleared by centrifugation at 13000 g at 4°C for 15 min and the protein concentration was determined by the method of Bradford.

2.2.3.3 SDS-polyacrylamide gel electrophoresis

For SDS-polyacrylamid gel electrophoresis, the Mini-PROTEAN®3 System (*BioRad*, München, Germany) was used to cast the gels and perform the electrophoresis. 8, 10 or 12% SDS-polyacrylamide gels were employed.

An inner and an outer glass plate were assembled in a casting tray. For the separating gel, 2.5 ml 1.5 M Tris-HCl pH 8.8; 50 µl 20% (w/v) SDS; 100 µl 10% (w/v) ammonium persulfate, 4 µl TEMED and acrylamide/bisacrylamide (30:0.8) up to the desired concentration (8, 10 or 12%) were mixed and adjusted with water to a final volume of 10 ml. The gel solution was poured into the gel chamber and overlaid with isopropanol. After polymerization, the isopropanol was removed and the stacking gel was poured onto the separation gel (6.8 ml water; 1.7 ml acrylamide/bisacrylamide (30:0.8); 1.25 ml 1 M Tris-HCl, pH 6.8; 50 µl 20% (w/v) SDS; 100 µl 10% (w/v) ammonium persulfate, 10 µl TEMED). A comb was inserted into the stacking gel and the gel was allowed to polymerize. After polymerization, the comb was removed, the gel chamber was disconnected from the casting tray and inserted into an electrophoresis chamber. Protein samples were mixed with an equal volume of 2× SDS-PAGE sample buffer (4% sodium dodecyl sulfate, 0.16 M tris pH 6.8, 20% glycerol, 4% β-mercaptoethanol, 0.002% bromophenol blue), heat denatured for 5 min at 95°C and loaded onto the gel. Electrophoresis was carried out in 1× running buffer (25 mM Tris, 200 mM glycin, 0.1% (w/v) SDS) at 150 V until the bromophenole blue front reached the bottom of the gel.

2.2.3.4 Western Blotting

After proteins were separated by SDS-PAGE, they were electrically transferred onto pre-soaked in methanol Immobilon membrane at 30 V overnight in transfer buffer (25 mM Tris, 200 mM glycin, 10% methanol). After transfer, the membrane was incubated in blocking solution: 5% (w/v) non-fat milk in PBST (137 mM NaCl, 2.7 mM KCl, 6.5 mM Na_2HPO_4, 1.5 mM K_2HPO_4, 0.2% Tween 20) at RT for 1 h with shaking, to reduce unspecific binding of antibodies to the membrane. The blocking solution was removed and the membrane was incubated with the primary antibody, diluted in blocking solution, at RT for 1.5 h or, for phosphorylation-specific antibodies, in 5% BSA in PBST at 4°C overnight (the optimal working dilution was determined empirically). After 3 washes of 10 min each in PBST, the membrane was incubated for 1 hour with a HRP-conjugated secondary antibody, diluted 1:2000 in blocking solution. After incubation with the secondary antibody, the membrane was washed 3 times 10 min each in PBST. Equal volumes of ECL solution I (100 mM Tris-HCl, pH 8.5, 2.5 mM luminol, 400 µM coumaric acid) and ECL solution II (100 mM Tris-HCl, pH 8.5, 0.02% H_2O_2) were mixed and applied to the membrane. The volume was adjusted so that the liquid completely covered the surface of the membrane. After

1 minute incubation, excess of the ECL solution was removed, the membrane was exposed to ECL Hyperfilm and developed in a Kodak M35 X-OMAT Processor.

2.2.3.5 Immunoprecipitation

Cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed in NP-40 buffer (150 mM NaCl, 50 mM Tris pH 8.0, 5 mM EDTA, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride). The protein extract was cleared by centrifugation at 13000 rpm at 4°C for 15 min and the protein concentration was determined by the method of Bradford. 1 μg of the antibody, pre-coupled to Protein A sepharose, was added to 400 μg of cell lysate and the mixture was incubated on a rotating wheel at 4°C for 1.5 hours. The protein/antibody complexes were washed three times with NP-40 lysis buffer. 1× SDS sample buffer (2% sodium dodecyl sulfate, 0.08 M tris pH 6.8, 10% glycerol, 2% β-mercaptoethanol, 0.001% bromophenol blue) was added to the beads and the samples were heat denatured for 5 min at 95°C before they were loaded onto a 10% SDS-PAGE gel.

2.2.3.6 Ubiquitylation assay (Rodriguez et al., 2000)

U2OS cells were transfected with a plasmid encoding His-tagged ubiquitin. 36 hours after transfection, cells were harvested, washed twice in ice-cold PBS and lysed in 6 ml guanidinium lysis buffer (6 M guanidinium-HCl, 0.1 M Na_2HPO_4/NaH_2PO_4 pH 8, 0.01 M Tris pH 8, 5 mM imidazole, 10 mM β-mercapthoethanol). 75 µl Ni^{2+}-NTA agarose beads were added to the lysate and the mixture was incubated with end-over-end rotation at RT for 4 hours. The beads were consecutively washed with the following buffers: guanidinium buffer (6 M guanidinium-HCl, 0.1 M Na_2HPO_4/NaH_2PO_4, pH 8, 0.01 M Tris pH 8, 10 mM β-mercaptoethanol), Urea pH 8 buffer (8 M urea, 0.1 M Na_2HPO_4/NaH_2PO_4, pH 8, 0.01 M Tris pH 8, 10 mM β-mercaptoethanol), buffer A (8 M urea, 0.1 M Na_2HPO_4/NaH_2PO_4, pH 6.3, 0.01 M Tris pH 6.3, 10 mM β-mercaptoethanol), buffer A plus 0.2% Triton X-100 and buffer A plus 0.1% Triton X-100. Ubiquitylated proteins were eluted with 75 μl of elution buffer (200 mM imidazole in 5% SDS, 0.15 M Tris pH 6.7, 30% glycerol, 0.72 M β-mercaptoethanol) for 30 min at RT under constant shaking. The eluate was diluted 1:1 with 2× SDS-PAGE sample buffer and subjected to SDS-PAGE. The proteins were transferred to Immobilon-P blotting membrane and probed with the anti-p53 antibody DO-1.

2.2.3.7 Preparation of GST fusion proteins

50 ml of LB medium (10 g tryptone, 5 g yeast extract and 5 g NaCl for 1 l; autoclaved) were inoculated with 5 ml of an overnight culture of transformed *E. coli* BL21 and incubated at 37°C with vigorous shaking for 1-2 h until the culture reached OD_{600} of 0.6 –

0.8. Then isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM to induce expression of the GST-fusion proteins. After an additional incubation for 5 h at 37°C, bacteria were collected by centrifugation, resuspended in 2.5 ml of PBS and lysed by mild sonification. To ensure the solubilization of the proteins, Triton X-100 was added to a final concentration of 1%, and the lysate was incubated for 30 min at RT with end-over-end rotation. The debris was removed by centrifugation for 10 min at 13000 rpm and the GST-fusion proteins were further purified. 10 µl of glutathione sepharose 4B was added to 1.5 ml of bacterial lysate and incubated for 2 hours at 4°C with end-over-end rotation. The sepharose was washed three times with 1 ml PBS and the proteins were eluted 3 times with 20 µl glutathione elution buffer (50 mM Tris-HCl, pH 8.0, 10 mM glutathione).

2.2.3.8 Kinase assays

1 µg of bacterially expressed GST-Mdm2 fusion proteins, Mdm2 protein immunoprecipitated from cells or 1 µg of tau protein (*Upstate*) were incubated with 50 ng of recombinant GSK 3β, or with GSK 3β immunoprecipitated from mammalian cells, in the presence of 7 mM MOPS, pH 7.3, 20 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, 10 µM ATP and 250 µCi/ml [^{32}P]-γ-ATP for 30 min at 30°C. The reaction was stopped by adding 2× SDS sample buffer (4% sodium dodecyl sulfate, 0.16 M Tris pH 6.8, 20% glycerol, 4% β-mercaptoethanol, 0.002% bromophenol blue) and separated by SDS-PAGE. The proteins were transferred to Immobilone-P blotting membrane and exposed to MP Hyperfilm.

For phosphorylation of primed Mdm2 with GSK 3β, 2 µg of bacterially expressed GST-Mdm2 were incubated with 50 units of recombinant CKIδ in 1x CKIδ buffer (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 5 mM DTT) and 100 µM ATP for 3 hours at 30°C, to ensure complete phosphorylation of the substrate. The reaction was heated for 15 min to 70°C to inactivate CKIδ and 0.5 µg of pre-phosphorylated GST-Mdm2 were subjected to phosphorylation by GSK 3β as described.

To measure GSK 3 activity in crude cell lysates (Ryves et al., 1998), cells were washed with ice-cold PBS and scraped into 1 ml NP-40 lysis buffer (150 mM NaCl, 50 mM Tris pH 8.0, 5 mM EDTA, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride, 50 mM NaF, 5 mM β-glycerolphosphate). The lysate was cleared by centrifugation at 13000 rpm for 10 min and 12.5 µl of protein extract was mixed with 6 µl phospho-Glycogen Synthase Peptide-2 (*Upstate*, 4 mg/ml), 6.5 µl ATP mix (200 mM HEPES, pH 7.5; 50 mM $MgCl_2$, 8 mM DTT, 400 µM ATP, 250 µCi/ml [γ-^{32}P]-ATP) and incubated at 30°C for 15 min. 20 µl of the sample were spotted onto P81 ion-exchange paper. The P81-paper was washed three times for 5 min in 0.75% phosphoric acid and once for 5 min in acetone. Bound radioactivity was quantified by Cherenkov counts in a scintillation counter.

2.2.3.9 *In vivo* labelling cells with ^{32}P-orthophosphate

Cos-7 cells were transfected by jet-Pei method with pDWM659 Myc-Mdm2. 48 hours after transfection, cells were washed twice with 1× TBS (25 mM Tris-HCl, pH 7.4, 137 mM NaCl, 2.7 mM KCl), and pre-incubated for 1 hour with 8 ml of phosphate-free DMEM (*Invitrogen*) containing 10% dialyzed FCS. The medium was replaced with 8 ml fresh phosphate-free DMEM, 2 – 3 mCi of [^{32}P]-orthophosphate were added per 175 cm^2 plate and the cells were incubated for an additional 3 hours. The cells were lysed in 1 ml of NP-40 buffer (150 mM NaCl, 50 mM Tris pH 8.0, 5 mM EDTA, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride) and lysed for 20 minutes on ice. Cell debris was removed by centrifugation at 4°C for 15 min at 13000 rpm and the p53 and Mdm2 proteins were immunoprecipitated from the supernatant as described in 2.2.3.5.

2.2.3.10 Two-dimensional peptide mapping

[^{32}P]-orthophosphate-labelled proteins (see 2.2.3.9) or GST-fusion proteins phosphorylated by GSK 3 *in vitro* (see 2.2.3.8) were subjected to two-dimensional chymotryptic peptide mapping according to Blattner et al., 2002. Phosphorylated proteins were separated by SDS-PAGE, the gel was dried and exposed to MP Hyperfilm. The p53 and Mdm2 proteins were excised from the gel and the gel pieces were homogenised in 500 µl of fresh "ambic" solution (4 mg/ml NH_4HCO_3). An additional 500 µl "ambic" solution, 10µl 20% SDS and 20µl β-mercaptoethanol were added and the gel pieces were heated for 5 min to 100°C. The proteins were eluted overnight with end-over-end rotation. The next day, the supernatant was transferred to a new reaction tube and the proteins were precipitated with 250 µl 100% ice-cold trichloroacetic acid (TCA) and 20 µg RNAse A for 1 hour on ice. The protein pellet was collected by centrifugation at 10000 rpm for 10 min, and washed with 100% cold ethanol. The protein pellet was air-dried for 10 min. To oxidase cysteines into cysteic acid, protein pellet was resuspended in fresh performic acid (prepared by incubating for 1 hour the mix of 450 µl 98% formic acid and 50 µl 30% hydrogen peroxide at RT). After 1 hour incubation on ice, 300 µl water was added and the samples were lyophilised. The dried protein was resuspended in 50 µl „ambic" solution by hard vortexing and digested with 10 µg chymotrypsin (sequencing grade, *Sigma*) at 37°C overnight. The next day, 10 µg of chymotrypsin were added and the samples were incubated at 37°C for at least 2 more hours. 300 µl water were added and the sample was lyophilised. Addition of water and lyophilisation was repeated 2-3 times until all salt was removed. Peptides were resuspended in 50 µl of "pH 1.9 buffer" (580 mM formic acid, 1.36 M acetic acid) by vortexing and the debris was removed by brief centrifugation. The supernatant was transferred into a new tube, lyophilised, resuspended in 5 µl "pH 1.9 buffer" and spotted onto cellulose plates. 5 µl of 40% "pH 1.9 buffer", 5 mg/ml DNP-lysine and 1 mg/ml xylene cyanol were loaded as a marker. Electrophoresis was performed in

"pH 1.9 buffer" for 1.5 hours at 500 V at 4°C. Plates were dried and assembled in chromatography chambers. Chromatography was performed in Phosphopeptide Chromatography Buffer (isobutyric acid (62.5%, vol/vol), *n*-butanol (1.9%, vol/vol), pyridine (4.8%, vol/vol), and glacial acetic acid (2.9%, vol/vol) in deionized water) for 5 – 6 hours. After chromatography, the plates were dried and exposed to MP-Hyperfilm.

2.2.3.11 Immunofluorescence staining

Cells, grown on coverslips, were washed twice with PBS (137 mM NaCl, 2.7 mM KCl, 6.5 mM Na_2HPO_4, 1.5 mM K_2HPO_4) and fixed for 20 min with 3.7% paraformaldehyde in PBS at RT. The coverslips were washed 3-4 times with PBS and the cells were permeabilized in 0.1% Triton-100 in PBS for 5 min. The coverslips were washed twice with PBS and blocked for 1 hour with 1% BSA in PBS (PBS-BSA). The blocking solution was aspirated and 250 µl of primary antibody diluted in PBS-BSA were added and incubated for 1 h. The coverslips were washed 3 times with PBS and incubated with 150 µl of a Cy2- or Cy3-coupled secondary antibody, diluted in PBS-BSA, for 1 hour. The coverslips were washed 5 times with PBS and mounted onto glass slides with Hydromount. The slides were dried overnight in the dark and analyzed by laser scanning microscopy.

Preparation of the paraformaldehyde solution

1.85 g of paraformaldehyde (3.7%) were dissolved in 2.5 ml distilled water at 80°C for 2-3 min. 5 drops of 1 M NaOH were added and the solution was allowed to cool. The solution was adjusted to 50 ml with PBS and used immediately.

3. RESULTS

3.1 GSK 3 phosphorylates Mdm2 *in vitro* and *in vivo*

The p53 protein is a critical regulator of cell death. To allow cell proliferation, the p53 protein needs to be kept under tight control. This control is performed mainly by the Mdm2 protein that binds to the N-terminal transactivation domain of the p53 protein, ubiquitylates it and targets it for rapid degradation by cellular proteasomes (Momand et al., 1992; Fang et al., 2000). Degradation of the p53 protein, however, requires phosphorylation of the central domain of the Mdm2 protein. Particularly, phosphorylation of serine 238, 240, 244, 251, 254, 258 and 260 of the Mdm2 protein has been shown to be critical for its function (Blattner et al., 2002). Despite their importance for the regulation of p53 protein levels, kinases that phosphorylate the central region of the Mdm2 protein have not been identified when I started with my PhD. The goal of my PhD work was therefore to identify kinases that phosphorylate this important region of the Mdm2 protein. Intriguingly, two serines in the central domain of the Mdm2 protein (serine 240 and serine 254) conform to a classical GSK 3 (Glycogen Synthase Kinase 3) consensus site (Fig. 3.1).

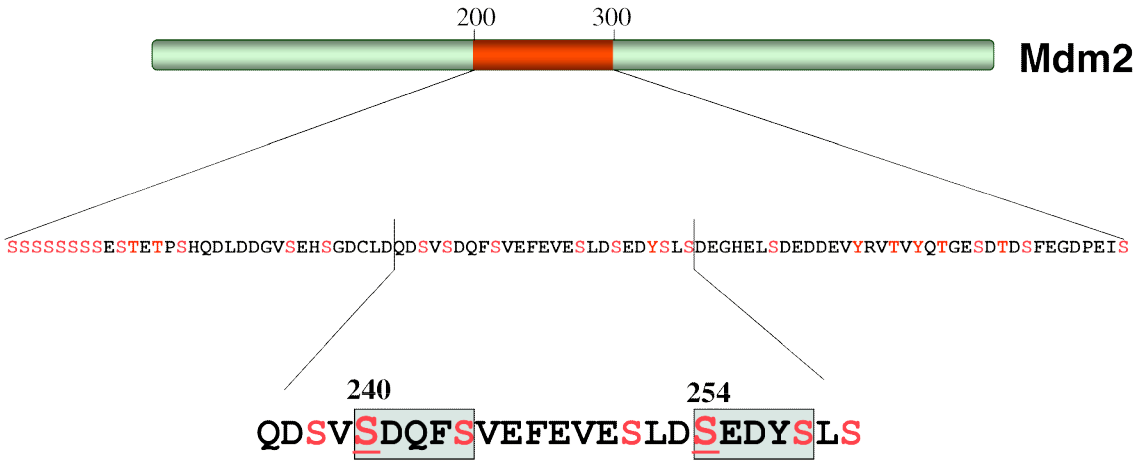
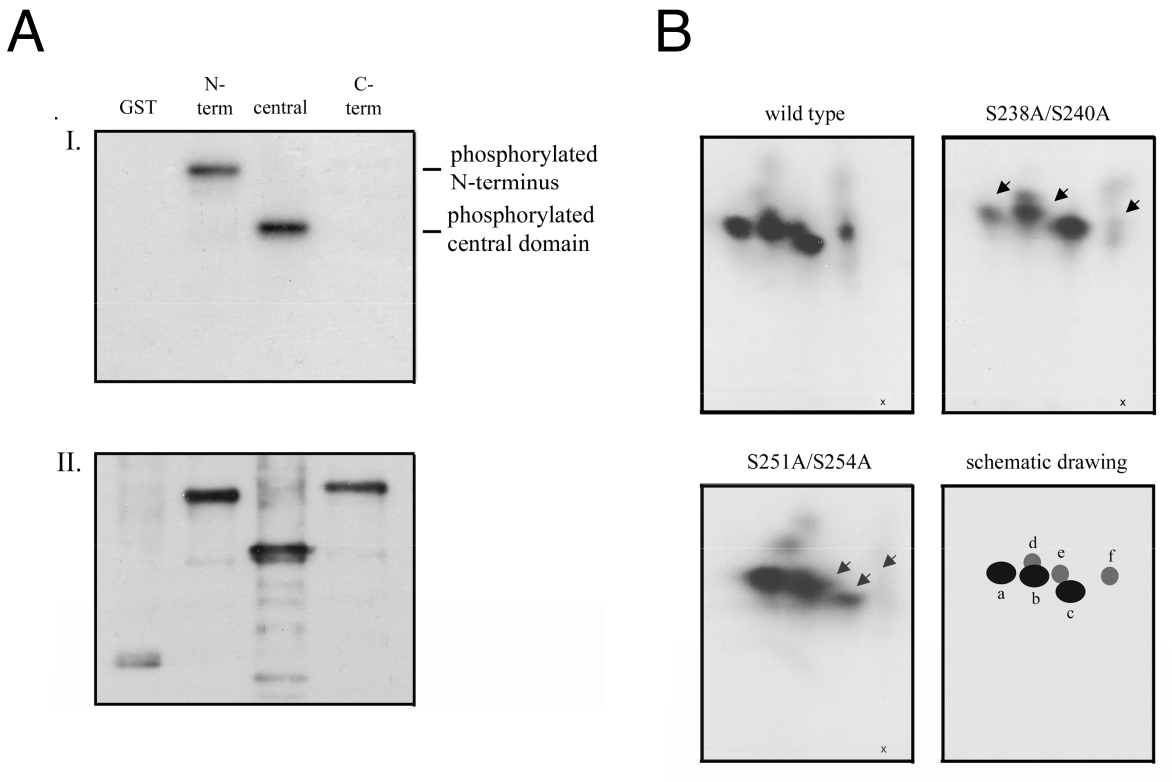


Figure 3.1. GSK3 consensus sites in the central domain of Mdm2. Two potential GSK 3 phosphorylation sites in the central region of Mdm2 are marked in boxes. Phosphorylatable aminoacids marked in red.

