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# TUMOR SUPPRESSOR P53 AND TRANSCRIPTIONAL CHANGES IN CELLULAR STRESS RESPONSES TO UV RADIATION

LEENA LATONEN

DEPARTMENT OF VIROLOGY, HAARTMAN INSTITUTE,
MOLECULAR AND CANCER BIOLOGY RESEARCH PROGRAM, BIOMEDICUM HELSINKI,
AND
HELSINKI GRADUATE SCHOOL OF BIOTECHNOLOGY AND MOLECULAR BIOLOGY,
UNIVERSITY OF HELSINKI, FINLAND

ACADEMIC DISSERTATION

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***Thesis supervisor***

Dr. Marikki Laiho
Department of Virology, Haartman Institute and
Molecular and Cancer Biology Research Program, Biomedicum Helsinki
University of Helsinki
Helsinki, Finland

***Thesis reviewers***

Prof. John E. Eriksson
Department of Biology
University of Turku
Turku, Finland

Dr. Jorma J. Palvimo
Institute of Biomedicine
Biomedicum Helsinki
University of Helsinki
Helsinki, Finland

***Thesis opponent***

Dr. David W. Meek
Biomedical Research Centre
Ninewells Hospital and Medical School
University of Dundee
Dundee,Scotland, UK

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## Summary

Solar ultraviolet (UV) radiation promotes skin tumorigenesis by inducing DNA damage. UV radiation activates cellular stress responses involving induction of transcription factor p53 and adaptive changes in transcriptional patterns of the cells. p53 is the most often mutated gene in human cancers, indicating its essential role in tumor suppression. Upon UV radiation, amongst many other types of stress, p53 halts cell proliferation and induces damage repair. However, if a particular cell has suffered from extensive, unrepairable amount of damage, p53 launches apoptosis to prevent multiplication of the damaged genome. If these protective cellular responses are compromized, accumulation of mutations may lead to genomic instability and tumorigenesis.

In this study, the effects of UV radiation on p53 and cellular transcriptional changes have been studied in human skin fibroblasts. Low doses of UV radiation induced transient cell cycle arrest, correlating with rapid, but transient, stabilization of p53. In contrast, high doses of UV radiation caused apoptosis with a slower, but sustained, increase in p53. This was apparently due to a lack of induction of Mdm2, a negative regulator of p53. Regulation of p53 target genes upon UV radiation was highly dose-dependent. PI-3-kinases, which phosphorylate N-terminal p53 residues, were without a significant impact on UV-induced p53 activity. Instead, C-terminal acetylation of p53 was time- and dose-dependently regulated. Redox regulation of p53 after genotoxic insults affected its affinity towards a subset of target genes, due to a redox state-dependent ability of p53 residue Cys277 to contact DNA bases. p53 stabilized by proteasome inhibition was found to be transcriptionally active, but to differ from UV-stabilized p53 in its interaction with Mdm2, posttranslational modifications, and subnuclear localization. PI-3-kinases were found to regulate basal expression of Mdm2, and translocation of p53 and Mdm2 into nucleoli after inhibition of the proteasome function.

In a large scale study of transcriptional changes upon UV radiation, 460 transcripts were found to be significantly regulated. The majority (89%) of these were repressed, supporting the idea that UV radiation induces prominent, dose-dependent downregulation of transcription. However, target gene-selective transrepression took also place. Dose-dependent induction of cell cycle arrest and apoptosis by UV radiation are transcriptionally highly distinct responses, and only 5% of the significantly regulated genes were common to both low and high doses of radiation. The low dose induced transient changes in transcript patterns followed by a recovery, whereas after the high dose, the changes occurred more slowly and were persistent. A clustering analysis and functional classification of the UV-regulated targets indicated involvement of changes in transcriptional and translational machinery, and inflammatory, anti-proliferative, and anti-angiogenic transcripts in the different biological responses. Several UV-regulated unknown genes were identified to be studied further for their role in cellular stress responses.

# Table of contents

## List of original publications

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals.

I Latonen L, Taya Y, Laiho M. UV-radiation induces dose-dependent regulation of p53 response and modulates p53-HDM2 interaction in human fibroblasts. *Oncogene* 20, 6784-6793 (2001).