To investigate whether the Mdm2 protein can be phosphorylated by GSK 3 I first tested whether GSK 3 phosphorylates the Mdm2 protein *in vitro*. Therefore I fused the aminoterminal part (aa 1 to 206), the central part (aa 220 to 305) and the carboxyterminal part (aa 296 to 491) of the Mdm2 protein to the the glutathione S-transferase gene and expressed the fusion proteins in bacteria. I purified the GST-Mdm2 fusion proteins by adsorption to glutathione sepharose and incubated 1 μg of the fusion protein with 50 ng of recombinant GSK 3β and γ-[^{32}P]-ATP for 30 minutes.



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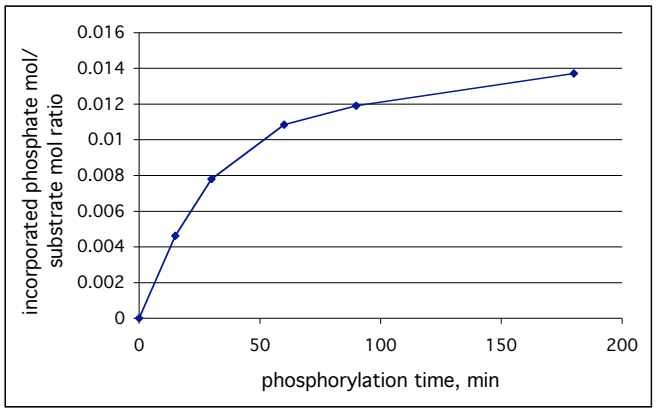


Figure 3.2. GSK 3β phosphorylates Mdm2 *in vitro*. A: GST and GST fused to parts of the Mdm2 protein (N-term: aa 1-206 of Mdm2; central: aa 220-300 of Mdm2; C-term; aa 296-491 of Mdm2) were incubated with GSK 3β and [γ-^{32}P]-ATP. Proteins were separated by SDS-PAGE, transferred to a blotting membrane and exposed to X-ray film (I). The membrane was hybridised with an anti-GST antibody and developed by ECL (II). **B:** GST-Mdm2 fusion proteins of the central domain (aa 220-305) of wild type or mutant Mdm2 with the indicated substitutions were incubated with GSK 3β and [γ-^{32}P]-ATP. Phosphorylated proteins were eluted from the gel, digested with chymotrypsin and separated by electrophoresis and chromatography. The origin (x) where phosphopeptides were loaded is indicated. Arrows point to mutation-dependent changes in the phosphorylation pattern. **C:** Time course of Mdm2 phosphorylation. GST fused to full length Mdm2 was incubated with GSK 3β and [γ-^{32}P]-ATP for the indicated times. Proteins were separated by SDS-PAGE, excised from the gel and the ratio of incorporated phosphate to substrate was measured. The experiment was performed twice. The average ratio was calculated and plotted.

The proteins were separated by SDS-PAGE, transferred to Immobilone P and exposed to X-ray film. As shown in Fig. 3.2 A,I, GSK 3 phosphorylated the N-terminal domain and the central domain of the Mdm2 protein under these conditions. In contrast, the GST part alone or the C-terminal part of the Mdm2 protein were not phosphorylated. To monitor that equal amounts of substrates were applied, I incubated the membranes with an anti-GST antibody and developed the Western blot by ECL. Fig. 3.2 A,II shows that equal amounts of substrates were employed in the assay.

Since serine 240 and 254 conform to a classical GSK 3 consensus phosphorylation site, I wondered whether GSK 3 phosphorylates the Mdm2 protein at these sites. To approach this question, I used mutants of the Mdm2 protein, where serines 238 and 240 or serines 251 and 254 were replaced with an alanine, and cloned them into the pGEX 4T-2 vector to prepare GST-fusion proteins. The corresponding GST-Mdm2-fusion proteins were expressed in bacteria, purified by adsorption to glutathione sepharose and phosphorylated by recombinant GSK 3β. To map the phosphorylation sites, the phosphorylated GST-Mdm2 fusion proteins were purified by gel electrophoresis, digested with chymotrypsin and separated in two dimensions by electrophoresis and chromatography.

The two-dimensional peptide analysis of wild type Mdm2 revealed six distinct peptides that were phosphorylated by GSK 3 *in vitro* (Fig. 3.2 B). When serine 238 and serine 240 were replaced with an alanine, two peptides became invisible due to the absence of incorporated radioactive phosphate (peptide “e” and “f”). In addition, phosphorylation of another peptide (peptide “a”) was severely reduced. Similarly, substitution of serine 251 and serine 254 with an alanine brought about loss of phosphorylation of peptide “f” and decreased phosphorylation of peptides “c” and “e” (Fig. 3.2 B).

In summary, these results clearly show that GSK 3 is able to phosphorylate the central domain of the Mdm2 protein, at least *in vitro*. Moreover, GSK 3 phosphorylated the Mdm2 protein at the two GSK 3 consensus sites within the central conserved domain. Interestingly, since GSK 3 phosphorylated bacterially expressed Mdm2 protein, it is obviously capable to phosphorylate Mdm2 even in the absence of a primed phosphorylation.

A few substrates are phosphorylated by GSK 3 at sites that differ from a classical GSK 3 consensus site (Cho and Johnson, 2003; Qu et al., 2004). GSK 3 phosphorylates these substrates with high efficiency even in the absence of priming phosphorylation. To test whether the Mdm2 protein belongs to one of these GSK 3 substrates with a non-classical phosphorylation motif, I determined the efficiency of Mdm2 phosphorylation by GSK 3 in the absence of prior phosphorylation. Therefore, I incubated purified GST-MDM2 (full length) protein with recombinant GSK 3β and radioactive γ-[^{32}P]-ATP for different time periods, separated the proteins by SDS-PAGE, excised the phosphorylated substrate

from the gel and determined the amount of incorporated radioactive phosphate in a scintillation counter (Fig. 3.2 C).

This approach revealed that the phosphorylation efficiency was very low. After 3 hours of incubation, only 1% of the Mdm2 molecules were phosphorylated by GSK 3β, indicating that non-primed Mdm2 is a not a good substrate for GSK 3β.

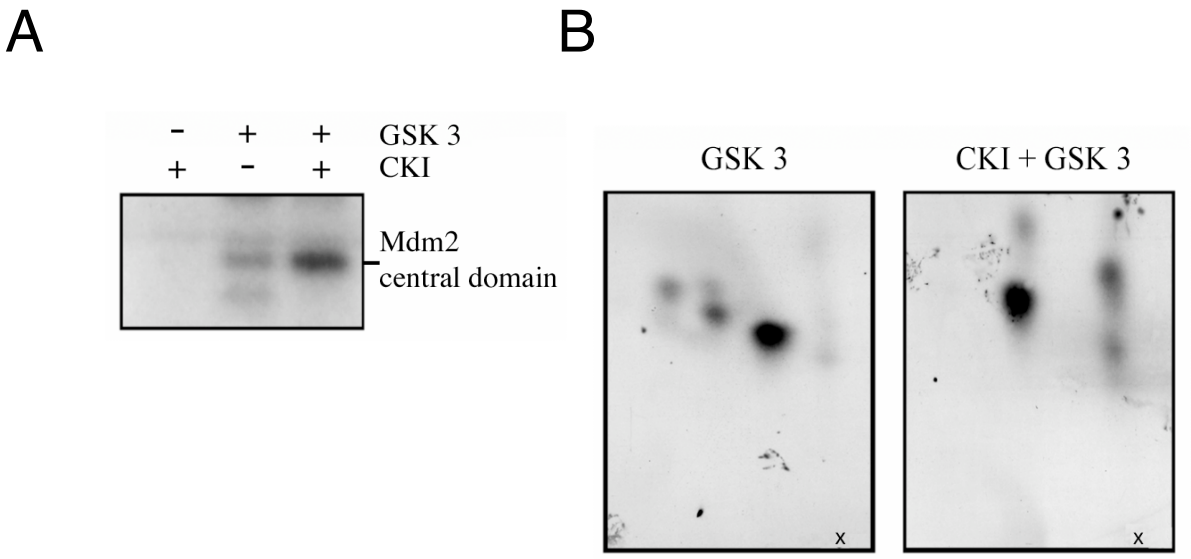


Figure 3.3. Phosphorylation of Mdm2 by GSK 3β is enhanced after priming by CKIδ. A: GST fused to the central domain Mdm2 was phosphorylated with GSK 3β or Mdm2 was first phosphorylated with CKIδ in the presence of cold ATP. After 30 minutes incubation CKIδ was heat-inactivated and Mdm2 was incubated with recombinant GSK 3β and [γ-^{32}P]-ATP or in the absence of GSK 3β as a control of CKIδ inactivation. Proteins were separated by SDS-PAGE and exposed to X-ray film. **B:** Phosphorylated proteins were eluted from the gel, digested with chymotrypsin and separated by electrophoresis and chromatography. The origin (x) where phosphopeptides were loaded is indicated.

Phosphorylation of substrates by GSK 3 is enhanced 100 – 1000 fold when the (n+4) aminoacid is already phosphorylated. This priming phosphorylation brings the serine or threonine that is to be phosphorylated into the correct position within the active site of GSK 3 (Thomas et al., 1999). Markus Winter, a former diploma student has shown that CKIδ phosphorylates the Mdm2 protein at serine 244, which is one of the priming sites for GSK 3 (Winter et al., in press; Fig. 3.1). Since the phosphorylation rate of unprimed Mdm2 by GSK 3β was rather low, I tested whether phosphorylation efficiency can be improved by pre-phosphorylating the Mdm2 protein with CKIδ. I incubated the purified GST-MDM2 fusion protein (aa 220 to 300) for 3 hours with recombinant CKIδ in the presence of non-radioactive phosphate. I inactivated the kinase by heating the sample to 65°C for 15 minutes, diluted it 4 times with GSK 3 kinase buffer and incubated it with recombinant GSK 3β and radioactive γ-[^{32}P]-ATP. As shown in Figure 3.3 A, pre-phosphorylation of Mdm2 by CKIδ significantly enhanced phosphorylation by GSK 3β in comparison to non-

primed Mdm2. Moreover, when I eluted the phosphorylated Mdm2 from the gel and analyzed it by two-dimensional peptide mapping, I observed, that the pattern of Mdm2 phosphorylation was completely different. While in the absence of CKIδ phosphorylation of the central domain with GSK 3β occurred at three major peptides located at a diagonal, phosphorylation of primed Mdm2 by GSK 3 resulted in only one major peptide (Fig. 3.3 B).
The result that GSK 3β phosphorylated Mdm2 *in vitro* indicated that GSK 3β might also phosphorylate Mdm2 under physiologic conditions. Nevertheless, the large excess of substrates and kinases in a test tube frequently leads to false positive results. It was therefore important to show that GSK 3 phosphorylates the Mdm2 protein also in a living cell. To investigate whether GSK 3 phosphorylates Mdm2 under physiologic conditions, I used two different approaches. I analyzed the phosphorylation pattern of the Mdm2 protein that was derived from control cells and from cells that had been treated with alsterpaullone, a potent inhibitor of GSK 3 and I phosphorylated Mdm2 that was derived from control cells or inhibitor-treated cells with recombinant GSK 3. If Mdm2 is a physiologic substrate for GSK 3, only Mdm2 from cells that had been treated with the inhibitor should be phosphorylated by recombinant kinase under these conditions since only in the presence of the inhibitor the Mdm2 protein remained unphosphoylated (Fig. 3.5 A).
For the analysis of the phosphorylation pattern of the Mdm2 protein in living cells, I transfected Cos-7 cells with myc-tagged Mdm2, and incubated the cells for 4 hours with [^{32}P]-orthophosphate in the presence or absence of the GSK 3 inhibitor alsterpaullone. After harvesting the cells, I immunoprecipitated the Mdm2 protein and purified it further by gel electrophoresis. I digested the Mdm2 protein with chymotrypsin and separated the resulting peptides by electrophoreses and chromatography.
The two-dimensional peptide map of the Mdm2 protein showed many peptides with incorporated radioactive phosphate, indicating that the Mdm2 protein is heavily phosphorylated *in vivo* (Fig 3.4 I). Interestingly, treatment of cells with alsterpaullone resulted in the loss of peptide “a” of the Mdm2 protein, indicating that this peptide is normally phosphorylated by GSK 3 (Fig. 3.4 I).
To further prove that peptide “a” is phosphorylated by GSK 3, I transfected Cos-7 cells with myc-tagged Mdm2 and wild type GSK 3β or with myc-tagged Mdm2 together with a GSK 3β where arginine 96 is replaced with an alanine. This mutant form of GSK 3β is inactive towards primed substrates. Since the Mdm2 protein is heavily phosphorylated at GSK 3 consensus sites in the central domain (Blattner et al., 2002) and since the phosphorylation of non-primed Mdm2 by GSK 3β occurs at a very low rate, the Mdm2 protein most likely constitutes a primed substrate for GSK 3. The R96A mutant of GSK 3β should therefore be unable to phosphorylate the Mdm2 protein and eventually even acts in a dominant negative fashion. I incubated the cells with [^{32}P]-orthophosphate,

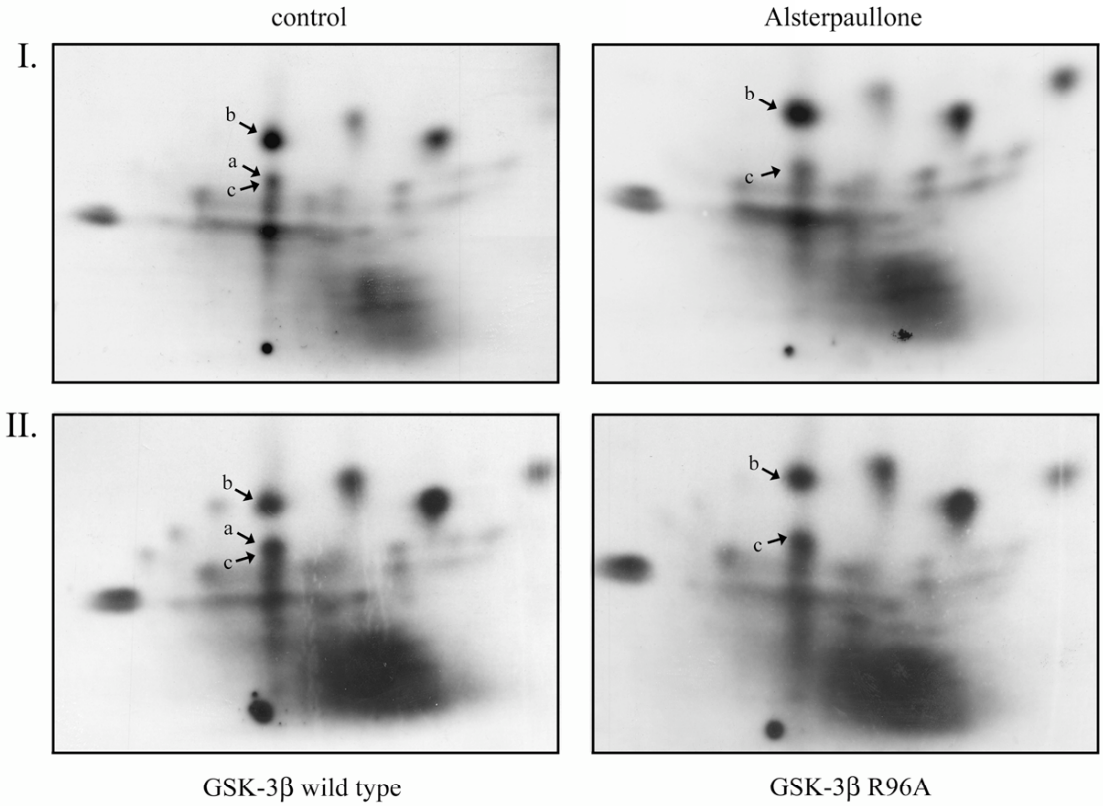


Figure 3.4. Two-dimensional peptide map of Mdm2 phosphorylated *in vivo*. (I) Cos-7 cells were transfected with myc-tagged wild type Mdm2. 48 hours after transfection, cells were incubated for 4 hours with [^{32}P]-orthophosphate in the absence or presence of alsterpaullone. (II) Cos-7 cells were co-transfected with myc-tagged Mdm2 and wild type GSK 3β or the R96A mutant. 48 hours after transfection, cells were incubated for 4 hours with [^{32}P]-orthophosphate. Myc-tagged Mdm2 was precipitated with the 9E10 antibody, resolved by SDS-PAGE, eluted and digested with chymotrypsin. The resulting peptides were separated by electrophoresis and chromatography. (a, b and c are individual peptides of the Mdm2 protein. Peptide "a" is partly overlapping with peptide c.)

precipitated and purified the Mdm2 protein and performed a two-dimensional peptide analysis.

Overexpression of wild type GSK 3β increased the phosphorylation of peptide "a". Consistently, transfection of Mdm2 together with the dominant negative GSK 3β mutant resulted in the loss of peptide "a" (Fig. 3.4, II). These data indicate that GSK 3 phosphorylates Mdm2 also in living cells.

Because of the complexity and the bad resolution of the two dimensional peptide maps of the Mdm2 protein, I complemented the study by a second approach. I transfected cells with Myc-tagged wild type Mdm2 or a Myc-tagged mutant of Mdm2 where I had deleted the central domain (aa 200 to 300). I treated the cells with the GSK 3 inhibitor alsterpaullone or left them untreated for control. After harvesting, I immunoprecipitated the Mdm2 protein from the cells and phosphorylated it by recombinant GSK 3β in the

presence of radioactive γ-[^{32}P]-ATP. I separated the proteins by SDS-PAGE, transferred them to Immobilon P blotting membrane and exposed the membrane onto an X-ray film. To monitor that equal amounts of the Mdm2 protein were loaded onto the gel, the membranes were incubated with an anti-Myc antibody after exposure onto an X-ray film and the western blots were developed by ECL.

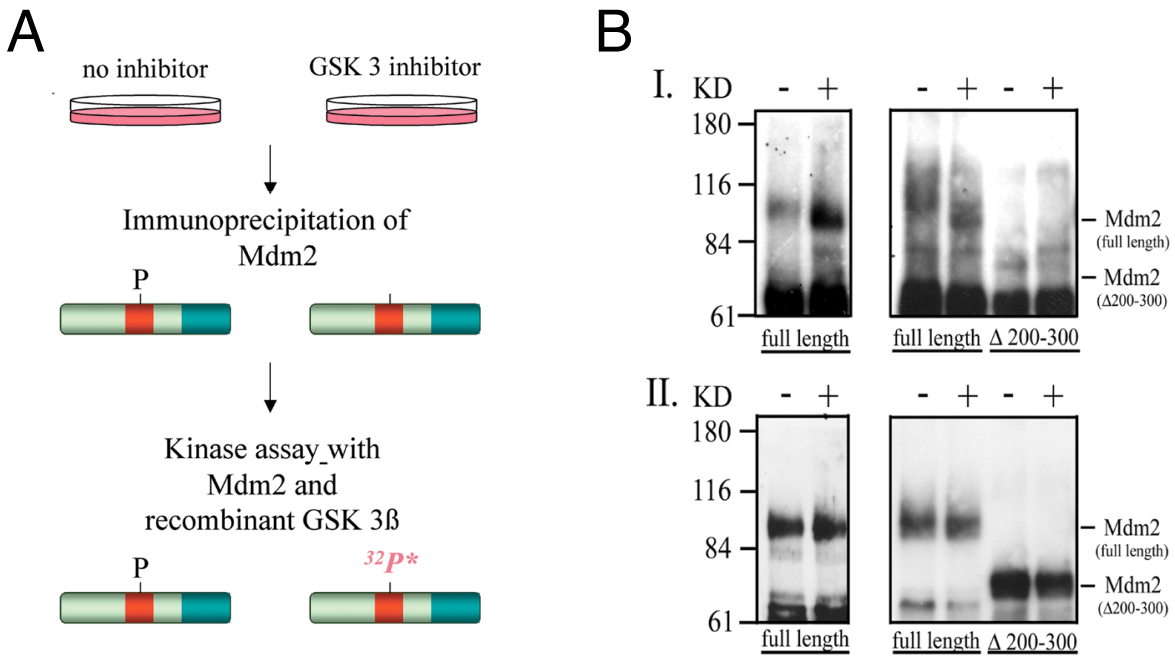


Figure 3.5. GSK 3 phosphorylates Mdm2 *in vivo*. A: Schematic representation of the experiment. **B:** U2OS cells were transfected with a cDNA expressing myc-tagged wild type or mutant Mdm2 (Δ200-300). 36 hours after transfection, cells were treated for 4 hours with Alsterpaullone (+) or left untreated for control (-). Cells were lysed and myc-tagged Mdm2 was precipitated with the 9E10 (anti-myc) antibody, incubated with [γ-^{32}P]-ATP with or without GSK 3β, separated by SDS-PAGE, transferred to nitrocellulose and analysed by autoradiography (I). After exposure, the membrane was hybridised with the anti-Myc antibody and developed by ECL (II).

Indeed, in this phosphorylation assay, the Mdm2 protein that was derived from inhibitor-treated cells was phosphorylated stronger by recombinant GSK 3β than the Mdm2 protein that had been derived from cells that had been left untreated for control. (Fig. 3.5 B,I). Importantly, phosphorylation of the Mdm2 protein, where I had deleted the central domain (Δ200-300), remained very low independently of whether the cells had been treated with the GSK 3 inhibitor alsterpaullone or not (Fig. 3.5 B). Fig. 3.5 B,II shows that equal amounts of the Mdm2 protein were loaded on the gel.

The result of this assay supports the conclusion from the two-dimensional peptide map. Moreover, it shows that the sites that are phosphorylated by GSK 3 are located within the central domain of the Mdm2 protein.

In attempt to identify the amino acids that are phosphorylated by GSK 3 within the central domain of the Mdm2 protein, I created single or double point mutations and deletions of

the Mdm2 protein, where serine 227 and 230, serine 238 and 240, serine 244, serine 251 and 254, serine 258 and 260, serine 267, serine 284 and 288 or serine 296 were replaced with an alanine or where amino acids 201 to 235, 236 to 260 or 261 to 300 were deleted. These Mdm2 mutants were transfected into U2OS cells and phosphorylated by recombinant GSK 3 as described above (Fig. 3.5 A). However, I received no conclusive results since all Mdm2 mutants were phosphorylated by recombinant GSK 3β in a similar fashion (data not shown), possibly indicating that GSK 3 phosphorylates Mdm2 at several sites within the central domain.
In summary, the results from the two dimensional peptide analysis and from the kinase assays strongly suggest that GSK 3 phosphorylates the Mdm2 protein within the central domain *in vitro* and *in vivo*, eventually concomitantly at several sites. Nevertheless, despite the indication that Mdm2 is a physiological substrate of GSK 3, the possibility that the Mdm2 protein is phosphorylated by an alternative kinase that is regulated by GSK 3 cannot be entirely excluded.

To further support the conclusion that GSK 3β phosphorylates the Mdm2 protein directly, I investigated whether GSK 3β associates with the Mdm2 protein. Therefore I transfected U2OS cells with Myc-tagged Mdm2 and wild type GSK 3β. After harvesting, I immunoprecipitated GSK 3β, separated GSK 3β/Mdm2 complexes by SDS-PAGE and transferred them to Immobilon P membrane. I incubated the membrane with anti-GSK 3β and anti-Myc antibodies and developed the western blots by ECL. Unfortunately, I was unable to detect an interaction of Mdm2 and wild type GSK 3β (data not shown).

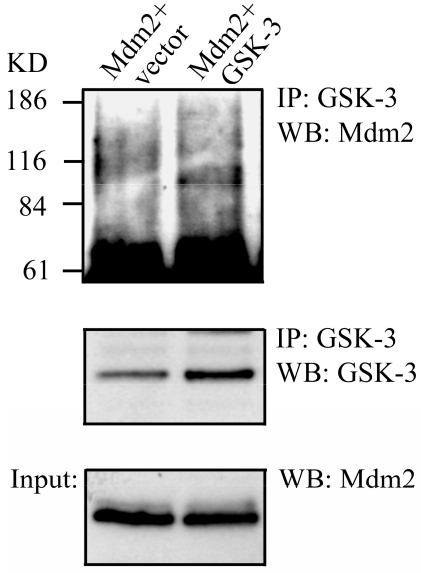


Figure 3.6. Mdm2 interacts with GSK 3β. U2OS cells were transfected with myc-tagged Mdm2 and GSK 3 (R96A) or myc-tagged Mdm2 and a vector control. 36 hours after transfection, GSK 3β was precipitated, the complexes were resolved by SDS-PAGE, transferred to a blotting membrane and probed for Mdm2 and GSK 3β. An aliquot of the cell lysate was analysed to determine Mdm2 expression levels.

The interaction of kinases and substrates is frequently very transient since the proteins dissociate immediately after the enzyme reaction. In consequence, I reasoned that the mutant form of GSK 3β (R96A) that is unable to phosphorylate the Mdm2 protein should longer associate with the Mdm2 protein and thus the interaction might be easier detectable. To test this prediction, I transfected U2OS cells with Myc-tagged Mdm2 and the GSK 3β mutant R96A or vector alone. Again, I immunoprecipitated GSK 3β, separated GSK 3β/Mdm2 complexes by SDS-PAGE, transferred them to Immobilon P membrane and incubated the membrane with antibodies directed against GSK 3β and the Myc-tag and developed the western blots by ECL. Under these conditions, I was able to detect the interaction of the mutant form of GSK 3β with the Mdm2 protein. However, the interaction of endogenous wild type GSK 3 with the Mdm2 protein was again not detectable (Fig. 3.6).

3.2 Inhibition of GSK 3 leads to p53 accumulation

Phosphorylation of several serines in the central domain of the Mdm2 protein is vital for p53 degradation (Blattner et al., 2002). Among these serines are the two GSK 3 consensus sites. If GSK 3 is a kinase that phosphorylates these serines *in vivo,* one would expect that inhibition of GSK 3 interferes with the degradation of the p53 protein. To test this hypothesis, I treated a human osteosarcoma U2OS cell line that expresses wild type p53 with a range of GSK 3 inhibitors: lithium chloride (Jope, 2003), alsterpaullone (Leost et al., 2000), bisindolylmaleimide IX (Hers et al., 1999) and bisindolylmaleimide I (Hers et al., 1999). After harvesting, I separated proteins by SDS-PAGE, transferred them onto Immobilon P blotting membrane and incubated the membrane subsequently with the anti-p53 antibody DO-1 and with the anti-PCNA antibody PC-10, which served as a loading control.

Inhibition of GSK 3 resulted in the accumulation of p53 protein levels. Within less than two hours, an increase in p53 protein levels was detectable in the presence of each of the GSK 3 inhibitors. p53 protein levels remained high and accumulated even further in the course of the experiment (Fig. 3.7 A), indicating that the activity of GSK 3 is required to maintain physiological low levels of the p53 protein.

Although I used four different inhibitors of GSK 3, I could not completely exclude the possibility that the p53 protein accumulated due to an unspecific activity of the GSK 3 inhibitors. To show that the increase in p53 protein levels is specifically due to inhibition of GSK 3, I used two different approaches. Firstly, I transfected a p53-negative lung carcinoma cell line (H1299) with p53 in the presence or absence of Mdm2 and GSK 3β. After harvesting, I separated the cellular lysates by SDS-PAGE, transferred the proteins onto a membrane and incubated the membrane with an antibody directed against p53 and with an antibody directed against PCNA, for a loading control.

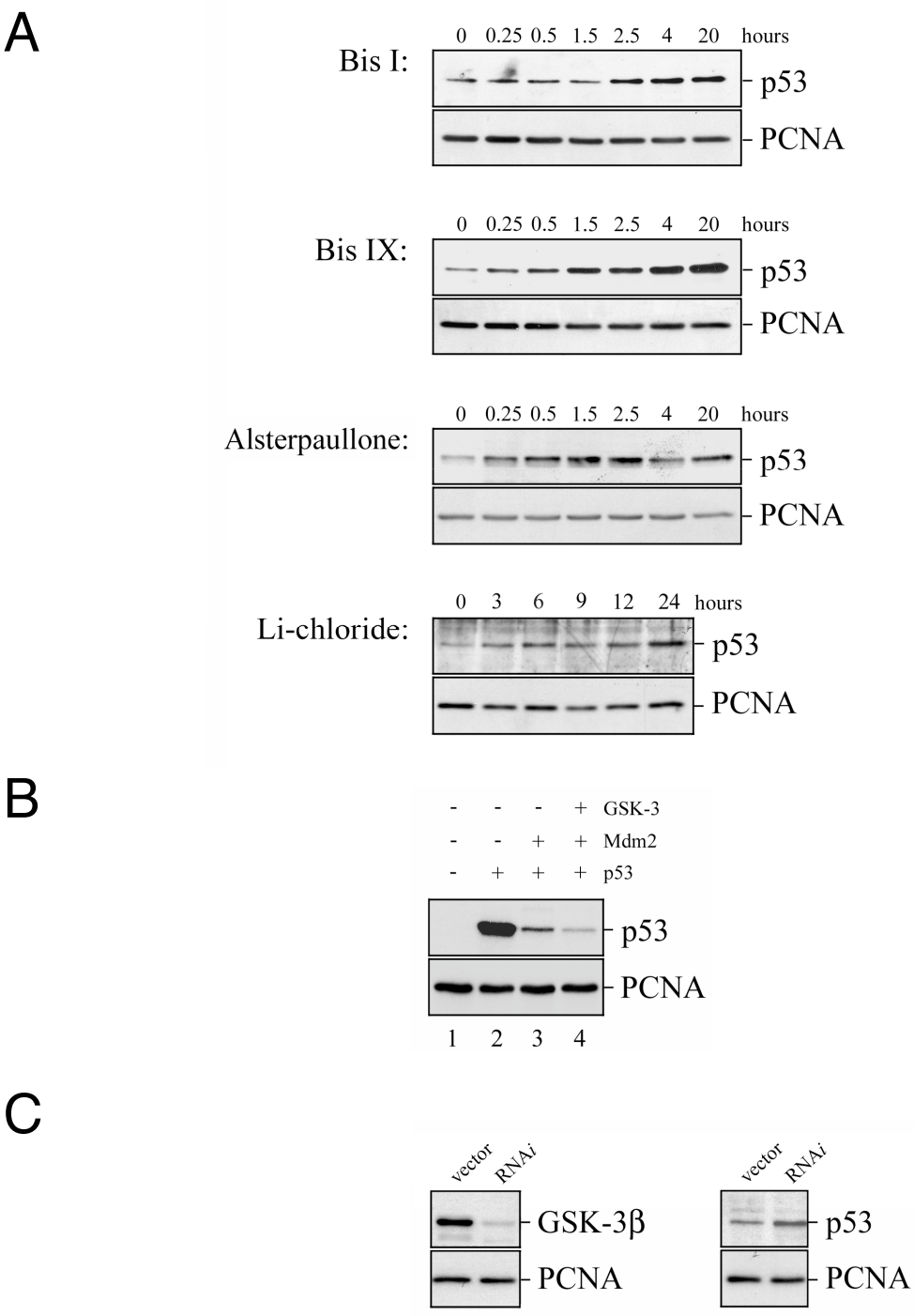


Figure 3.7. Inhibition of GSK 3 leads to the accumulation of p53. A: U2OS cells were incubated with bisindolylmaleimide I, bisindolylmaleimide IX, alsterpaullone or lithium chloride. Cellular extracts were separated by SDS-PAGE and transferred to Immobilon P membrane. The membrane was probed with the anti-p53 antibody DO-1 and the anti-PCNA antibody PC-10. Western blots were developed by ECL. **B:** H1299 cells were transfected with pcDNA-p53 (lanes 2-4), pcDNA-mdm2 (lanes 3-4) and pcDNA3-GSK 3β (lane 4). 36 hours after transfection, cells were harvested and probed for the presence of p53 and PCNA. **C:** U2OS cells were co-transfected with pSuper-GSK 3β expressing GSK 3β RNA*i* and a cDNA encoding a puromycin resistance gene. Cells were selected for 3 days with 1.5 μg/ml puromycin, lysed and probed for p53, GSK 3β and PCNA.

In accordance with published data, p53 protein levels were reduced when Mdm2 was co-expressed with p53 (Fig. 3.7 B; Blattner et al., 2002). Importantly, co-transfection of GSK 3β further reduced p53 protein levels, indicating that GSK 3 promotes p53 degradation (Fig. 3.7 B).

In a second approach, I transfected U2OS cells with a plasmid that expresses siRNA for GSK 3β and a puromycin-resistance coding vector. To select for transfected cells, I cultured the cells in the presence of 1.5 μg/ml puromycin. After three days of selection, I harvested the cells, separated the proteins by SDS-PAGE, transferred them onto a blotting membrane and incubated the membrane with an anti-p53 and an anti-GSK 3β antibody, and an anti-PCNA antibody for loading control.

Figure 3.7 C shows that GSK 3β protein levels were reduced after expression of GSK 3β siRNA. Moreover, and most importantly, p53 protein levels were significantly elevated in cells that lack GSK 3β.

These results clearly show that GSK 3 is an important regulator of p53. Under normal growth conditions, GSK 3 helps to maintain p53 protein levels low. Conversely, inhibition of GSK 3 leads to the accumulation of the p53 protein.

3.3 Accumulated p53 is transcriptionally active

Ways to regulate p53 abundance are currently under intense investigation. The observation that the p53 protein accumulated in response to GSK 3 inhibition, opens new possibilities for cancer therapy, since it allows to raise p53 protein levels by administering small molecules that specifically inhibit GSK 3. For a potential use in therapy, it is, however, important that the inhibition of GSK 3 does not interfere with the capacity of p53 to activate transcription of its target genes.

To test whether inhibition of GSK 3 interferes with the transactivation function of p53, I incubated NIH/3T3 fibroblasts with the GSK 3 inhibitor lithium chloride. I harvested the cells after zero, two, five, ten or twenty-four hours and prepared polyA$^+$ RNA by adsorption to oligo(dT)cellulose. I separated the RNA by formaldehyde-agarose gel electrophoresis and transferred it to a Hybond-N+ membrane. I hybridized the membrane consecutively with ^{32}P-labeled cDNAs encoding the open reading frame of *p53*, *p21/waf1*, *bax*, *puma*, *mdm2* and a 1 kb *PstI*-fragment of *GAPDH* and exposed it to X-ray film.

In consistency with an earlier report (Mao et al., 2001), I observed a strong increase in *p21* mRNA levels within 4 hours after addition of lithium chloride. *mdm2* RNA levels were also elevated after inhibition of GSK 3 whereas *bax* and *puma* mRNA levels were not significantly altered. Hybridization of the membrane with the *GAPDH* cDNA shows that comparable amounts of mRNAs were loaded onto the gel (Fig. 3.8).

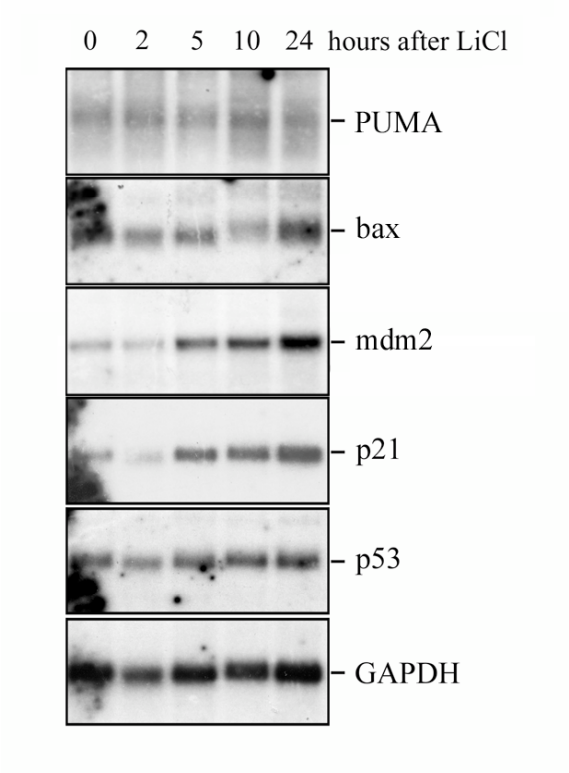
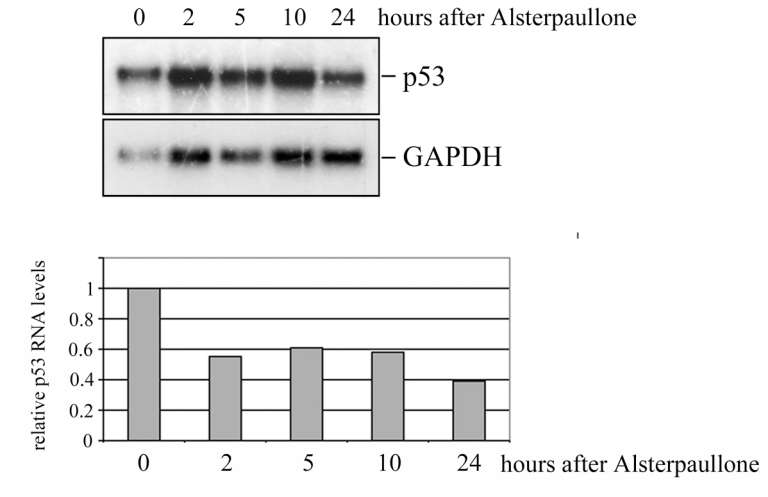


Figure 3.8. GSK 3 inhibition leads to the accumulation of transcriptionally active p53. NIH 3T3 cells were treated with LiCl and harvested after the indicated times. Poly(A)$^+$ RNA was prepared, separated on a agarose-formaldehyde gel and transferred to a Hybond N+ membrane. The membrane was sequentially probed with [^{32}P]-labeled cDNAs encoding *PUMA*, *bax*, *mdm2*, *p21*, *p53* and *GAPDH*, and exposed against X-ray films.