II Latonen L, Kurki S, Pitkänen K and Laiho M. p53 and MDM2 are regulated by PI-3-kinases on multiple levels under stress induced by UV radiation and proteasome dysfunction. *Cellular Signalling* 15, 95-102 (2003).

III Buzek J, Latonen L, Kurki S, Peltonen K, Laiho M. Redox state of tumor suppressor p53 regulates its sequence-specific DNA binding in DNA-damaged cells by cysteine 277. *Nucleic Acids Research* 30, 2340-2348 (2002).

IV Gentile M*, Latonen L*, Laiho M. UVC radiation induced cell cycle arrest and apoptosis are transcriptionally highly divergent responses. *Nucleic Acids Research* 31, 4779-4790 (2003).

*These authors contributed equally to this work

# Abbreviations

| | |
|---|---|
| 6-4PP | (6-4)-photoproduct |
| ARF | alternative reading frame |
| ATM | Ataxia telangiectasia mutated |
| ATR | Ataxia telangiectasia-related |
| bp | base pair |
| C | carboxy |
| CDK | cyclin-dependent kinase |
| CK | casein kinase |
| CKI | cyclin-dependent kinase inhibitor |
| CPD | cyclobutane-type pyrimidine dimer |
| CTD | C-terminal domain |
| DSB | double strand break |
| ECM | extracellular matrix |
| GGR | global genomic repair |
| GTF | general transcription factor |
| HAT | histone acetyl-transferase |
| HDAC | histone deacetylase |
| HPV | human papilloma virus |
| KO | knock-out |
| IR | ionizing radiation |
| MAP | mitogen-activated protein |
| Mdm2 | Murine double minute 2 |
| MEF | mouse embryonal fibroblast |
| N | amino |
| NER | nucleotide excision repair |
| NES | nuclear export signal |
| NLS | nuclear localization signal |
| PI-3 | phosphoinositide-3 |
| Rb | retinoblastoma |
| RE | response element |
| RNAP | RNA polymerase |
| ROS | reactive oxygen species |
| TAD | transactivation domain |
| TAF | TBP-associated factor |
| TBP | TATA-binding protein |
| TCR | transcription-coupled repair |
| TF | transcription factor |
| UV | ultraviolet |
| UVB | ultraviolet B radiation |
| UVC | ultraviolet C radiation |
| wt | wild type |
| XP | xeroderma pigmentosum |

# Introduction

Multicellular organisms have developed through disciplined control of cell division, growth, and death, achieved by tightly regulated changes of gene expression able to adapt to environmental challenges. The information stored in our genes lies at the heart of proper organismal development and homeostasis, and thus has to be preserved. Several mechanisms have evolved to maintain genomic integrity in order to ensure prevention, removal, or destruction of undesired changes in our genetic information. These mechanisms are not perfect, however, and errors resulting from both normal metabolic processes in the cell and exogenous causes are sometimes left unrepaired. This imperfection guarantees continuous evolution, but for a single cell, changes in the genetic information may pose a threat to continued homeostasis and normal functions. At organismal level, failure of these control mechanisms are at the cost of disease and malfunctions of organs. Maybe the most captivating outcome from failures of this kind is cancer: the escape of cells from surrounding restraints, resulting in cells with indefinite ability to proliferate. Ironically enough, one of the most destructive scourge of modern man has - even though just on the cellular level - achieved something people are so often craving for: the ability to outwit, outplay, and outlive.

Centrally cast in the play of cancer is tumor suppressor p53. This “guardian of the genome” is suggested to maintain genomic integrity by making decisions whether, in case of genomic insult, the damage is reversible and the cell is allowed to survive. If yes, the necessary actions are undertaken within the cell to repair the damage. If not, p53 serves as the executioner triggering the destruction mechanisms which result in the death of an irreparably damaged cell, thus preventing possible propagation of an altered genome.

p53 is challenged on a daily basis by sunlight. UV radiation, even though necessary for the birth and maintenance of life on earth, is the most prominent physical carcinogen in our natural environment. It possesses an ability to damage our DNA, thus inducing mutations to our genome. p53 is able to respond to this damage in a manner still poorly understood at the molecular level. In addition to activation of p53, other stress-responsive events take place when cells are subjected to UV light. In this thesis, the activation of p53 and transcriptional regulation in the cellular responses to UV radiation have been studied using human skin fibroblasts as a model. The aim was to gain information on how cellular UV responses normally occur, to be able to identify how they have failed in cancer.