3.4 GSK 3 regulates p53 degradation

To further consolidate the hypothesis that GSK 3 regulates p53 stability by phosphorylating the central domain of the Mdm2 protein, I explored the mechanism that leads to the accumulation of the p53 protein in response to GSK 3 inhibition in more detail. According to the principle “DNA makes RNA makes protein”, protein expression may be regulated at the level of transcription, translation or protein degradation. To determine the regulation level of p53 expression after GSK 3 inhibition, I first determined the levels of *p53* mRNA by Northern blotting. I incubated U2OS cells with 10 μM of the GSK 3 inhibitor alsterpaullone and harvested the cells after zero, two, five, ten and twenty-four hours. I purified polyA$^+$ RNA by adsorption to oligo(dT)cellulose, separated the RNA by agarose-formaldehyde gel electrophoresis, transferred it onto a Hybond-N+ membrane and hybridized the membrane consecutively with ^{32}P-labeled cDNAs encoding the coding region of the *p53* gene and a *PstI* fragment of the *GAPDH* gene for a loading control. I exposed the membrane against an X-ray film and determined *p53* and *GAPDH* mRNA levels densitometrically, normalized *p53* mRNA levels by using the values obtained for the *GAPDH* mRNA and plotted the normalized *p53* values (Fig. 3.9 A).

A



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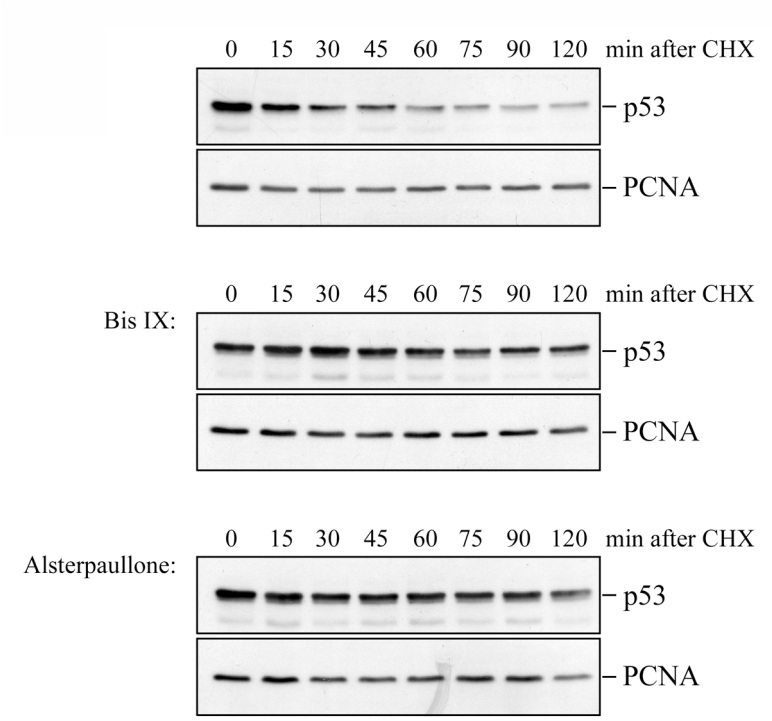


Figure 3.9. Inhibition of GSK 3 prevents p53 degradation I. A: U2OS cells were treated with alsterpaullone for the indicated times. Poly(A)$^+$ RNA was prepared, resolved on an agarose-formaldehyde gel and transferred to Hybond N+ membrane. The membrane was sequentially probed with [^{32}P]-labelled cDNAs encoding *p53* and *GAPDH*, and exposed against X-ray films. *p53* mRNA levels were normalized by *GAPDH* and plotted. Relative p53 mRNA amount in untreated cells was set to 1. **B:** U2OS cells were treated with alsterpaullone and bisindolylmaleimide IX (Bis IX) for 4 hours or left untreated for control. At the indicated times, cycloheximide (CHX) was added. p53 and PCNA protein levels were determined as described in the legend to Figure 3.7.

Within the time frame of this experiment, there was no induction of *p53* mRNA levels detectable. Instead, at two hours after addition of the GSK 3 inhibitor, I observed a slight reduction of *p53* mRNA levels (Fig. 3.9 A). This result shows that the increase in p53 protein levels in response to alsterpaullone does not result from transcriptional activation, but is due to posttranscriptional regulation.

After transcription, *p53* mRNA is translated into protein which is then degraded via the ubiquitin-proteasome pathway. In response to a diverse array of insults such as irradiation, hypoxia and nucleotide depletion p53 protein levels are increased due to rescue of the p53 protein from proteasomal degradation. Therefore, I investigated whether this principle also applies to the regulation of p53 protein levels in response to GSK 3 inhibition.
To test this concept, I treated U2OS cells with the GSK 3 inhibitors alsterpaullone or bisindolylmaleimide IX or left them untreated for control. One hour after addition of the GSK 3 inhibitors, I added cycloheximide to the cells to block ongoing protein synthesis. I harvested at the cells at 15 minutes intervals, separated the lysates by SDS-PAGE, and determined p53 protein levels by western blotting.
In consistency with previous reports, I observed a half-life of the p53 protein of about 20 to 30 min in the absence of GSK 3 inhibitors (Fig. 3.9 B; Blattner et al., 1999a; Maltzman and Czyzyk, 1984). Treatment of cells with the GSK 3 inhibitor alsterpaullone or bisindolylmaleimide IX, however, prolonged the half-life of the p53 protein considerably. During the whole time frame of the experiment, p53 protein levels didn't significantly decrease (Fig. 3.9 B), indicating that inhibition of GSK 3 elevates p53 protein levels by blocking p53 protein degradation.

To further support the conclusion that GSK 3 inhibition raises p53 protein levels by blocking p53 degradation by proteasome, I inhibited GSK 3 in the presence or absence of the proteasome inhibitor MG132.
I harvested the cells at 30 minutes, one, two, four, six and eight hours after GSK 3 inhibition, separated the proteins by SDS-PAGE and determined p53 protein levels by western blotting. To analyze whether GSK 3 inhibition may block p53 degradation by decreasing the levels of Mdm2, the major ubiquitin-ligase for p53, I incubated divided the membrane into two parts and hybridized the upper part of the membrane with an antibody directed against the Mdm2 protein.
As expected, inhibition of GSK 3 increased p53 protein levels only in the presence of functional proteasomes (Fig. 3.10) demonstrating that the increase in p53 abundance after GSK 3 inhibition is solely due to rescue from proteasomal degradation and not e.g. increased translation.

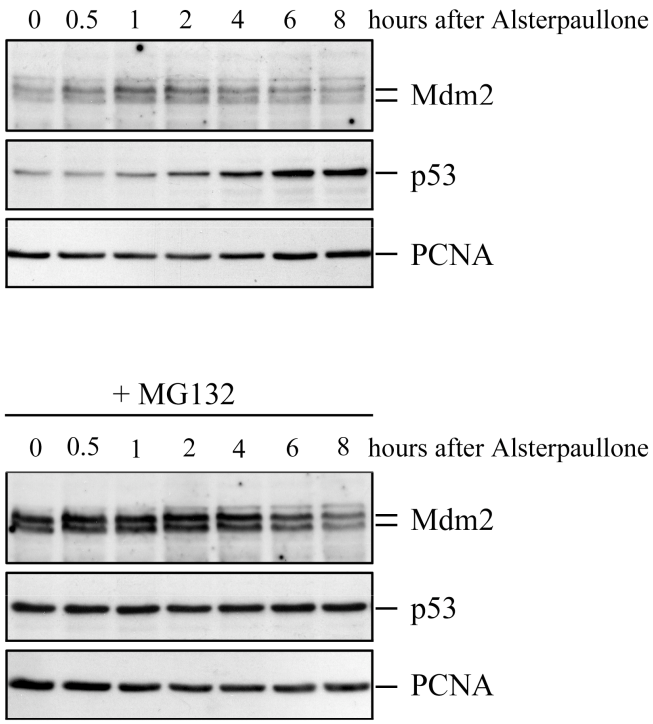


Figure 3.10. Inhibition of GSK 3 prevents p53 degradation II. U2OS cells were incubated with the proteasome inhibitor MG132 for 4 hours or left untreated for control. Alsterpaullone was added and the cells were harvested after the indicated time. p53, Mdm2 and PCNA protein levels were determined by Western blotting.

Interestingly inhibition of GSK 3 not only induces p53 accumulation, but also modulates Mdm2 protein levels. Within the first two hours after drug addition, Mdm2 protein levels were slightly increased. Longer exposure to GSK 3 inhibitors, however, decreased Mdm2 expression significantly (Figure 3.10). Nevertheless, since p53 accumulated prior to Mdm2 downregulation, the reduction in the Mdm2 protein levels cannot account for the block in the p53 protein degradation.

Proteasomal degradation of p53 depends on its ubiquitylation by E3 ubiquitin ligases. Apart from Mdm2, three other ubiquitin ligases, Pirh2, Cop1 and topors, are able to ubiquitylate p53 in cells (Leng et al., 2003; Dornan et al., 2004; Rajendra et al., 2004). Inactivation of these alternative ubiquitin ligases could therefore contribute to the stabilization of p53 in response to GSK 3 inhibition. However, if my concept that the p53 protein accumulates in response to GSK 3 inhibition because the Mdm2 protein is underphosphorylated is correct, then depletion of Mdm2 should prevent this accumulation. To test this principle, I transfected U2OS cells with a plasmid encoding siRNA for Mdm2 to knock-down Mdm2 expression. This plasmid also encodes a neomycin resistance gene which allows selection for transfected cells. Because of the low transfection efficiency of U2OS cells, I selected the transfected cells by addition of 300 μg/ml neomycin.

Unfortunately, all cells died within the selection period of seven days, probably because p53 accumulates in the absence of Mdm2 and directs cells into apoptosis (Jones et al., 1995; Montes de Oca Luna et al., 1995). I therefore decided to analyse the transfected cells at earlier time points. Since neomycin needs several days to kill cells, it was necessary to sort the transfected cells from the non-transfected ones. I therefore transfected the pMACS K^k.II vector, which expresses the truncated mouse MHC class I molecule H-$2K^k$ as a selection surface marker, and the plasmid encoding Mdm2 siRNA. One day after transfection, I treated the cells with alsterpaullone or left them untreated for control. After 2 hours, I purified the transfected cells with MACSelect kit using magnetic beads coupled to antibodies directed against H-$2K^k$ selection marker. I lysed the selected cells, separated the proteins by SDS-PAGE and determined the levels of Mdm2, p53 and PCNA, for loading control.

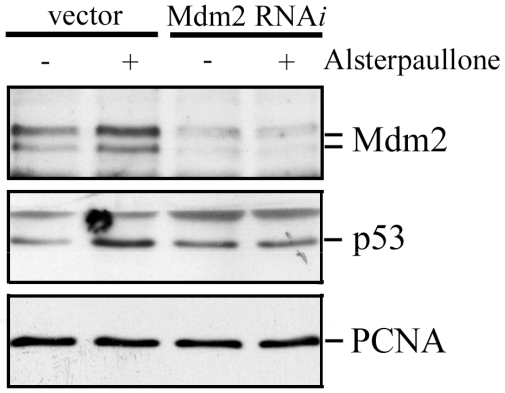


Figure 3.11. Accumulation of p53 after GSK 3 inhibition depends on the presence of Mdm2. U2OS cells were transfected with pMACS K^k.II vector and pSuper.gfp/neo encoding Mdm2 siRNA, or pSuper.gfp/neo vector for control. 24 hours after transfection, cells were treated with alsterpaullone for 2 hours. Transfected cells were separated from non-transfected cells by magnetic beads (*Miltenyi Biotech*) and Mdm2, p53 and PCNA protein levels were determined by western blotting.

As shown in Figure 3.11, Mdm2 protein levels were significantly reduced when Mdm2 siRNA was transfected. Importantly, in these Mdm2-depleted cells, inhibition of GSK 3 did not alter p53 protein levels. Only in the cells transfected with pMACS K^k.II plasmid and empty vector, p53 protein levels were significantly increased (Fig. 3.11). These data strongly argue that GSK 3 regulates the p53 protein in a Mdm2-dependent manner. Interestingly, one day after transfection of the vector coding siRNA for Mdm2, the p53 protein levels were not yet elevated, suggesting that other ubiquitin ligases are able to substitute the Mdm2 protein in its absence, but since GSK 3 inhibition didn't change the p53 protein levels in the absence of the Mdm2 protein, these ubiquitin ligases are obviously not regulated by GSK 3.

Apart from p53, several other cellular proteins, e.g. E2F-1, Numb or the androgen receptor, can be targeted by Mdm2 for proteasomal degradation (Fig. 1.7; Blattner et al.,

1999; Yogosawa et al., 2003; Lin et al., 2002). If GSK 3 inhibition interferes with Mdm2-dependent degradation of p53, one would expect that the turnover of other Mdm2 substrates should be altered as well.

To test this prediction, I therefore determined the expression of the E2F-1 protein, another Mdm2 substrate. I treated U2OS cells with alsterpaullone, harvested the cells after zero, three, eight and twenty-four hours, separated the cell lysates by SDS-PAGE and analyzed E2F-1, p53, β-catenin and PCNA protein levels by western blotting.

As shown in Figure 3.12, the presence of alsterpaullone increased E2F-1 protein levels in a similar way to the p53 protein although the increase in p53 protein levels preceded the accumulation of E2F-1. This discrepancy may, however, be due to differences in protein expression and protein half-life. I also observed an increase in β-catenin expression under these conditions indicating that alsterpaullone was functional (Fig. 3.12; Frame and Cohen, 2001).

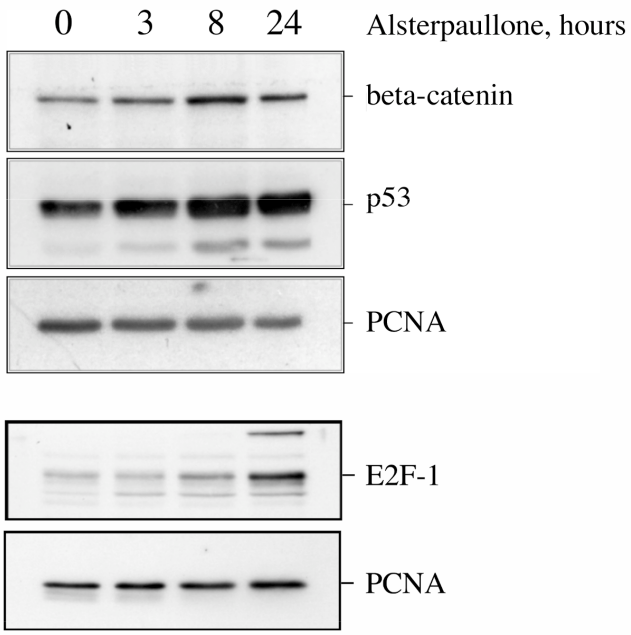


Figure 3.12. Inhibition of GSK 3 leads to the accumulation of E2F-1. U2OS cells were treated with alsterpaullone or left untreated for control. After the indicated times, cells were harvested. E2F-1, β-catenin, p53 and PCNA protein levels were determined by western blotting.

3.5 Inhibition of GSK 3 does not interfere with p53 ubiquitylation

Mdm2 mediates ubiquitylation and degradation of the p53 protein. As described in the introduction, several prerequisites are required for successful p53 ubiquitylation and degradation, e.g. localization of the p53 and Mdm2 proteins in the same compartment, interaction of the p53 and Mdm2 proteins and Mdm2 ubiquitin ligase activity. These factors are tightly regulated via multiple posttranslational modifications of p53 and Mdm2 (Fig. 1.8) and via protein-protein interactions (Fig. 1.7).

Having established that GSK 3 phosphorylates the central domain of the Mdm2 protein where phosphorylation is essential for p53 degradation, I explored if mechanisms other than phosphorylation of the central domain of Mdm2 could account for the accumulation of the p53 protein in response to inhibition of GSK 3.

One of the plausible mechanisms that could interfere with Mdm2 mediated degradation of the p53 protein would be alteration of the phosphorylation pattern of the p53 protein at one of sites that are critical for Mdm2-mediated turnover of the p53 protein.
To test whether GSK 3 phosphorylates the p53 protein directly, I incubated Cos-7 cells for 4 hours with [^{32}P]-orthophosphate in the presence or absence of the GSK 3 inhibitor alsterpaullone. I immunoprecipitated the p53 protein, purified it further by SDS-PAGE and subjected it to two-dimensional peptide analysis as described in section 3.1 for Mdm2.
Comparison of the two-dimensional peptide maps from alsterpaullone-treated and non-treated cells showed no alteration in the phosphorylation pattern of the p53 protein (Fig. 3.13). Since the p53 protein was derived from cells where the Mdm2 protein phosphorylation was altered (Figure 3.4), I can exclude the possibility that the inhibitor was nonfunctional. Therefore, I conclude that GSK 3 doesn't phosphorylate the p53 protein *in vivo* under normal physiological conditions (Figure 3.13).

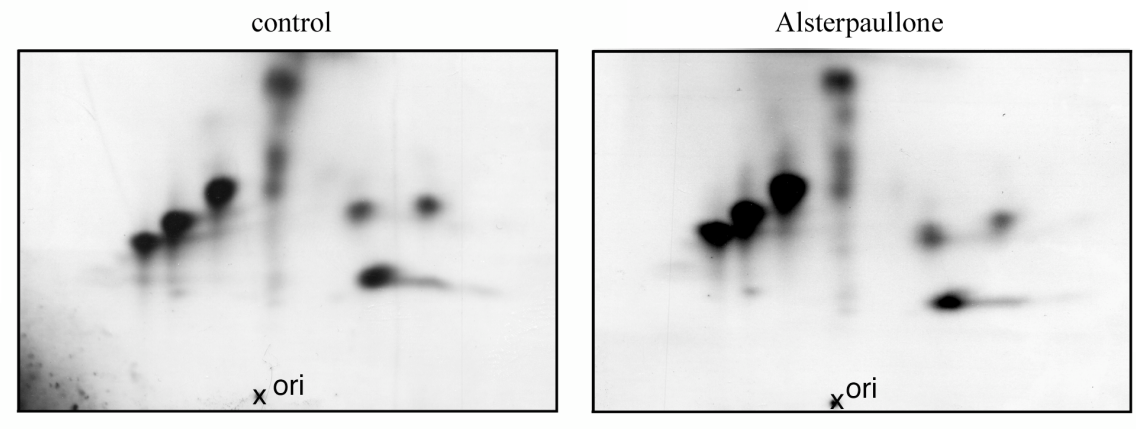


Figure 3.13. p53 is not phosphorylated by GSK 3. Cos-7 cells were transfected with myc-tagged pcDNA3-mdm2. 48 hours after transfection, cells were incubated for 4 hours with [^{32}P]-orthophosphate in the absence or presence of alsterpaullone (see Fig. 3.4). p53 was precipitated using the DO-1 antibody coupled to Protein A sepharose and p53-antibody complexes were resolved by SDS-PAGE. p53 was eluted from the gel, digested with chymotrypsin, and the resulting peptides were separated by electrophoresis and chromatography.