# Review of the literature

## REGULATION OF EUKARYOTIC GENE EXPRESSION

Human genome seems to contain approximately 30000 genes, majority of which code for proteins holding individual characteristics (The genome international sequencing consortium 2001, Venter *et al*. 2001). To achieve a proper orchestra of functional proteins from this pool of genes within any type of a cell, at any given moment throughout development and life of an organism, expression of genes has to be controlled in a very detailed manner. Regulation of gene expression occurs virtually at every possible level on the route to construct a protein according to the information on its gene: transcribing the sequence of deoxyribonucleic acid (DNA) to a messenger ribonucleic acid (mRNA), processing of the mRNA molecule, transportation of it to the cytoplasm, and the translation of it on ribosomes. The main level of restriction is, according to current knowledge, initiation of transcription.

### *Chromatin structure*

To fit the extensive 2 m of human DNA to a cell nucleus, it is first wrapped around histone proteins, which in their non-acetylated form have a positive charge and thus bind DNA well. Nucleosome units of histone octamers (containing two each of H2A, H2B, H3, and H4) wrapping 146 bp of DNA are linked by 20 bp of DNA and an additional histone (H1), and are further coiled and folded to form chromatin fibers (Workman and Kingston 1998, Lee and Young 2000). The downside of this tight packing is the nonaccessibility of DNA to the transcription machinery. In order to transcribe a particular sequence, positive regulation has to occur to relieve the structural inhibition (Kornberg 1999). Nucleosomal structure is loosened by histone acetylation to decrease the affinity of histones to DNA (Sterner and Berger 2000). This is carried out by histone acetyltransferases (HATs) like p300, CBP (*C*REB-*b*inding *p*rotein), and p/CAF (*p*300/*C*BP-*a*ssociated *f*actor), which render DNA accessible to other proteins, especially transcription machinery (Workman and Kingston 1998, Chen *et al*. 2001). Chromatin structure is affected also by other posttranslational modification of histones, including acetylation, phosphorylation, methylation, and ubiquitination (van Leeuwen and Gottschling 2002). Other mechanisms to relieve nucleosomal structures are histone octamer sliding or jumping along DNA, and ATP-dependent histone remodelling (Workman and Kingston 1998). In addition, availability of genomic sequences to transcriptional machinery is regulated by epigenetic mechanisms, including silencing of genes by methylation of DNA (Jaenisch and Bird 2003).

### *RNA polymerase II-regulated promoters*

Protein-coding genes in eukaryotic cells are transcribed by RNA polymerase II (RNAPII). Human RNAPII machinery is composed of a 12-subunit core and general transcription factors (GTFs; TFIIA, TFIIB, TFIID, TFIIE, TFIIF, TFIIH), which are necessary for the enzyme to be able to associate with a template (Woychik and Hampsey

2002). Each protein in the RNAPII machinery has a specific function to govern, for example, interaction of the core complex with transcriptional regulators, stabilization of the complex, selection of the start site, and regulation of the elongation rate. To start transcription, an active RNAPII complex has to form on the promoter of the gene, a regulatory DNA region upstream (5') of the protein coding sequence. A promoter consists of a core promoter, which binds the RNAPII complex, and gene-specific transcriptional regulator-binding sites. The core promoter includes transcriptional start site and often a TATA box 25-30 bp upstream of the start site (Lee and Young 2000). When exposed, the TATA box is recognized by TATA-binding protein (TBP), a subunit of TFIID, which allows sequential docking of the RNAPII complex on the promoter and thus eventually leads to initiation of transcription (Pugh 2000).

Although the complex of RNAPII and GTFs is sufficient to drive basal gene transcription *in vitro*, numerous coregulatory proteins are required for efficient transcription *in vivo* (Woychik and Hampsey 2002). An orchestra of additional transcription factors regulates a given promoter by binding to it and either promoting or inhibiting the formation of an active RNAPII complex. Transcription factors also recruit or inhibit histone-modifying complexes. Expression of a particular gene at a given moment depends on the sum of these different modulatory events (Figure 1). Central to this regulation is the composition and context of the promoter: its transcription factor-binding sites, arrangement of these sites relative to each other influencing protein-protein interactions, and addition of architectural proteins (Lee and Young 2000, Gill 2001). Also other regulatory DNA elements, including enhancers and silencers located either upstream or downstream and up to 85 kb away from the start site, can influence the expression of a gene in *trans* through the regulatory factors they bind (Lee and Young 2000).