Another mechanism that could trigger the accumulation of the p53 protein in response to GSK 3 inhibition would be spatial separation of the p53 and Mdm2 proteins. To investigate whether GSK 3 inhibition alters the sub-cellular localization of the p53 or Mdm2 proteins, I grew U2OS cells on coverslips, treated the cells with the GSK 3 inhibitor alsterpaullone harvested the cells after zero, two and four hours and fixed the cells with paraformaldehyde. To monitor p53 and Mdm2 localization, I incubated the slides with an

anti-p53 or anti-Mdm2 primary antibody, washed the slides and incubated them further with a fluorochrome-coupled secondary antibody.

In the control cells, I detected a rather weak but exclusively nuclear staining for the p53 and Mdm2 proteins (Fig. 3.14, I) but already at two hours after addition of alsterpaullone, nuclear abundance of p53 was significantly increased. For the Mdm2 protein, no change was detectable while the staining for the Mdm2 protein was still exclusively nuclear. After additional two hours of incubation, the intensity of the nuclear fluorescent signal for the p53 protein was even superior while the signal for the Mdm2 protein was comparable with the signal in control cells. In summary, both p53 and Mdm2 proteins were localized to the nucleus in the presence and absence of alsterpaullone. No cytoplasmic staining for the p53 or Mdm2 proteins was evident. Notably, the p53 protein accumulated in the presence of alsterpaullone despite the presence of the Mdm2 protein in the same cellular compartment (Fig. 3.14, I).

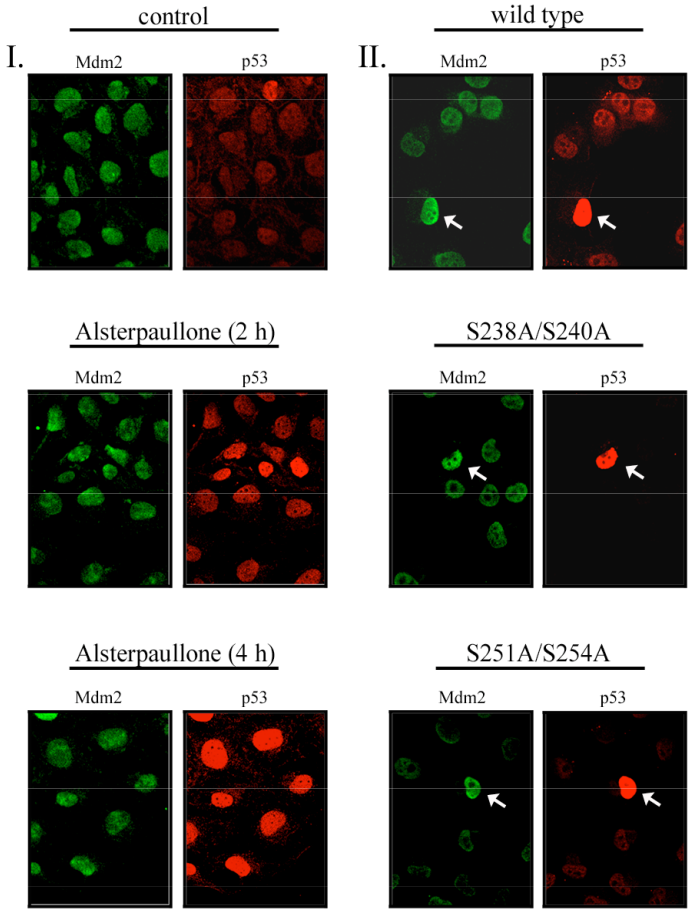


Figure 3.14. GSK 3 inhibition does not change the intracellular localization of p53 and Mdm2. (I) U2OS cells were treated with alsterpaullone for 2 and 4 hours, harvested and incubated with the 4B2 (mouse anti-Mdm2) and CM-1 (rabbit anti-p53) antibody followed by incubation with Cy2-coupled anti-mouse IgG and Cy3-coupled anti-rabbit IgG secondary antibodies. The Mdm2 protein is stained in green, the p53 protein in red. (II) H1299 cells were transfected with pcDNA3-p53 and wild type or mutant pcDNA3-mdm2. Twenty-four hours after transfection, cells were harvested and analysed for the expression of Mdm2 and p53 by immunofluorescence staining. Mdm2 is stained in green, p53 in red.

The fact that the p53 protein accumulated in the presence of Mdm2 reveals that the capacity of Mdm2 to target p53 for degradation must have been inactivated by other means. A conceivable possibility would be that the association of the p53 protein with the Mdm2 protein might be hampered when GSK 3 is inactivated. The p53 protein and the Mdm2 protein form stable complexes and this complex formation is important for the ability of the Mdm2 protein to target the p53 protein for degradation (Haupt et al., 1997). To investigate whether the p53 protein and the Mdm2 protein remain associated after GSK 3 inhibition, I transfected H1299 cells with p53 and Mdm2 coding plasmids. 24 hours after transfection, I treated cells with the GSK 3 inhibitors alsterpaullone (A), bisindolylmaleimide IX (B) or left them untreated for control. To obtain similar levels of the p53 protein in all samples despite the stabilization of p53 in the presence of GSK 3 inhibitors, I treated the cells with the proteasome inhibitor MG132. After harvesting the cells, I immunoprecipitated the p53 protein with the rabbit polyclonal anti-p53 antibody CM-1 (Midgley et al., 1992), separated the p53/antibody complexes by SDS-PAGE, transferred the proteins to a blotting membrane and determined the level of associating Mdm2 by incubating the membrane with an the 4B2 antibody, which is directed against Mdm2. For control, aliquots of cellular lysates were separated by SDS-PAGE and analyzed by western blotting for the expression of p53 and Mdm2.

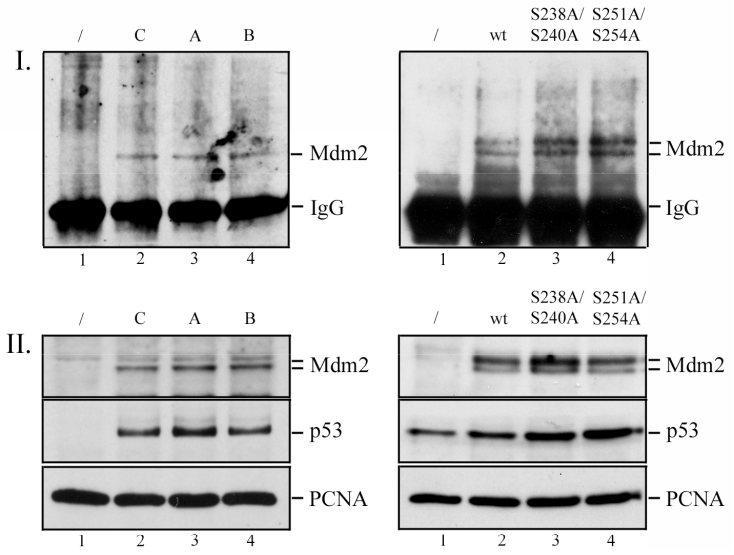
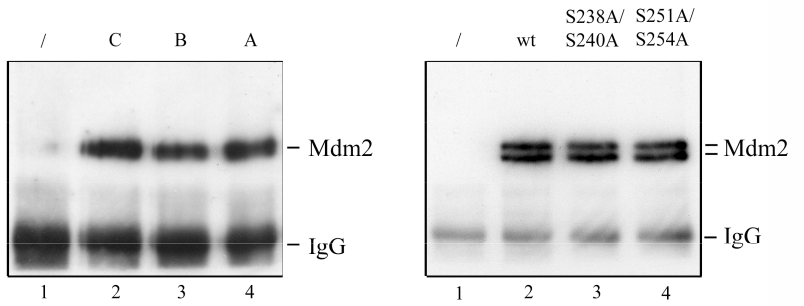


Figure 3.15. GSK 3 inhibition does not influence p53-Mdm2 interaction. *left panel* H1299 cells were transfected with pcDNA3-p53 and pcDNA3-mdm2 (lane 2-4). 24 hours after transfection, cells were treated with the proteasome inhibitor MG132 for 5 hours. 2 hours prior to harvest, alsterpaullone (A) or bisindolylmaleimide IX (B) were added (*experiment was done by Christine Blattner*). *right panel:* H1299 cells were transfected with pcDNA3-p53 (lane 1) or with pcDNA3-p53 and wild type pcDNA3-mdm2 (lane 2) or mutant mdm2 (lane 3 and 4). 24 hours after transfection, cells were treated with the proteasome inhibitor MG132 for 5 hours. (I) p53 was precipitated using the CM-1 anti p53-antibody and complexes were separated by SDS-PAGE gel. Proteins were blotted onto a membrane and probed with the 4B2 (anti-Mdm2) antibody. Western blots were developed by ECL. (II) Cellular lysate was separated on a SDS PAGE gel and probed for the presence of Mdm2, p53 and PCNA.

The Mdm2 protein was detectable with this assay in p53-immunoprecipitates both in control cells and in cells treated with the GSK 3 inhibitors. Moreover, there was not a slight difference between the differently treated cells with regard to the amount of co-precipitating Mdm2 protein (Fig. 3.15, I, *left panel*). Figure 3.15, II, *left panel* shows that equal amounts of p53 and Mdm2 were expressed in the differently treated cell samples.
This result demonstrates that the activity of GSK 3 does not influence the interaction of the p53 and Mdm2 proteins.

A



B

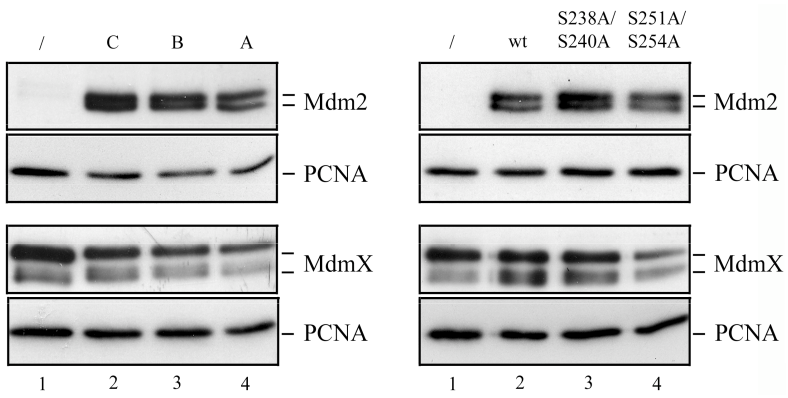


Figure 3.16. GSK 3 inhibition does not influence Mdm2/MdmX interaction. *left panel:* U2OS cells were transfected with pcDNA3-HA-mdmX together with pcDNA3 (lane 1) or with pcDNA3-HA-mdmX and pcDNA3-mdm2 (lane 2-4). 24 hours after transfection, cells were treated with the proteasome inhibitor MG132 for 5 hours. 2 hours prior to harvest, alsterpaullone (A) or bisindolylmaleimide IX (B) were added. *right panel:* U2OS cells were transfected with pcDNA3-HA-mdmX and pcDNA3 (lane 1) or with pcDNA3-HA-mdmX and wild type pcDNA3-mdm2 (lane 2) or mutant pcDNA3-mdm2 (lane 3 and 4). 24 hours after transfection, cells were treated with the proteasome inhibitor MG132 for 5 hours and cells were lysed. **A:** MdmX/Mdm2 complexes were immunoprecipitated with an anti-HA antibody coupled to Protein A sepharose. The protein-antibody complexes were washed and separated on a SDS-PAGE gel. Proteins were transferred to a blotting membrane and probed with the 4B2 (anti-Mdm2) antibody. **B:** Total cellular lysate was separated on a SDS-PAGE gel and probed for the presence of Mdm2, MdmX and PCNA.

Since the p53 accumulated despite the interaction with and colocalization of the Mdm2 and p53 proteins in the same cellular compartment, other mechanisms must be responsible for p53 accumulation. For instance, the interaction of the Mdm2 protein with

the MdmX protein could be regulated by GSK 3. The MdmX protein stimulates Mdm2 activity to ubiquitylate and degrade the p53 protein (Gu et al., 2002). Dissociation of the Mdm2 and MdmX proteins should therefore reduce the ubiquitylation of the p53 protein.
To investigate whether inhibition of GSK 3 influences the interaction of the Mdm2 and MdmX proteins, I transfected U2OS cells with Mdm2 and HA-tagged MdmX. 24 hours after transfection, I treated the cells with the GSK 3 inhibitors alsterpaullone and bisindolylmaleimide IX or left them untreated for control, harvested the cells and prepared cell lysates. The HA-tagged MdmX protein was immunoprecipitated from the lysate with an antibody directed against the HA epitope and the complexes were resolved by SDS-PAGE. Co-precipitating Mdm2 protein levels was determined by incubating the Western blot membranes with the 4B2 anti-Mdm2 antibody. For control of the Mdm2 and MdmX protein expression, aliquots of cellular lysates were separated by SDS-PAGE and analyzed by western blotting (Fig. 3.16 B, *left panel*).
As shown in Fig. 3.16 A, *left panel*, comparable amounts of Mdm2 associated with Mdm2 in the presence and absence of the GSK 3 inhibitors. I therefore conclude that the association of the Mdm2 protein with the MdmX protein is independent of the activity of GSK 3.

In addition to the dissociation of Mdm2-p53 or Mdm2-MdmX complexes, other, so far unknown, mechanisms could influence p53 ubiquitylation and therefore block p53 degradation in response to GSK 3 inhibition. To address this possibility, I determined whether inhibition of GSK 3 reduces p53 ubiquitylation.
I transfected U2OS cells with His-tagged ubiquitin. The addition of the His-tag to the ubiquitin-protein allows the purification of ubiquitylated proteins by adsorption to Nickel-agarose since the interaction is strong enough to be retained in highly chaotropic buffers. I treated the cells with the GSK 3 inhibitors alsterpaullone and bisindolylmaleimide IX or left them untreated for control. To obtain similar levels of the p53 protein in all samples despite the stabilization of p53 in the presence of GSK 3 inhibitors, I also treated the cells with the proteasomal inhibitor MG132 for 5 hours prior to lysis. Two hours after the addition of the GSK 3 inhibitors I harvested the cells and lysed them in a 6 M guanidinium buffer, which immediately blocks protein degradation. I purified ubiquitylated proteins by adsorption to Ni-agarose, separated them by SDS-PAGE and transferred them onto a blotting membrane. To monitor the levels of ubiquitylated p53 protein, I hybridized the membrane with the anti-p53 antibody DO-1. For control of the p53 protein levels, aliquots of cellular lysates were separated by SDS-PAGE and analyzed by western blotting (Fig. 3.17, II, *left panel*).
Figure 3.17, I, *left panel* shows that the p53 protein was still ubiquitylated in the presence of GSK 3 inhibitors. Moreover, independent on the presence or absence of the GSK 3

inhibitors, comparable amounts or the p53 protein were ubiquitylated and also the pattern of ubiquitylation was unaltered (Fig. 3.17, I, *left panel*). This result shows that inhibition of GSK 3 does not interfere with the ubiquitin-ligase activity of the Mdm2 protein and demonstrates that the p53 protein accumulates in response to GSK 3 inhibition despite its ubiquitylation. Therefore, inhibition of GSK 3 must interfere with a step in p53 degradation that is beyond p53 ubiquitylation.

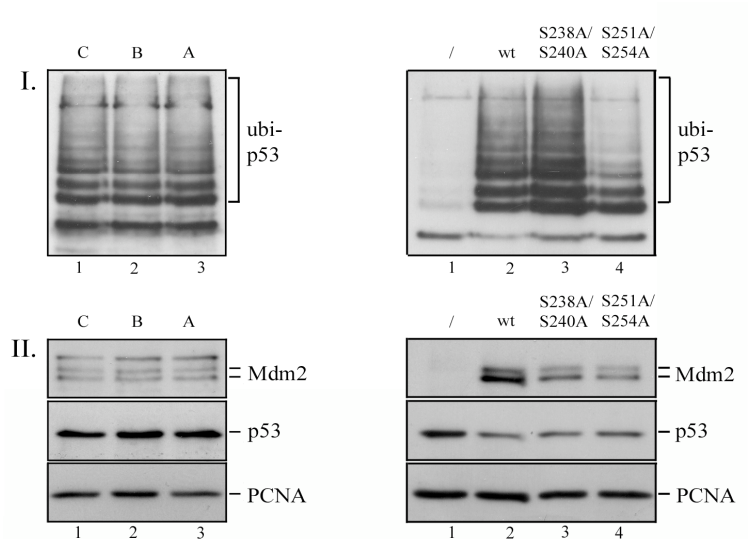


Figure 3.17. GSK 3 inhibition does not change p53 ubiquitylation. *left panel:* U2OS cells were transfected with a plasmid encoding His-tagged ubiquitin. 24 hours after transfection, cells were treated with the proteasome inhibitor MG132 for 5 hours. 2 hours prior to harvest, alsterpaullone (A) or bisindolylmaleimide IX (B) were added or cells were left untreated for control (C): *right panel:* H1299 cells were transfected with a plasmid encoding His-tagged ubiquitin and pcDNA3-p53 (lane 1) or with a plasmid encoding His-tagged ubiquitin, pcDNA3-p53 and wild type pcDNA3-mdm2 (lane 2) or mutant mdm2 (lane 3 and 4). 24 hours after transfection, cells were treated with the proteasome inhibitor MG132 for 5 hours. Aliquots of the cells were analysed for the presence of Mdm2, p53 and PCNA by Western blotting (II). The remaining cells were lysed in guanidinium lysis buffer. Ubiquitylated proteins were purified by adsorption to Ni^{2+}-NTA-agarose beads, separated on a SDS-PAGE gel, transferred to a blotting membrane and probed for the presence of p53 (I).

3.6 GSK 3 regulates the Mdm2-proteasome interaction

Several reports support the hypothesis that in addition to its ubiquitin ligase activity, Mdm2 fulfills a function beyond p53 ubiquitylation in the pathway that leads to p53 degradation (Argentini et al., 2001; Zhu et al., 2001; Blattner et al., 2002; Brignone et al., 2004). This function is associated with the central domain of the Mdm2 protein since deletion mutants of the central domain or point mutations of serine 238, 240, 244, 251, 254, 258 or 260 into an alanine allow p53 ubiquitylation but the ubiquitylated p53 protein is not degraded. One of the hypotheses for the nature of this function is that the central domain of Mdm2 may directly interact with the proteasome thus bringing ubiquitylated p53 protein to the place of its degradation.

To test whether the Mdm2 protein interacts with cellular proteasomes, I transfected U2OS cells with Myc-tagged Mdm2, treated the cells with the GSK 3 inhibitor alsterpaullone or left them untreated for control and harvested the cells two hours after drug addition. I precipitated the proteasomes from cell lysates with an antibody against the S6b protein, one of the regulatory AAA ATPases of 19S proteasome. I separated the S6b-antibody complexes by SDS-PAGE and determined the level of co-precipitated Mdm2 protein by western blotting. For control of the Mdm2 protein expression, aliquots of cellular lysates were separated by SDS-PAGE and analyzed by western blotting (Fig. 3.18 B).

In these experiments, I repeatedly obtained a weak but reproducible signal for the Mdm2 protein in the absence of GSK 3 inhibitors, indicating that the Mdm2 protein interacts with the proteasome (Fig. 3.18 A). Inhibition of GSK 3 significantly reduced the signal for the co-precipitated Mdm2 protein (Fig. 3.18 A), indicating that GSK 3-dependent phosphorylation of Mdm2 is important for the interaction of the Mdm2 protein with cellular proteasomes.

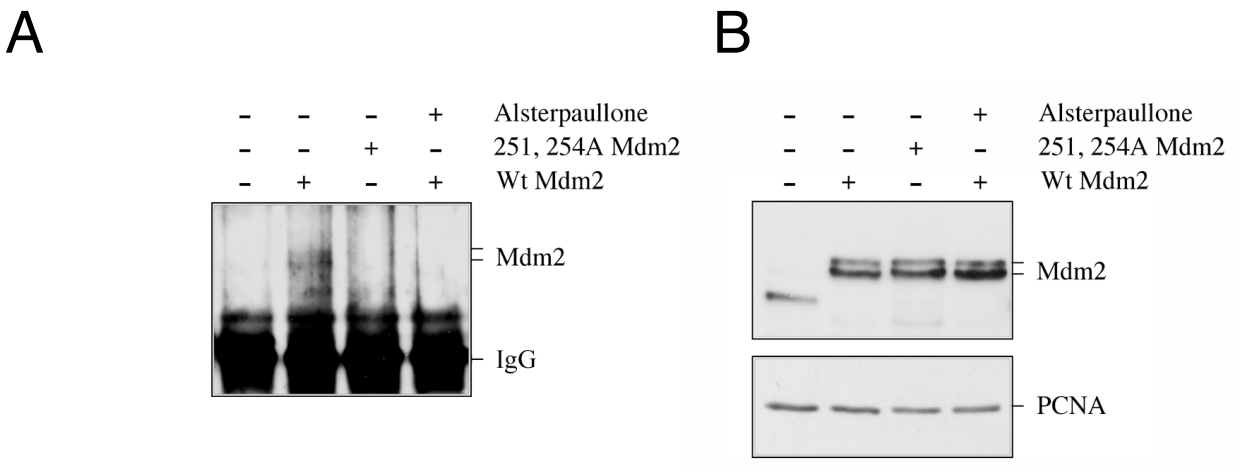


Figure 3.18. GSK 3 inhibition blocks the interaction of the Mdm2 protein with the proteasome. U2OS cells were transfected with pcDNA3-Myc-Mdm2 wild type or mutant where serines 251 and 254 are mutated into alanine. 2 hours prior to harvest, alsterpaullone was added. **A:** Mdm2/proteasome complexes were immunoprecipitated with an anti-S6b antibody coupled to Protein A sepharose. The protein-antibody complexes were washed and separated by SDS-PAGE. Proteins were transferred to a blotting membrane and probed with anti-Myc (Mdm2) antibody. **B:** Cellular lysates were separated by SDS-PAGE and probed for the presence of Mdm2 and PCNA.

3.7 Mutants of Mdm2 where GSK 3 consensus sites are mutated into alanine ubiquitylate p53 but do not promote p53 degradation

GSK 3 inhibition did not interfere with the interaction of the p53 and Mdm2 proteins or their localization, with the interaction of the Mdm2 and MdmX proteins or with Mdm2 ubiquitin ligase activity but it blocked the interaction of the Mdm2 protein with the proteasome. If the reason for the accumulation of the p53 protein in response to GSK 3

inhibition is indeed underphosphorylation of the GSK 3 consensus sites in the central domain of the Mdm2 protein in the absence of GSK 3 activity, one would expect that replacing GSK 3 phosphorylation sites in the Mdm2 protein with non-phosphorylatable residues should lead to the same effect.

To test this hypothesis, I transfected p53 negative H1299 cells with p53 and wild type Mdm2 or Mdm2 where serine 238 and 240 or serine 251 and 254 were replaced with an alanine (S238/240A Mdm2, S251/254A Mdm2). Twenty-four hours after transfection, I harvested the cells, separated the lysates by SDS-PAGE and determined p53, Mdm2 and PCNA levels by western blotting.

In consistence with published data, co-expression of wild type Mdm2 with p53 significantly reduced p53 protein levels due to targeting of the p53 protein for proteasomal degradation. In contrast, mutant Mdm2 protein (S238/240A Mdm2, S251/254A Mdm2) was not able to mediate p53 protein degradation (Fig. 3.19; Blattner et al., 2002).

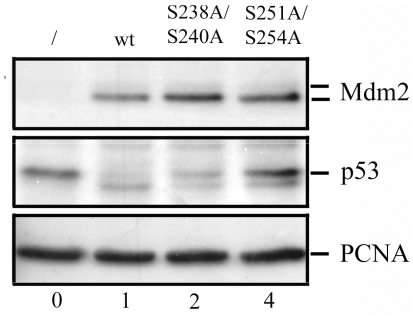


Figure 3.19. Hypophosphorylated Mdm2 does not degrade p53. H1299 cells were transfected with pcDNA3-p53 (lanes 1-4) and wild type pcDNA3-mdm2 (lane 2) or pcDNA3-mdm2 possessing the indicated point mutations (lane 3 and 4). 24 hours after transfection, cells were harvested and analysed for p53, Mdm2 and PCNA by western blotting.

To investigate whether the interaction of the p53 protein with the Mdm2 protein, their localization, p53 ubiquitylation or the interaction of the Mdm2 protein with the MdmX protein is affected by the mutations, I transfected H1299 cells with p53 and wild type Mdm2, S238/240A Mdm2 or/and S251/254A Mdm2. I harvested the cells 24 hours after transfection and analyzed the cellular lysate as described in section 3.5.

In complete agreement with the results I obtained when I inhibited GSK 3, mutation of the GSK 3 consensus sites in the Mdm2 protein did not affect the localization of the p53 protein or the Mdm2 protein (Fig. 3.14, II). It did also not alter the interaction of both proteins, (Fig. 3.15, *right panel*), or the interaction of the Mdm2 protein with the MdmX protein (Fig. 3.16, *right panel*) nor p53 protein ubiquitylation (Fig. 3.17, *right panel*).

To determine whether the mutations in the Mdm2 protein prevent the association of the Mdm2 protein with cellular proteasomes as it occurred in the presence of GSK 3 inhibitors, I transfected U2OS cells with Mdm2 where serine 251 and 254 are replaced by

an alanine (S251,254A Mdm2). I harvested and precipitated the proteasomes from cell lysates with an antibody against the S6b protein. I separated the S6b-antibody complexes by SDS-PAGE and determined the level of co-precipitated Mdm2 protein as described in section 3.6. In contrast to wild type Mdm2, mutated Mdm2 did not associate with the proteasome (Fig. 3.18), indicating that phosphorylation of GSK 3 consensus sites in the central domain of Mdm2 is critical for association of Mdm2 with the proteasome.
As a summary, these results strongly argue that the accumulation of p53 in response to GSK 3 inhibition is related to the hypophosphorylation of the central domain of the Mdm2 protein.

3.8 GSK 3 is inhibited after ionizing irradiation

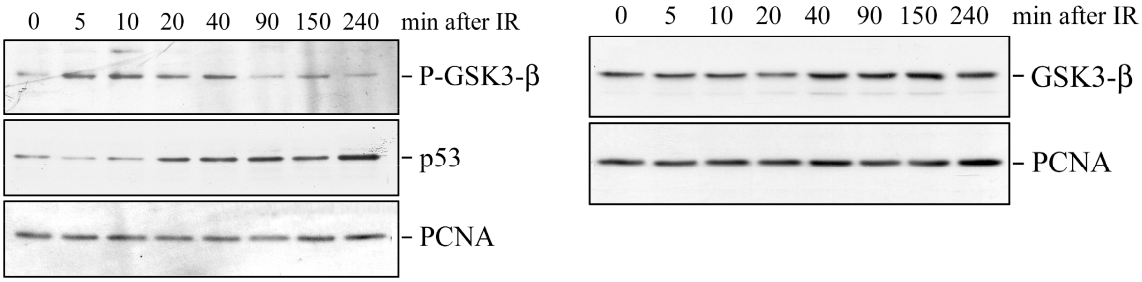
Ionizing irradiation (IR) leads to hypophosphorylation of the central domain of the Mdm2 protein although the mechanism(s) are as yet unclear (Blattner et al., 2002). During my work, I observed that GSK 3 most likely phosphorylates the Mdm2 protein at some of the residues that are hypophosphorylated after IR. If GSK 3 was indeed a physiologic Mdm2 kinase, one would expect that phosphorylation of the Mdm2 protein by GSK 3 must be somehow prevented after IR. One possibility to prevent phosphorylation of the Mdm2 protein by GSK 3 is to inhibit GSK 3 activity. In cells, there are two distinct ways to inactivate GSK 3. GSK 3 can be inactivated either by disruption of multiprotein complexes that contain GSK 3 as it occurs after activation of the Wnt-signalling pathway or via phosphorylation of serine 9 in GSK 3β (serine 21 in GSK 3α) as it happens after activation of different signaling pathways (Fig. 1.9; Sutherland et al., 1993; Sutherland and Cohen, 1994). Since the possibility to inactivate GSK 3 in response to IR by phosphorylation of Ser9 was more likely event I analyzed whether GSK 3 is phosphorylated at Ser9 in response to IR.
I irradiated the human osteosarcoma U2OS cell line with 7.5 Gray by using a Co^{60}-gamma-source and harvested the cells at different time points after irradiation. I prepared protein extracts, separated them by SDS-PAGE, transferred the proteins to a blotting membrane, cut the membrane into half and incubated the lower part of the membrane consecutively with an antibody directed against phosphorylated Ser9 of GSK 3β, and PCNA, for loading control. To monitor total levels of GSK 3β, I incubated the membrane with an anti-GSK 3β antibody. The upper part of the membrane, I hybridized with an Ab-2 antibody directed against the p53 protein.
In consistency with the hypophosphorylation of the central domain of the Mdm2 protein (Blattner et al., 2002), GSK 3β was phosphorylated within 5 minutes after IR. Phosphorylation was retained for 1.5 to 2 hours and declined thereafter to basal levels. The total GSK 3β protein levels did not change significantly under these conditions (Fig. 3.20 A). The p53 protein levels began to raise at twenty or forty minutes after IR and

reached their maximum at four hours after IR. This result shows that phosphorylation of GSK 3β at Ser9 clearly preceeds the accumulation of the p53 protein (Fig. 3.20 A).

To have confidence that the phosphorylation of GSK 3β after IR corresponds indeed to its inhibition, I measured GSK 3 activity after IR.

I irradiated U2OS in a Co^{60}-gamma-source and harvested the cells after different time periods. I incubated the cellular lysates with the pre-phosphorylated GSK 3 substrate phospho-glycogen synthase peptide-2 and radioactive γ-[^{32}P]-ATP. After 15 minutes of incubation, I applied the sample onto the P81 phosphocellulose paper, which binds phosphorylated substrate, and determined the amount of incorporated radioactive phosphate in the scintillation counter.

A



B

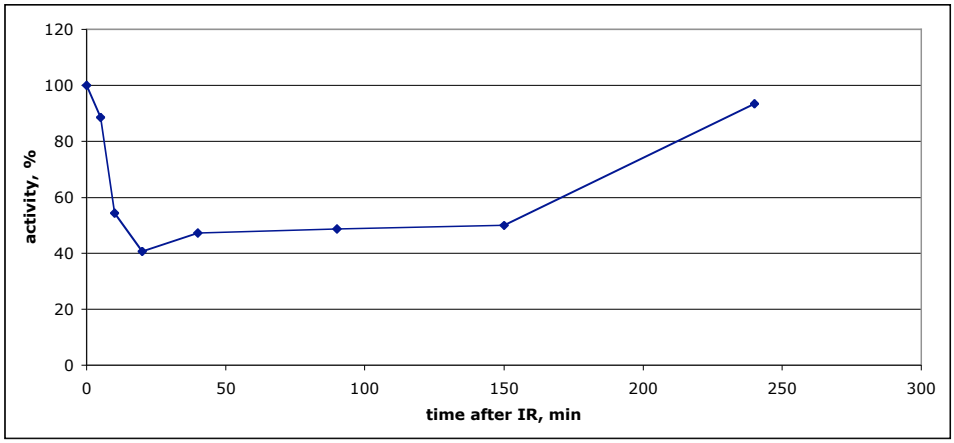


Figure 3.20. GSK 3β is inactivated towards primed substrates after ionizing irradiation. U2OS cells were irradiated and harvested after the indicated times. **A:** Proteins were separated by SDS PAGE and transferred to a blotting membrane. The membrane was probed for phospho-Ser9-GSK 3β, total GSK 3β, p53 and PCNA. **B:** The activity of GSK 3β towards primed substrates. Cellular lysates were incubated at 30°C with phospho-glycogen synthase peptide-2 and γ-[^{32}P]-ATP. After 15 minutes incubation, substrate was washed on phosphocellulose paper P81 and the amount of incorporated phosphate was measured in scintillation counter. The average of two experiments was calculated and plotted.

In consistency with earlier report (Turenne et al., 2001), GSK 3 was inactivated in response to ionizing irradiation. The inactivation was evident as early as 10 min after irradiation and was retained for 2-3 hours. At four hours post-irradiation, GSK 3 activity was fully restored. Thus, inactivation of GSK 3 is completely consistent with the phosphorylation at Ser9 (Figure 3.20).

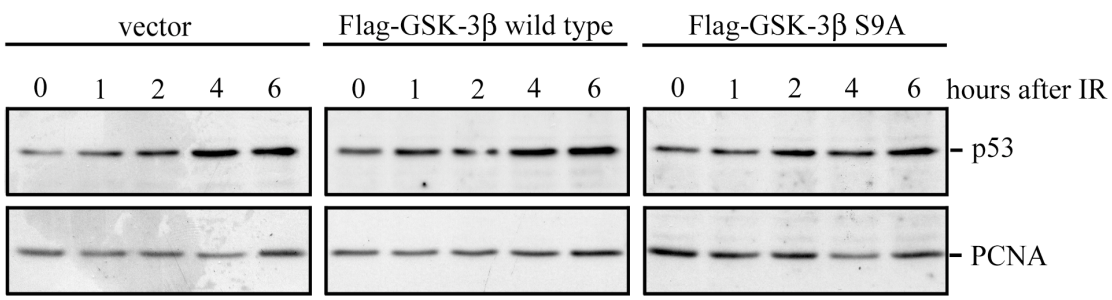
3.9 Inactivation of GSK 3 contributes to p53 accumulation after IR

The delay between the phosphorylation of GSK 3 at Ser9 and the accumulation of the p53 protein would be consistent with a contributory role of the phosphorylation of GSK 3 and in consequence its inactivation, and the accumulation of the p53 protein. If both processes are causally related, then cells expressing a constitutively active form of GSK 3 should accumulate less of the p53 protein after ionizing irradiation than normal cells.

To test this principle, I generated a Flag-tagged mutant form of GSK 3β where Ser9 was replaced with an alanine by site-directed mutagenesis. This mutation makes the kinase refractory to inactivation by Ser9 phosphorylation (Eldar-Finkelman et al., 1996). For control, I also generated a Flag-tagged form of wild type GSK 3β. I transfected U2OS cells with the Flag-tagged wild type GSK 3β, with the Flag-tagged mutant GSK 3β or with the empty vector and grew the cells for 10 days in cell culture medium supplemented with 300 μg/ml neomycin. Expression of GSK 3β was confirmed by western blot analysis with anti-Flag antibodies. I irradiated the selected cells with 7.5 Gray in a Co^{60}-gamma-source, harvested them at zero, one, two, four and six hours after irradiation and determined p53 protein levels by western blotting. I quantified the signal for the p53 and PCNA proteins densitometrically and normalized the signal that I obtained for the p53 protein by the signal that I obtained for the PCNA protein.

In support for the involvement of the GSK 3 protein in the p53 response to IR, the cell line that expressed the constitutively active mutant form of GSK 3β (S9A) accumulated significantly less of the p53 protein in response to IR in comparison to cells that had been transfected with the vector alone (Fig. 3.21). To my surprise, overexpression of wt GSK 3β also reduced the accumulation of the p53 protein (Fig. 3.21). In fact, there was hardly any difference between cells expressing wild type and mutant GSK 3. The basis for the behavior of cells that express either wild type or mutant GSK 3 is not entirely clear. Nevertheless, the reduction in p53 accumulation in response to IR in cells expressing wild type or mutant GSK 3 corresponds precisely to the level of GSK 3 activity which is not significantly reduced in response to IR when cells overexpress wild type GSK 3 or a mutant where Ser9 is replaced with an alanine (Karen Böhme, unpublished observation). In summary, these data suggest that inactivation of GSK 3β in response to IR is required for the full accumulation of the p53 protein.

A



B

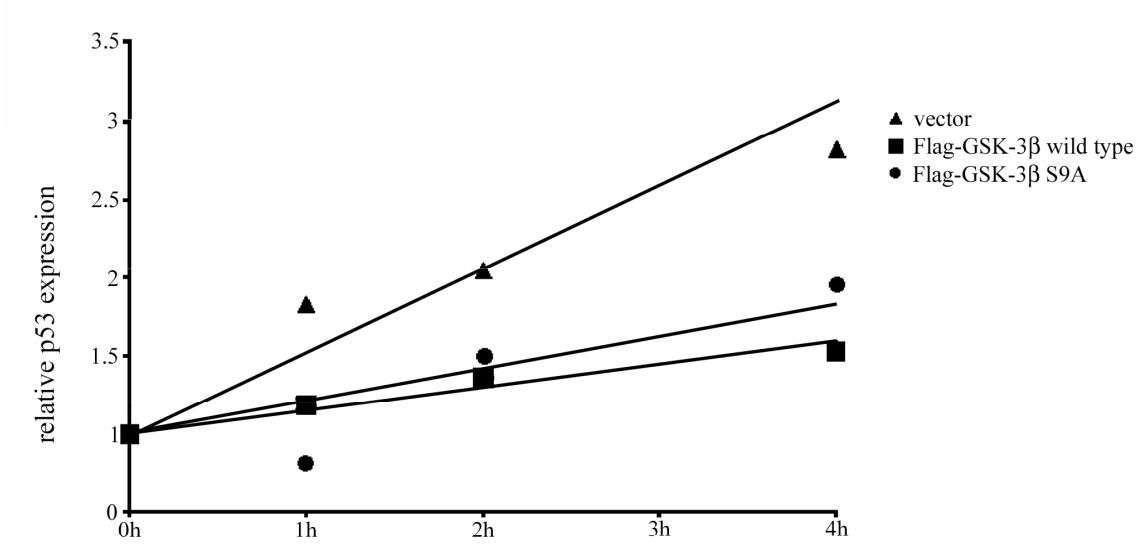


Figure 3.21. Expression of a constitutive active GSK 3 mutant reduces p53 accumulation after ionizing irradiation. U2OS cells were transfected with Flag-tagged wild type GSK 3β or with mutant GSK 3β where serine 9 was replaced with an alanine. Transfected cells were selected for 10 days with 300 μg/ml neomycin, irradiated and harvested after the indicated time. Proteins were separated by SDS-PAGE and transferred to Immobilon P membrane. The membrane was probed for p53 and PCNA. **A:** Western blot. **B:** p53 and PCNA expression of four independent experiments were quantified and mean values of relative p53 expression were plotted. Relative p53 expression of non-irradiated cells was set as 1.