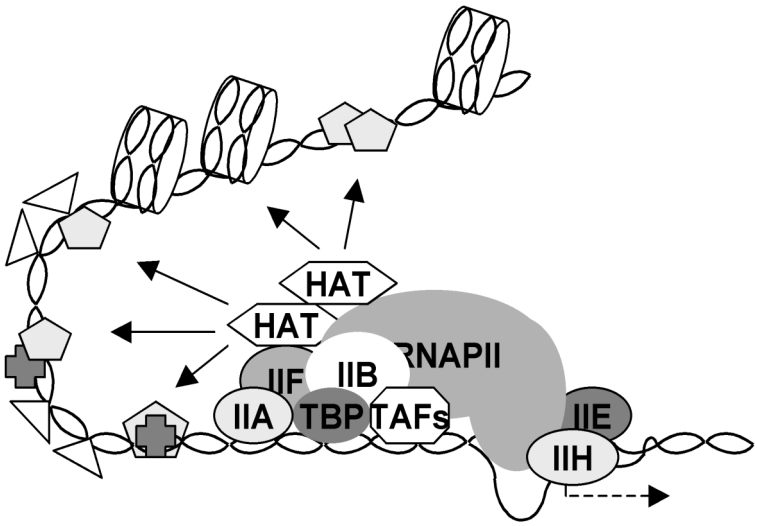



**Figure 1**. A model of eukaryotic RNAPII transcription initiation complex. RNAPII requires general transcription factors (TFII) to associate with template DNA and to become active. Histone acetyl-transferases (HAT) are needed to acetylate nucleosomal histones (sylinders) to relieve chromatin structure, and to acetylate several transcription associatory factors. Sequence-specific transcriptional activators (octagons) and repressors (crosses) either promote or inhibit the formation of an active transcription initiation complex. In addition, several structural proteins are required to obtain a favourable local structure on DNA through e.g. DNA bending (triangles). Modified from Carey 1998, Fry and Peterson 2001.

### *Transcription factors*

The normal and adaptive gene expression responses are mediated by specific transcription factors, the regulation of which forms basis to control gene expression. The set of active transcription factors at a given moment in any cell varies depending on e.g. cell type, state of differentiation, environmental signals, and stress (Fry and Farnham 1999). Transcription factors are often latent becoming functional during a physiological response (Kornberg 1999). Their activity can be regulated by several means, usually by changing either protein localization, pattern of posttranslational modifications, or specific protein-protein interactions (Kornberg 1999, Sterner and Berger 2000, Freiman and Tjian 2003). Transcriptional activators have at least two domains: one to bind DNA sequence-specifically and one to recruit or stimulate activity of chromatin-modifying complexes or transcription initiation apparatus (Lee and Young 2000). One activator usually regulates several genes. Transcriptional repressors can be divided into two sub-groups: general and gene-specific (Lee and Young 2000). The general repressors often interact with TBP inhibiting its ability to bind to promoters, thereby hindering the formation of an active RNAPII complex. Many gene-specific transcriptional repressors bind to transcriptional activators inhibiting their functions, or compete with them for binding sites on the promoters (Lee and Young 2000, Courey and Jia 2001).

## MAINTENANCE OF GENOMIC INTEGRITY IN EUKARYOTES

DNA is not chemically inert, and it faces both endogenous and exogenous challenges constantly (de Laat *et al*. 1999). Endogenous changes, arising from normal DNA metabolism, are probably the greatest source of DNA errors. In DNA replication, they occur at the rate of 1 error/$10^{10}$ nucleotides synthesized; a rate which is greatly enhanced in case of defective proofreading (Jiricny 1998). Different types of DNA lesions can be caused by exogenous effectors, such as chemical compounds and irradiation, e.g. single and double strand breaks by ionizing radiation (IR), inter- and intrastrand crosslinks by chemical agents such as cytostaticum cisplatin, and certain base modifications by UV radiation (de Boer and Hoeijmakers 2000). As a result of DNA damage, transcription and DNA replication are hampered, and thus cell cycle arrest, cell death, or decrease in genomic instability are induced (de Boer and Hoeijmakers 2000). At organismal level, DNA lesions cause carcinogenesis, genetic disorders, and aging (Hanawalt 1998, Hoeijmakers 2001). To preserve genetic information intact, DNA has to adapt to the challenges. To achieve this, a variety of enzymatic DNA fidelity control and repair mechanisms have evolved. Through these, DNA damage is recognized by proteins which initiate series of events leading to elimination of the lesions and to the restoration of intact DNA (Hanawalt 1998, Hoeijmakers 2001).

### *The cell cycle*

Eukaryotic cells multiply in the cell cycle, which is composed of four phases (Figure 2). In the synthesis (S) phase, the genome is copied and eventually divided between the two daughter cells in mitosis (M). In between there are two gap periods (G1 and G2), during which cells prepare themselves for S and M phases. When cells are not dividing, they exit the cell cycle to stay in a quiescent state (G0). The progression through different steps in the cell cycle is tightly regulated by fluctuating activities of protein complexes composed of cyclins and their respective kinases (cyclin-dependent kinases; CDKs) specific for particular phases of the cell cycle (Johnson and Walker 1999, Nurse 2000) (Figure 2). The activity of cyclin/CDK complexes is regulated by multiple mechanisms, especially by oscillating protein synthesis and degradation of cyclins, inhibitory phosphorylations on CDKs, and by binding of factors of the two cyclin-kinase inhibitor (CKI) families (Arellano and Moreno 1997). INK4 (*in*hibitor of cd*k4*/6) family consists of four members, $p16^{Ink4a}$, $p15^{Ink4b}$, $p18^{Ink4c}$, and $p19^{Ink4d}$, which have specific activities towards inhibiting cyclin D-associated kinase activity (Vidal and Koff 2000). Members of the Cip/Kip-family of inhibitors, $p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$, preferentially inhibit activity of CDK2, but bind also CDK4-containing complexes (Johnson and Walker 1999, Vidal and Koff 2000).

The initial "start" of cell proliferation is progression from G1 to S phase. During mid- to late G1 phase, a cell monitors for growth-promoting and -inhibiting signals. Provided that the cellular environment is favourable for proliferation, the cell decides to enter S phase by bypassing the restriction point (R) (Figure 2). Under nonstressed circumstances, this commitment to replicate DNA and divide is irreversible until the next G1 phase (Bartek and Lukas 2001a). G1/S transition is controlled by the retinoblastoma (Rb) protein and its homologs p107 and p130 (Weinberg 1995, Bartek and Lukas 2001a). Rb in its hypophosphorylated state binds and inhibits E2F transcription factors. Phosphorylation of Rb by cyclin/CDK complexes in late G1 releases E2F factors to drive expression of genes necessary for G1/S transition (Weinberg 1995). Another important factor for G1/S transition is transcription factor Myc which induces several factors promoting S phase entry (Pelengaris *et al*. 2002). During the cell cycle, a cell monitors successful completion of the previous phase before proceeding to the next one. These key transitions are often referred to as the cell cycle checkpoints (Hartwell and Weinert 1989).