DISCUSSION

4.1 The Mdm2 protein is a physiological substrate for GSK 3

In the last years, the significance of the central domain of the Mdm2 protein for the regulation of the p53 protein was underestimated. However, more recently the central domain of Mdm2 received more attention and several labs are now trying to unravel its function. It is clear that along with the N-terminus, which associates with the p53 protein and the C-terminal RING finger, which harbors the ubiquitin ligase function of the Mdm2 protein, the central domain is critical for p53 ubiquitylation and degradation (Argentini et al., 2001; Zhu et al., 2001; Kawai et al., 2003; Meulmeester et al., 2003; Blattner et al., 2002). Though the precise mechanism, by which the central domain contributes to p53 ubiquitylation and degradation is as yet unclear. Several laboratories proposed a model in which the central acidic domain of the Mdm2 protein fulfills a function that is required for degradation of ubiquitylated p53 (Glockzin et al., 2003; Brignone et al., 2004). This model is supported by the observation that a number of deletion and point mutations in the central domain interfere with the degradation of the p53 protein, although they still allow its ubiquitylation.

Efficient degradation of the p53 protein depends on the phosphorylation of several serines in the Mdm2 protein, which are located in the middle of the central domain (Ser 238, Ser240, Ser244, Ser251, Ser254, Ser258, Ser260; Blattner et al., 2002). When these serines are replaced with an alanine, the p53 protein accumulates though it is fully ubiquitylated. Importantly, this region of the Mdm2 protein is hypophosphorylated in response to ionizing irradiation suggesting a role of the phosphorylation of the central domain in p53 regulation in response to DNA damage (Blattner et al., 2002). The acidic domain is heavily phosphorylated under normal conditions, and a few kinases have been shown to phosphorylate it (Zhang und Prives, 2001; Gotz et al., 1999; Winter et al., in press) but before I began with my PhD work no kinase was known that phosphorylates any of the serines that are crucial for the degradation of the p53 protein, which are serine 238, serine 240, serine 244, serine 251, serine 254, serine 258 and serine 260 of mouse Mdm2. In accordance to described observations kinases that phosphorylate this region must be active under normal physiological conditions, they are likely to be inhibited in response to DNA damage and the inhibition of the kinases should lead to p53 accumulation.

At the beginning of my PhD work, I found that glycogen synthase kinase 3 suites the mentioned requirements. Glycogen synthase kinase 3 (GSK 3) has initially been identified due to its activity in glycogen metabolism. It is now also well known for its activity in the Wnt signaling pathway (Patel et al., 2004). In the following years of my PhD work, I have addressed the question whether GSK 3 is a physiological kinase for the Mdm2 protein.

Along with CKI, GSK 3 is the only kinase requiring prior phosphorylation of its consensus site which is [S/T]xxx[S/T](*P*) (Fiol et al., 1987; x is any aminoacid). The presence of a phosphorylated serine or threonine at the +4 position increases phosphorylation efficiency by 100-1000 fold (Thomas et al., 1999). This property of GSK 3 makes the identification of GSK 3 substrates more complex since *in vitro* phosphorylation assays with bacterially expressed proteins are hardly conclusive. In accordance to it, when I expressed the Mdm2 protein in bacteria and phosphorylated it with recombinant GSK 3 protein, only 1 % of the molecules were phosphorylated. Using *in vitro* kinase assays I found that recombinant GSK 3β phosphorylated N-terminal and central domains of the Mdm2 protein. Moreover, two GSK 3 consensus sites that are present in the central domain were phosphorylated by GSK 3 as determined by two-dimensional peptide analysis.

While I was working on my PhD, Markus Winter, a former diploma student of our group, showed that CKIδ phosphorylates serine 244 of the Mdm2 protein (Winter et al., In press). Serine 244 lies at the +4-position within one of the two GSK 3 consensus sites of the Mdm2 protein. The finding that CKIδ phosphorylates the Mdm2 protein within the GSK 3 consensus site then allowed a two-step *in vitro* phosphorylation, first with the priming kinase CKIδ, which created the phosphorylated +4 residue for GSK 3, and then with GSK 3β itself. Pre-phosphorylation of bacterially expressed Mdm2 by CKIδ remarkably enhanced subsequent phosphorylation by GSK 3β thus supporting the idea that the Mdm2 protein may be an authentic GSK 3 substrate.

After having established that GSK 3 phosphorylates the Mdm2 protein *in vitro*, I addressed the question whether GSK 3 also phosphorylates the Mdm2 protein in a living cell. The obvious and most straightforward approach would be to use phosphorylation specific antibodies that are directed against the putative GSK 3 phosphorylation sites in the Mdm2 protein. Unfortunately, antibodies which recognize these residues are not available. As an alternative approach, I mapped the phosphopeptides of the Mdm2 protein and compared the phosphopeptide maps from cells that I had treated with the GSK 3 inhibitor alsterpaullone with phosphopeptide maps from control cells. As expected from the presence of the consensus site and the *in vitro* phosphorylation data, the two maps differed in the phosphorylation pattern. Phosphorylation of a single peptide was absent in the phosphopeptide maps of inhibitor-treated cells or cells which were transfected with dominant negative mutant of GSK 3β indicating that the Mdm2 protein is indeed a physiologic substrate for GSK 3. The large number of multiply phosphorylated peptides and the complexity of the Mdm2 phosphopeptide maps made it, however, very difficult to draw a final conclusion from these maps. To definitely answer the question whether GSK 3 phosphorylates the Mdm2 protein *in vivo*, I used another approach. I immunoprecipitated the Mdm2 protein from cells and employed this pre-phosphorylated

Mdm2 protein in *in vitro* kinase assays. If Mdm2 is a physiologic substrate for GSK 3, only Mdm2 from cells that had been treated with the GSK 3 inhibitor should be phosphorylated by recombinant kinase since only in the presence of the inhibitor the Mdm2 protein remains unphosphoylated in cells (Fig. 3.5 A). As expected, Mdm2 derived from the GSK 3 inhibitor treated cells was phosphorylated stronger by recombinant GSK 3β further proving Mdm2 to be a physiological substrate of GSK 3. Importantly, deletion of the central domain of Mdm2 completely prevented *in vitro* phosphorylation by recombinant GSK 3β suggesting that GSK 3 phosphorylates Mdm2 *in vivo* within the central domain (aa 200-300). More precise mapping of the phosphorylation site, however, was not possible since neither single point mutations nor small deletion mutants of the parts of the central domain in Mdm2 were not able to block GSK 3 dependent phosphorylation suggesting the existence of several phosphorylation sites within the central acidic domain of Mdm2.

Despite the fact that the GSK 3 protein phosphorylates the Mdm2 protein *in vitro* and the observation that the Mdm2 protein is phosphorylated in GSK 3-dependent manner in cells, my results do not entirely exclude the possibility that the Mdm2 protein may be phosphorylated in the cell by another kinase whose activity depends on GSK 3. Nevertheless, the fact that the Mdm2 and GSK 3 proteins interact *in vivo* supports my assumption that GSK 3 phosphorylates the Mdm2 protein directly.

Since the level of phosphorylation of the central domain of the Mdm2 protein is reported to be critical for the degradation of the p53 protein (Blattner et al., 2002), p53 protein levels can be considered as an indication for the phosphorylation state of the Mdm2 protein, particularly if other effects of GSK 3 inhibition on p53 levels are excluded. Therefore, the effect of GSK 3 on p53 degradation was scrutiniously tested.

As expected, inhibition with structurally independent chemical inhibitors of GSK 3 led to p53 accumulation. Similarly, when I overexpressed GSK 3, p53 protein levels were reduced. When I determined the half-life of the p53 protein in the presence and absence of GSK 3 inhibitors, I observed a much longer half-life for the p53 protein in the presence of the GSK 3 inhibitor revealing that this increase is solely due to blocked degradation of p53. This view is supported by the fact that *p53* mRNA levels remained unchanged in the presence of GSK 3 inhibitor. When I knocked down Mdm2 expression by transfecting cells with Mdm2 siRNA, the p53 protein did no longer accumulate when GSK 3 was inhibited indicating the Mdm2 protein is required for the accumulation of the p53 protein in response to GSK 3 inhibition. The involvement of the Mdm2 protein is further supported by the observation that the E2F-1 protein, another Mdm2 substrate, also accumulates when GSK 3 is inhibited.

Since my results pointed to the involvement of the Mdm2 protein in the stabilization of the p53 protein in response to GSK 3 inhibition, I investigated whether principles other than hypophosphorylation of the Mdm2 protein could interfere with the degradation of the p53 protein in the absence of GSK 3 activity. In accordance to the principles of p53 degradation, which are discussed in detail in the introduction of my thesis (chapter 1.3; Fig. 1.4) several requirements need to be fulfilled to allow p53 degradation. The p53 and Mdm2 proteins must co-localize in the same cellular compartment and the two proteins must be able to associate. In addition, the ubiquitin ligase activity and the post-ubiquitylation function of Mdm2 must be present.
My results show that the inhibition of GSK 3 does not alter the intracellular localization of the p53 and Mdm2 proteins and it does not prevent their interaction. It also does not impair the interaction of the Mdm2 protein with the MdmX protein and it does not reduce the ubiquitin ligase activity of the Mdm2 protein. In fact, inhibition of GSK 3 led to the accumulation of ubiquitylated p53, indicating that the post-ubiquitylation function of the Mdm2 protein is most likely affected. Importantly, inhibition of GSK 3 followed the same lines with regarding to p53 degradation as mutations of serines 238/240 or 251/254 into alanine, which include the GSK 3 consensus sites.
The accumulation of ubiquitylated p53 protein and the similarity of the response to GSK 3 inhibition with the activity of Mdm2 mutants where serines in the GSK 3 consensus sites were replaced with an alanine strongly suggest that the Mdm2 is phosphorylated at least at one of these sites in a GSK 3-dependent manner.

In summary, I conclude that the phosphorylation of the central domain of the Mdm2 protein is the mechanism by which GSK 3 regulates p53 stability. Therefore, I assume that GSK 3 dependent phosphorylation of Mdm2 can be analyzed indirectly, by controlling p53 levels. Based on this assumption, I will discuss whether Mdm2 conforms to the criteria for being a physiological GSK 3 substrate.
A few years ago, Philip Cohen suggested 7 criteria that need to be met to regard a protein as a physiological substrate of GSK 3 (Frame and Cohen, 2001; Table 4.1). Occassionally, it is hardly possible to test all criteria due to the complexity of GSK 3 regulation (Frame and Cohen, 2001). In consequence, only a few of the suggested substrates of GSK 3 have been shown to meet all requirements. Nevertheless, these requirements are regarded as a strategy for identifying physiological substrates of GSK 3.

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

Mdm2 is phosphorylated *in vivo* at the serines 238, 240, 244, 251, 254, 258 and 260 (Blattner et al., 2002). As demonstrated by peptide mapping, several of these serines are phosphorylated by GSK 3 *in vitro*.

1) *The substrate should be shown, by phosphopeptide mapping and sequence analysis, to be phosphorylated by GSK3 in vitro at the same residue(s) that is (are) phosphorylated in vivo.* 2) *Phosphorylation should be abolished by mutagenesis of this (these) site(s) to non-phosphorylatable residue(s).* 3) *The phosphorylation of the endogenous protein in cells should decrease in response to one or more signals known to inhibit GSK 3; dephosphorylation should occur with similar kinetics to the inhibition of GSK 3.* 4) *The endogenous protein should become dephosphorylated at the relevant site(s) in vivo when cells are incubated with cell-permeant inhibitors of GSK 3.* 5) *Phosphorylation of the residues targeted by GSK 3 should affect the function of the protein in a manner consistent with physiological effects of the agonist(s) that regulate(s) GSK 3 activity.* *6) Phosphorylation of the protein at the GSK 3 site(s) should not occur in cells that carry targeted disruptions of the genes encoding GSK 3, and can be restored when GSK 3 is replaced.* *7) Dephosphorylation of the protein at the GSK 3 site(s) should not occur in response to the appropriate signal in cells that do not express one of the protein kinases that lies upstream of GSK 3;*

Table 4.1. Criteria for identifying physiological substrates of GSK 3 (Frame and Cohen, 2001).

2) *Phosphorylation should be abolished by mutagenesis of this (these) site(s) to non-phosphorylatable residue(s).*
The phosphorylation of Mdm2 by GSK 3 seems to occur at multiple sites and I failed to block the Mdm2 phosphorylation by mutating only one or two of the phosphorylation sites. However, the deletion of the central domain completely abolished phosphorylation of the Mdm2 protein by GSK 3 in accordance to the second criterion.

3) *The phosphorylation of the endogenous protein in cells should decrease in response to one or more signals known to inhibit GSK 3; dephosphorylation should occur with similar kinetics to the inhibition of GSK 3.*
There are a few pathways known to regulate GSK 3 activity. Among them, the insulin signaling and Wnt signaling are the most studied ones (Patel et al., 2004). These two

pathways represent two different ways of GSK 3 regulation. In the Wnt pathway GSK 3 activity is regulated via the formation of specific protein-protein complexes, in the insulin signaling pathway PKB phosphorylates GSK 3 at Ser 9 (Ser 21 for GSK 3α) thereby inhibiting GSK 3 activity towards the primed substrates (Patel et al., 2004). The inhibition is very transient as the insulin-induced PKB activation reaches the peak after 5 minutes and then it goes down to the basal level (Cross et al., 1995). The transient inhibition of GSK 3 is not sufficient to make any significant impact on Mdm2 phosphorylation and therefore, p53 stability. Physiologically relevant p53 regulation by GSK 3 should involve the stable inhibition of GSK 3, either indirectly, by inhibiting the priming kinase for Mdm2, which may be CKIδ for Ser244 or an unknown kinase, or by prolonged inhibition of GSK 3 activity via its phosphorylation at Ser9 (Ser21 for GSK 3α). In accordance to the requirements for an Mdm2 central domain kinase, GSK 3 should be inactivated after ionizing irradiation (IR). Indeed, it was reported previously that GSK 3 is inactivated in response to IR (Turenne and Price, 2001). In consistency with these data, I also observed that GSK 3β is inactivated after IR via the inhibitory phosphorylation at Ser9. This phosphorylation was stable for several hours and preceded p53 accumulation. Interestingly enough, after dephosphorylation (reactivation) of GSK 3β, p53 levels decreased as well (data not shown). Thus, p53 protein levels clearly correlated with GSK 3β activity. The kinetics of IR-induced dephosphorylation of the Mdm2 protein was not studied in this work in detail but it was shown before that it precedes the increase in p53 protein levels (Blattner et al., 2002) and it occurs at the same time as GSK 3 inactivation.

Therefore, according to the third criterion, it can be concluded that Mdm2 fits the requirement for a physiological GSK 3 substrate.

4) *The endogenous protein should become dephosphorylated at the relevant site(s) in vivo when cells are incubated with cell-permeant inhibitors of GSK 3.*

At the time of this study there were no specific inhibitors of GSK 3 known (Bain et al., 2003). Therefore, I tested several inhibitors of independent structure: lithium chloride, a classical inhibitor of GSK 3 that is used in therapy for bipolar disorder, bisindolylmaleimides I and IX and alsterpaullone (Hers et al., 1999; Leost et al., 2000; Jope, 2003). In all cases, treatment with GSK 3 inhibitors led to p53 accumulation. As a final prove of GSK 3 dependent p53 accumulation, I knocked-down GSK 3β expression by siRNA and again, I observed the accumulation of the p53 protein.

Phosphopeptide mapping of *in vivo* phosphorylated Mdm2 protein further supports the hypothesis that inhibition of GSK 3 with alsterpaullone or with a dominant negative mutant of GSK 3 (R96A) leads to the dephosphorylation of the Mdm2, in consistency with the accumulation of the p53 protein.

5) *Phosphorylation of the residues targeted by GSK 3 should affect the function of the protein in a manner consistent with physiological effects of the agonist(s) that regulate(s) GSK 3 activity.*
As it was discussed, GSK 3 activity is regulated by ionizing irradiation. The suggested GSK 3 phosphorylation sites are located in the central domain of Mdm2. IR leads to hypophosphorylation of the same residues resulting in p53 accumulation, which is consistent with my data on GSK 3 inhibition (Blattner et al., 2002).

6) *Phosphorylation of the protein at the GSK 3 site(s) should not occur in cells that carry targeted disruptions of the genes encoding GSK 3, and can be restored when GSK 3 is replaced.*
This criterion was not tested. GSK 3α /GSK 3β double knockout cell lines are not reported so far, and creating them was not within the goals of my PhD work. However, the knockout of GSK 3β with siRNA led to the accumulation of the p53 protein.

7) *Dephosphorylation of the protein at the GSK 3 site(s) should not occur in response to the appropriate signal in cells that do not express one of the protein kinases that lie upstream of GSK 3;*
The mechanism of GSK 3 phosphorylation and inactivation after IR is currently under investigation. So far no kinases are known that are responsible for GSK 3 inactivation after IR. The understanding of this mechanism will provide the tool to test the 7th criterion.

In summary, the Mdm2 protein meets 5 out of 7 criteria that are required to be regarded as a physiological substrate of GSK 3. The remaining 2 criteria are impossible to test at the moment due to the absence of the necessary research tools. Nevertheless, further investigations are required to identify the precise phosphorylation sites. The only possibility to reach this goal seems to be the use of phosphorylation specific antibodies against GSK 3 consensus sites. These antibodies, however, do not exist.