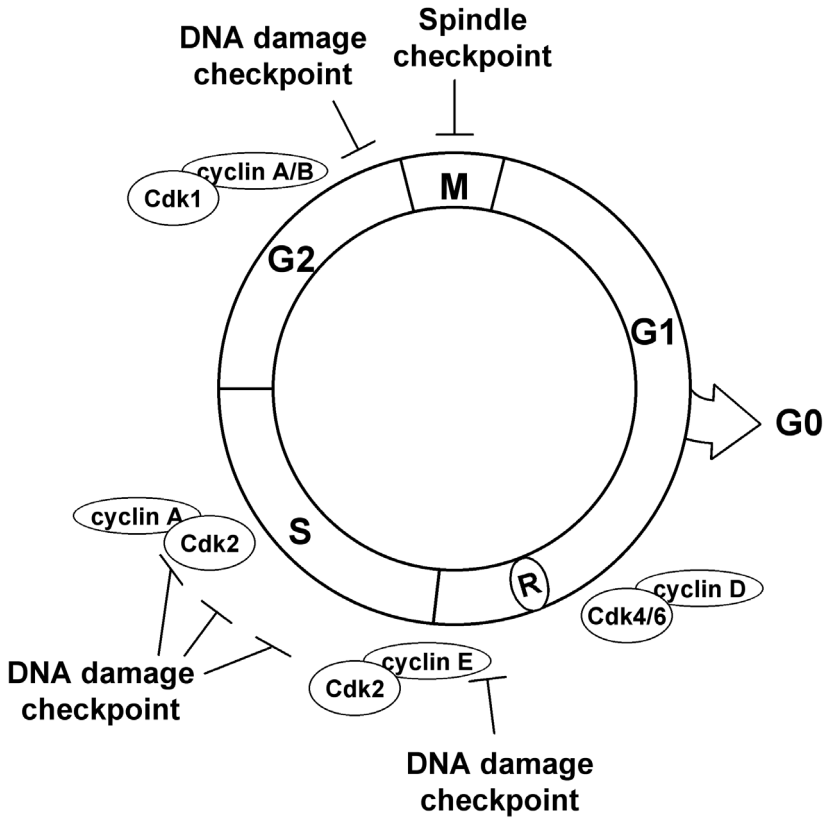



**Figure 2.** The cell cycle and DNA damage checkpoints. Cells multiply by dividing in the cell cycle driven by activities of different cyclin-CDK complexes (see text for details). The integrity of DNA is monitored in DNA damage checkpoints in late G1, late G2 and M (spindle checkpoint). R, restriction point.

### *DNA damage checkpoints*

Integrity of DNA is monitored during the cell cycle in DNA damage checkpoints which mostly overlap the cell cycle checkpoints (Zhou and Elledge 2000) (Figure 2). If either replication errors or DNA damage exists, cell cycle progression is arrested and DNA repair mechanisms are activated. The cell cycle arrest is maintained until the damage has been repaired, after which re-entry to the cell cycle is allowed (Hartwell and Weinert 1989, Bartek and Lukas 2001b). The main DNA damage checkpoints function in late G1 and late G2. During mitosis, the spindle checkpoint monitors for chromosome aberrations before chromosome segregation. In addition, an intra-S DNA damage surveillance exists to monitor for presence of DNA damage, especially double strand breaks (DSBs). This mechanism is often referred to as a checkpoint, but, in contrast to the other DNA damage checkpoints, the intra-S surveillance does not have the ability to halt cell cycle progression, only to slow it down (Bartek and Lukas 2001b).

The importance of DNA damage checkpoints is underlined by their evolutionary conservation (Zhou and Elledge 2000). They are composed of monitoring systems identifying DNA lesions, signal transducers communicating this information onwards, and effectors halting the cell cycle and repairing the lesions (Zhou and Elledge 2000, Bartek and Lukas 2001b). The upstream elements of the surveillance cascades seem to be mostly shared by different checkpoints, but the downstream effectors and their targets vary (Bartek and Lukas 2001b). Despite that most proteins needed for DNA repair are identified, it is still not finally established how the different types of damage are recognized and how the decisions on the cellular response are made (Hanawalt 1998).

The primary DNA damage sensors may include the Rad1-Rad9-Hus1 complex, Rad17, and the phosphoinositide-3-kinase (PI-3-kinase)-related proteins ataxia telangiectasia mutated (ATM) and ataxia telangiectasia-related (ATR) (Bartek and Lukas 2001a,b). The choice of transducers may depend on the damage type and the primary sensors. For example, ATM and ATR have overlapping functions, but the former is essential for IR-induced and the latter for UV-induced phosphorylation of several G1/S checkpoint proteins (Figure 3). First, a rapid cell cycle arrest is induced by activating the Chk1/Chk2 kinases, which then phosphorylate the Cdc25A phosphatase (Mailand *et al*. 2000). This leads to ubiquitination of Cdc25A and its rapid degradation by the proteasome, rendering the respective cyclinE/Cdk2 complex inactive through an inhibitory phosphorylation, and inhibiting entry into the S phase (Bartek and Lukas 2001a). Later, the same kinases function to maintain the cell cycle arrest through induction of tumor suppressor p53, while cyclin D is also downregulated, resulting in inhibition of the cyclinE/Cdk2 complexes by increased interaction with the CKI protein $p21^{Cip1}$ (Bartek and Lukas 2001a, Agami and Bernards 2002). ATM/ATR also regulate G2/M checkpoint via analogous cascades and the intra-S checkpoint by targeting several DNA repair-associated proteins, such as Brca1 (*br*east *ca*ncer *1*) and NBS1 (*N*ijmegen *B*reakage *S*yndrome *1*) (Abraham 2001, Bartek and Lukas 2001b, Pietenpol and Stewart 2002).

Several pathways exist to repair different types of DNA damage (de Boer and Hoeijmakers 2000). DSBs are repaired by homologous recombination-dependent repair or end-joining, and most small base modifications are repaired by base excision repair. Bulky, helix-distorting adducts caused by UV radiation, some chemicals, and oxidative damage are repaired by nucleotide excision repair (NER) in mammalian cells (de Laat *et al*. 1999). Lower eukaryotes can repair UV-induced DNA damage also by the photolyase enzyme (Thoma 1999). Considerable overlap exists in the substrate specificity and some proteins taking part in the different DNA repair processes.

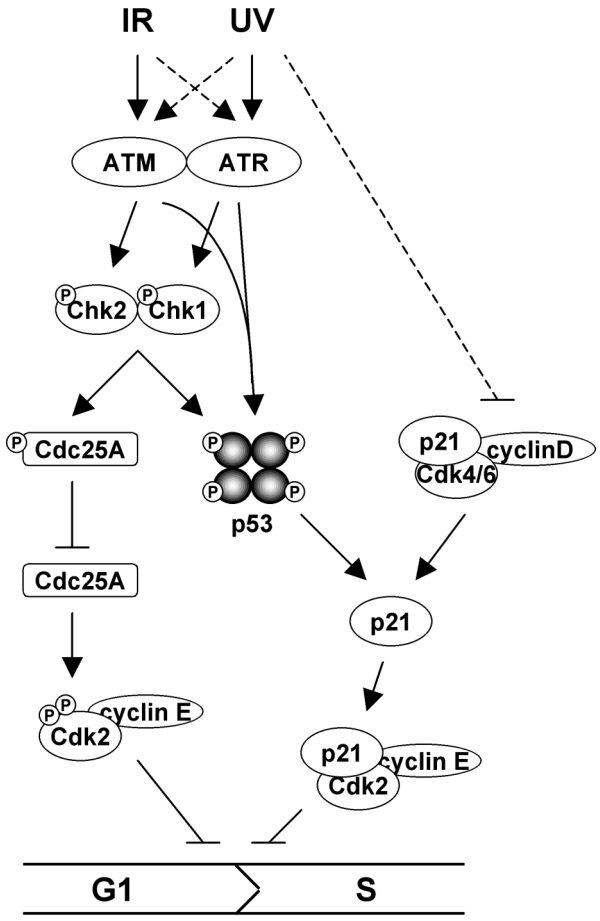



**Figure 3.** Signal pathways in DNA damage checkpoint controlling G1/S phase transition. DNA damage (IR or UV radiation) activates ATM and ATR kinases, which phosphorylate downstream targets Chk1/2 and p53. In addition, degradation of cyclin D is promoted by an unknown mechanism. These events lead to inactivation of cyclin E/Cdk2 complexes by inhibitory phosphorylation of Cdk2 or increased interaction with inhibitory protein $p21^{Cip1}$, and respective inhibition of G1/S transition.

### *Apoptosis*

An organized cell suicide program, apoptosis, is critical for organ development and tissue homeostasis in multicellular organisms (Baehrecke 2002). In addition, when cells are surmounted with irreparable amount of damage, the checkpoint functions can lead to activation of apoptosis to remove potentially hazardous cells from an organism. Contrary to necrosis, a pathological form of cell death, apoptosis allows a cell to die without inducing an inflammatory response in the surrounding tissue (Baehrecke 2002, Leist and Jäättelä 2001). Apoptotic process depends on numerous extra- and intracellular pro- and antiapoptotic signals which are integrated to activate apoptotic effectors only when necessary. Two separate, but crosstalking, mechanisms of apoptosis induction have been identified (Gupta 2001). In the first one, intracellular death messengers lead to disruption of mitochondrial membrane potential (Wang 2001). The following release of cytochrome c from mitochondria activates the apoptotic executors, cysteic proteases called caspases (Earnshaw 1999). Alternatively, signals from membrane-bound death receptors can lead to mitochondria-independent activation of caspases (Gupta 2001). The morphological apoptotic changes, including DNA fragmentation, nuclear condensation, loss of cell volume, and membrane blebbing (Kerr *et al*. 1972), usually result from the activity of caspases, but also caspase-independent pathways of programmed cell death exist (Leist and Jäättelä 2001). Finally, the remaining apoptotic bodies are engulfed by the surrounding cells (Baehrecke 2002).