4.2 Phosphorylation of the central region of the Mdm2 protein regulates its interaction with the proteasome.

A number of studies demonstrated that ubiquitylation of the p53 protein is not sufficient for its successful degradation. Overexpression of the hRad23 or MdmX proteins, or point mutations or specific deletions within the central domain of the Mdm2 protein do not interfere with p53 ubiquitylation, but hamper or completely block its degradation (Argentini et al., 2001; Zhu et al., 2001; Stad et al., 2001; Blattner et al., 2002; Glockzin et al., 2003). In my work, I demonstrated that inhibition of GSK 3 leads to the accumulation of ubiquitylated p53. This finding supports the hypothesis that the Mdm2 protein has another

function beyond p53 ubiquitylation that mediates degradation of ubiquitylated p53. This new function seems to depend on the central acidic domain of the Mdm2 protein. One of the hypotheses that I put forward describes this function as an interaction of the Mdm2 protein with the proteasome. This interaction is presumably required to bring ubiquitylated p53 to the place of its degradation. It is possible that the interaction of the Mdm2 protein with the proteasome is mediated by a third protein. At least, two proteins that are capable of binding polyubiquitin chains and the proteasome at the same time, the hRad23 proteins are claimed to target the p53 protein for degradation (Glockzin et al, 2003; Brignone et al., 2004). The hRad23 proteins directly interact with the Mdm2 and S5a protein, one of the regulatory subunits of the 19S proteasome. This interaction makes an attractive link between the Mdm2 protein and the proteasome. Moreover, the hRad23 proteins are the only proteins so far that proved to be multi-ubiquitin receptors for the proteasome (Verma et al., 2004). However, current evidence argues against the involvement of hRad23 in the interaction of the Mdm2 protein with the proteasome. First, silencing of hRad23 with siRNA increases the rate of p53 degradation rather then decreases it what one would expect from the model (Brignone et al., 2004). Second, a degradation-defective mutant form of the Mdm2 protein (Δ211-240 aa) displays an increased binding to hRad23 but it is not able to target the p53 protein for degradation (Brignone et al., 2004). It is therefore more likely that the Mdm2 protein interacts directly with the proteasome, without the involvement of bridging proteins. As discussed in the introduction, several E3 ubiquitin ligases associate directly with the proteasome and at least for one of them, pVHL, binding to the proteasome is critical for targeting its substrate for degradation (Tsuchiya et al., 1996). To analyze whether the Mdm2 protein interacts with the proteasome, I investigated whether the Mdm2 protein interacts with one of the AAA ATPase subunits of the 19S regulatory part of the proteasome. In my work, I observed a weak but reproducible interaction of the Mdm2 protein with the S6b subunit of the proteasome. Importantly, inhibition of GSK 3 with alsterpaullone or replacement of the potential GSK 3 phosphorylation site Ser254 with an alanine reduced the interaction of the Mdm2 protein with the proteasome significantly. This observation provides an important clue towards the mechanism how phosphorylation of the central domain of the Mdm2 protein may influence p53 degradation. Phosphorylation of the Mdm2 protein (by GSK 3 and/or other kinases) may regulate the direct interaction of the central domain of the Mdm2 protein with the proteasome. In this case inhibition of the phosphorylation would lead to the dissociation of the Mdm2 protein from the proteasome. Another plausible explanation for the reduction in the interaction of the Mdm2 protein with the proteasome would be the intrasteric inhibition of Mdm2 by its central domain when it is dephosphorylated. The Mdm2 protein is a very flexible protein that undergoes global conformational changes upon binding to different ligands, e.g. p53, zinc ions and RNA (Burch et al., 2000; Burch et al., 2004). Future

investigations should address the question whether phosphorylation of the central domain of the Mdm2 protein might regulate global conformation of the Mdm2 protein.
The possibility to regulate Mdm2 function via phosphorylation could provide a highly interesting possibility to regulate p53 function by small molecule kinase inhibitors. Such inhibitors are easy to develop in contrast to the drugs blocking protein-protein interactions since they are effective in their action and cell permeable. Since many tumors have defective p53 pathways, such inhibitors could present perspective drugs for activation of p53 to cure cancer.

4.3 The accumulation of ubiquitylated p53 leads to selective activation of *p21/waf1 and mdm2* transcription

An important aspect of GSK 3-dependent regulation of the p53 protein is its transcriptional activity. For a possible employment in tumor therapy, it is of utmost importance whether the p53 protein retains its capacity to activate transcription of its target genes when GSK 3 is inhibited. This is particularly interesting since inhibition of GSK 3 has been shown to reduce the capacity of p53 to activate gene transcription (Watcharasit et al., 2003). In consistency with earlier reports (Mao et al., 2001), I observed a strong increase of mRNA levels of p53 target gene *p21/waf1* in the presence of the GSK 3 inhibitor lithium chloride. Thus it appears as if the raise in the p53 protein level after GSK 3 inhibition is able to overcome the inhibitory effect of GSK 3 inhibition on p53-dependent transcription leading to a positive net result and the activation of p53 target genes. Interestingly, among the other p53 target genes, that I investigated, only *Mdm2* mRNA levels were increased. In contrast, transcription of the pro-apoptotic genes *bax* and *puma* was not altered after GSK 3 inhibition. This result opens the intriguing possibility that ubiquitylated p53 may specifically induce promoters of genes that are involved in growth arrest but not promotors of proapoptotic genes.
Mechanisms that contribute to the cellular decision whether a cell dies or arrests in response to p53 activation is currently intensely investigated (Wahl and Carr, 2001). Particularly the question what makes p53 to induce transcription from only a subset of the promoters is as yet unclear. The observation that the ubiquitylated p53 protein is transcriptionally active but induces only the *p21/waf1* and *mdm2* promoter could provide this long sought mechanism.
It is known that the promoters of proapoptotic genes (*DR5/Killer*, *PIG3*, *PTEN*, *FAS/apo1*, *PUMA* and *p53AIP1*) and of genes involved in growth arrest/DNA repair response (*p21*, *14-3-3σ* and *GADD45*) have different levels of loaded transcription preinitiation complexes in the absence of DNA damage (Espinosa et al., 2003). The *p21/waf1* promotor, for instance, is pre-loaded with paused RNA Pol II in normal cells. Once the p53 protein is activated in response to DNA damage, transcription starts (Espinosa et al., 2003). For

proapoptotic genes, however, the assembly of the transcription preinitiation complexes occurs after the DNA has been damaged and this process requires active p53 (Espinosa et al., 2003). A very recent report suggests that the C-terminus of the p53 protein is required for linear diffusion along the DNA (McKinney et al., 2004). It is likely that this linear diffusion allows the p53 protein to find its target sites in promoters. The same lysines that are acetylated and ubiquitylated are important for this function of the p53 protein (Weinberg et al., 2004). While acetylation or phosphorylation of the C-terminus have hardly any effect on the DNA-sliding activity of the p53 protein (McKinney et al., 2004), ubiquitin moieties are more bulky and could possibly block linear diffusion. Therefore, it is tempting to assume that the accumulation of ubiquitylated p53 interferes with the assembly of transcription preinitiation complexes on promotors of proapoptotic genes while it doesn't block the transcription from the *p21/waf1* promotor because this promoter is already preloaded with RNA Pol II in normal unstressed cells. According to this hypothesis, ubiquitylation of the p53 protein could represent the molecular switch that determines whether the cell becomes growth arrested or whether it undergoes apoptosis. DNA damage leads to p53 accumulation via several mechanisms, among which are the disruption of p53/Mdm2 interaction, their intracellular relocalization, inhibition of Mdm2 ubiquitin ligase activity via interaction with nucleolar proteins or the block of Mdm2-proteasome interaction via hypophosphorylation of the central domain of Mdm2 (Fei and El-Deiry, 2003; Ruby and Milner, 2003; Blattner et al., 2002; this work). Depending on the balance between the mechanisms, DNA damage may lead to the accumulation of ubiquitylated or non-ubiquitylated p53 protein, which as a consequence determines the cell fate by inducing transcription of a specific subset of p53 target genes.
In my work, I discovered a pathway that leads specifically to the accumulation of ubiquitylated p53 protein after DNA damage. It would be extremely interesting to see whether a selective block of such a pathway after DNA damage would change the cellular decision to live or to die.

4.4 GSK 3 contributes to the activation of the p53 protein in response to DNA damage

Although I have found that GSK 3 is phosphorylated at serine 9 and inactivated towards primed substrates in response to DNA damage and that this phosphorylation precedes p53 accumulation, it doesn't prove that GSK 3 inactivation is involved in the process that leads to the stabilization of the p53 protein in response to DNA damage. To prove the participation of GSK 3 in the process, I prevented DNA-damage induced phosphorylation of GSK 3 by replacing Ser9 in GSK 3β with a alanine. This mutant form of GSK 3 should be refractory to ionizing irradiation. I transfected either the mutant form (S9A), wild type GSK 3β or a vector control into U2OS cells. Importantly, when I irradiated the cells, the

accumulation of the p53 protein in response to DNA damage was partially blocked in cells that were transfected with mutant GSK 3 suggesting that GSK 3 inhibition contributes to the accumulation of the p53 protein in response to DNA damage. Surprisingly, the cells transfected with wild type GSK 3 did not accumulate p53 to the same levels as control vector transfected cells. This could be possibly explained by a limited phosphorylation of GSK 3 at Ser9 that is not sufficient to inactivate overexpressed GSK 3 in response to DNA damage. Indeed, when the activity of immunoprecipitated GSK 3 was measured, no significant inhibition of overexpressed wild type and mutant GSK 3 in response to DNA damage was observed (unpublished observation, Karen Böhme).

The link between p53 and GSK 3 has already been suggested in previous reports but in a different context. It was reported that GSK 3 directly interacts with the p53 protein after DNA damage stimulating its transcriptional activity (Watcharasit et al., 2002; Watcharasit et al., 2003). GSK 3 phosphorylation sites were identified in the p53 protein. While an initial study suggested Ser33 based on *in vitro* kinase assays (Turenne and Price, 2001), a more recent report identified two other serines in the p53 protein namely Ser315 and Ser376, that are phosphorylated by GSK 3 *in vivo* in response to endoplasmic reticulum (ER) stress (Qu et al., 2004). In my experiments I have not observed a GSK 3 dependent phosphorylation of the p53 protein at any of the sites. However, this does not contradict published data since phosphorylation of the p53 protein by GSK 3 was observed in response to ER-stress or DNA damage induced by the drug capmtothecin and not in unstressed cells (Turenne and Price, 2001; Qu et al., 2004). Jope and co-workers showed that DNA damage, induced by camptothecin, activated GSK 3 that was bound to the p53 protein (Watcharasit et al., 2002; Watcharasit et al., 2003). However, I have observed that ionizing irradiation leads to the phosphorylation of GSK 3 at Ser-9 and therefore, to its inactivation. Although the reports from Jope and co-workers and my results are contradictory, there may be a simple explanation. It is possible that the difference in the cellular reaction is caused by the difference in the nature of the stimuli. Ionizing irradiation initiates the formation of reactive oxygen radicals, single and double DNA strand breaks (Ward, 1988; Lyng et al., 2001), whereas camptothecin induces DNA damage by inhibiting DNA topoisomerase I (Thomas et al., 2004). It would therefore be conceivable that GSK 3 is not inactivated by the DNA double strand breaks but by oxidative stress which is generated by the insult (Shaw et al., 1998). However, I found that GSK 3 was also phosphorylated in response to treatment with the radiomimetic drug bleomycin (Iqbal et al., 1976), as well as in response to treatment with H_2O_2, an inducer of oxidative stress (data not shown). This result suggests that both, DNA damage and oxidative stress, can lead to GSK 3 inhibition.

The discrepancy in my results and observations of Jope and co-workers might be also explained by differential regulation of GSK 3 towards primed versus non-primed substrates. Actually, GSK 3 activity towards the non-primed substrates doesn't depend on the Ser9 phosphorylation (Frame et al., 2001). In fact, in my experiments phosphorylation of the non-primed protein Tau by GSK 3β was stimulated after irradiation (data not shown), while at the same time phosphorylation of the primed substrate phospho-Glycogen Synthase peptide was inhibited. This means that GSK 3β that is phosphorylated at Ser9 may be active towards non-primed substrates and inactive towards the primed substrates. Jope and co-workers have measured GSK 3β activity after DNA damage using non-primed GSK 3 substrate Tau protein (Watcharasit et al., 2002) which is then consistent with my results.

Importantly, the p53 protein seems to be a non-primed substrate for GSK 3 because it is phosphorylated very efficiently *in vitro* in the absence of a priming phosphorylation and the p53 *in vivo* phosphorylation sites identified so far are Ser-315 and Ser-376 which cannot be primed (Qu et al., 2004). The role of GSK 3 in the activation of the p53 protein therefore seems to be dual. First, GSK 3β becomes phosphorylated at serine 9 and inactivated within the first 30 minutes after DNA damage. This leads to the hypophosphorylation of the central domain of the Mdm2 protein. It is most likely that additional kinases, including GSK 3 priming kinases, phosphorylate the central domain of the Mdm2 protein. These kinases are certainly also inactivated leading to massive hypophosphorylation of the central domain of the Mdm2 protein. As a consequence, ubiquitylated p53 accumulates in the nucleus. At the same time, GSK 3 becomes activated towards non-primed substrates and stimulates the activity of accumulated p53 via the non-primed phosphorylation. Such regulation is rather unexpected and future investigations will show whether it may be true.

4.5 Model of p53 regulation by GSK 3: conclusion

GSK 3 inhibition is generally considered to have a pro-survival role. The inhibition of GSK 3 by lithium or small-molecule inhibitors protects primary neurons from death (Li et al., 2002; Cross et al., 2001) and this protective role is possibly mediated by inhibition of GSK 3-induced apoptosis (reviewed in: Grimes and Jope, 2001). My data suggest a novel mechanism by which GSK 3 could regulate cell survival and apoptosis, namely by phosphorylating the central domain of the Mdm2 protein. This phosphorylation stimulates the interaction of the Mdm2 protein with the proteasome and allows degradation of ubiquitylated p53 (Fig. 4.1).

At least one signal is discovered that blocks GSK 3 dependent degradation of the p53 protein. GSK 3β becomes phosphorylated and inactivated in response to ionizing irradiation and this inactivation of GSK 3β leads to Mdm2 hypophosphorylation and

contributes to p53 accumulation. The p53 protein that accumulates in response to GSK 3 inhibition is transcriptionally active and activates transcription of the *p21/waf1* and *mdm2* genes but not pro-apoptotic genes.

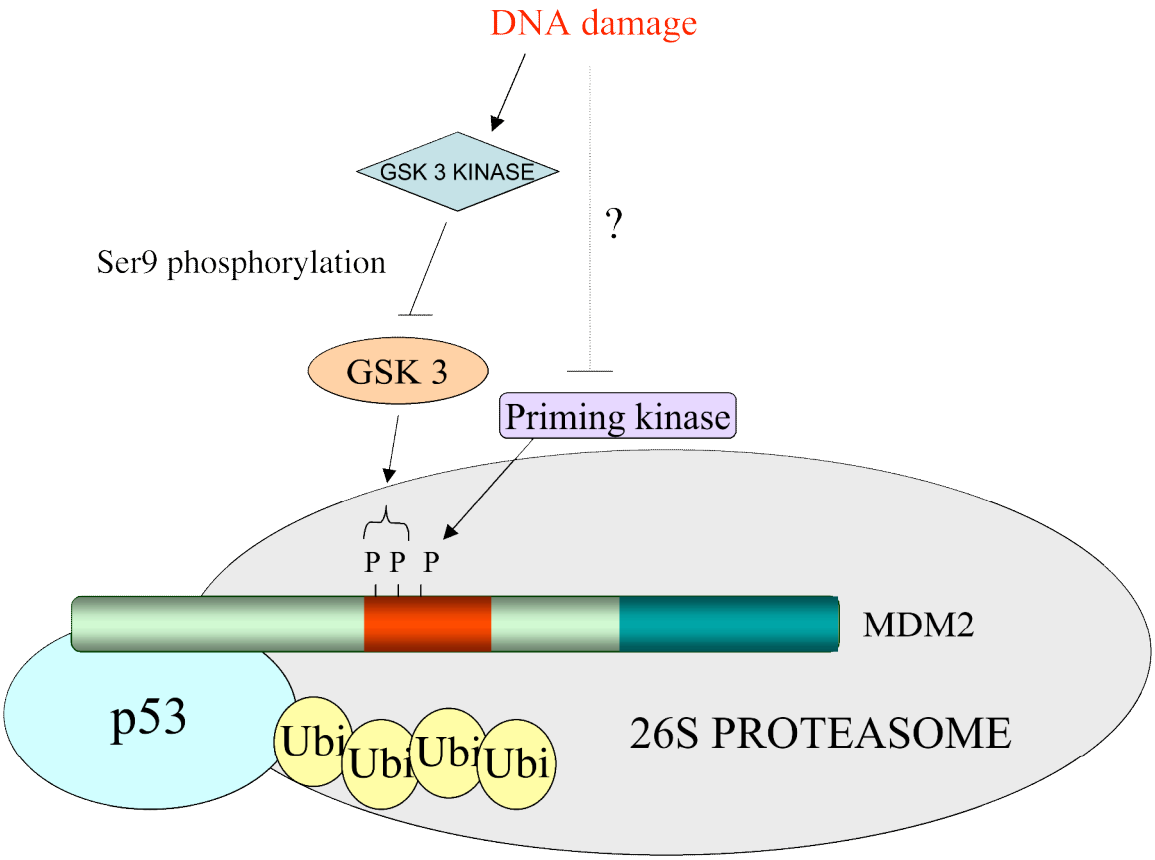


Figure 4.1. Model of p53 regulation by GSK 3. GSK 3 is a constitutively active kinase that phosphorylates the Mdm2 protein within the central domain. Phosphorylation of the Mdm2 protein is critical for the interaction of the Mdm2 protein with the proteasome and for targeting the ubiquitylated p53 protein for the proteasomal degradation. In response to DNA damage, GSK 3β becomes inactivated by phosphorylation of Ser9 that leads in turn to hypophosphorylation of the central domain of the Mdm2 protein. It blocks the interaction of the Mdm2 protein with the proteasome and leads to the accumulation of ubiquitylated p53 protein. Ubiquitylated p53 activates transcription of *p21* and *mdm2*.

This model of GSK 3-dependent degradation of the p53 protein raises many interesting questions that should be addressed in the future. First of all, the physiological consequences of the accumulation of the p53 protein in response to GSK 3 inhibition should be analyzed. It is possible that the increase in *p21* levels in response to p53 accumulation after GSK 3 inhibition initiates growth arrest. This idea is supported by the recent observation that prolonged inhibition of GSK 3 by lithium chloride leads to quiescence in WI-38 human fibroblasts (Zmijevsky and Jope, 2004). Interestingly, several reports demonstrated that the p53 protein protects fibroblasts from apoptosis after ionizing

and UV irradiation (Lackinger et al., 2001; McKay et al., 2000; McKay et al., 2001). It is therefore conceivable that GSK 3 might participate in this protective effect by regulating p53 abundance and transcription of p53 target genes in response to DNA damage. Another interesting question is whether regulation of GSK 3 activity by signaling pathways like Wnt signaling or insulin signaling can affect the p53 response after DNA damage and therefore influence whether cells arrest or die.

Multiple stress response pathways activate p53 protein that leads to cell-cycle arrest, DNA repair, cellular senescence or apoptosis. Since GSK 3 activity is regulated by different signaling cascades, it might provide a novel link between p53 stress responses and many cellular GSK 3-regulated pathways as diverse as glucose metabolism, protein synthesis, microtubule dynamics, cell motility and Wnt-signalling.

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