### *Cancer*

The checkpoint functions are not bullet proof, and over time DNA accumulates changes (Hoeijmakers 2001). This may lead to genomic instability and contribute to tumorigenesis when mutations occur and subsequently change behavior of genes controlling the cell cycle, DNA repair, cellular life span, apoptosis, and intercellular communication and interactions (Hanahan and Weinberg 2000). Incidence of many types of cancers in human population increases with age, and it has been proposed that over six consecutive changes must occur for a cancer to form (Fearon and Vogelstein 1990). To be able to proliferate despite the restraints posed by its environment, a tumor cell must obtain self-sufficiency in growth signals and insensitivity to growth-inhibitory signals. It must also be able to evade from apoptosis and obtain limitless replicative potential. At a later stage in tumor development, malignant growth requires sustained angiogenesis and ability to invade the surrounding tissue and metastasize (Hanahan and Weingberg 2000).

Alterations in two types of genes contribute most to formation of cancer. Proto-oncogenes promote tumorigenesis by a dominant gain of growth-promoting function. Tumor suppressor genes normally suppress carcinogenic progression; a function which is recessively lost to enable tumorigenesis. These often have opposing functions in the same, crucial cellular pathways. For example, the Rb pathway, critical for controlling normal cell proliferation and targeted in most - if not all - human cancers, harbors several oncogenes and tumor suppressor genes (Figure 4) (Ho and Dowdy 2002). The other major pathway targeted in human cancers is the tumor suppressor p53 pathway which functions in the damage checkpoints. Important proto-oncogenes include growth-promoting c-Myc, signaling molecule Ras, and anti-apoptotic Bcl-2 (*B-c*ell *l*ymphoma *2*) (Pelengaris *et al*. 2002, Coultas and Strasser 2003, Downward 2003). Several DNA repair- and apoptosis-promoting genes act as tumor suppressor genes, such as the mismatch repair genes MSH2 (*m*ut*S h*omolog *2*) and MLH1 (*M*ut*L h*omolog *1*), Apaf-1 (*a*poptotic *p*rotease *a*ctivating *f*actor *1*), and some of the caspases (Macleod 2000). Cell origin and tissue environment affect tumorigenesis, and many oncogenes and tumor suppressor genes are crucial mostly for a certain type of cancer originating from differentiated tissue, e.g. Brca1 for breast tumors, and APC (*A*denomatous *p*olyposis of the *c*olon) for colon cancer (Fearon and Vogelstein 1990, Macleod 2000).

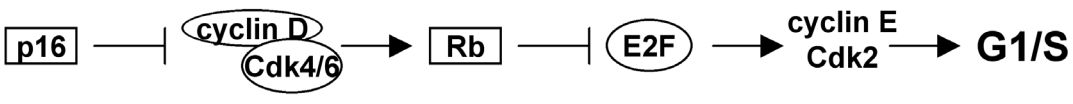



**Figure 4.** Proto-oncogenes and tumor suppressor genes often have opposite functions in the cellular pathways controlling cell proliferation and apoptosis. As an example, the Rb pathway controlling cell cycle entry is shown. The pathway contains several proto-oncogenes (ovals) and tumor suppressor genes (boxes), whose disturbed functions are associated with tumorigenesis.

## ULTRAVIOLET RADIATION

UV light is electromagnetic radiation emitted from the sun (or an artificial source) invisible to human eye. UV radiation is divided to three areas according to its wavelength: UVA 320-380 nm, UVB 290-320 nm, and UVC 190-290 nm (Figure 5). Energy content of the radiation is inversely correlated to its wavelength, rendering UVC the most potentially harmful part of UV light (Tyrrell 1994). The ozone layer absorbs solar UVC and most of UVB. At present, 1-10% of UV radiation on Earth surface is UVB and over 90% UVA, but the proportion of shorter wavelengths is increasing due to stratospheric ozone depletion (Lloyd 1993). UV radiation has many effects on skin, including inflammation, immunosuppression, alterations in the extracellular matrix (ECM), and accelerated aging (Norval 2001, Wlaschek *et al*. 2001). In addition, UV radiation harms the eyes especially by promoting age-related ocular diseases (Roberts 2001). The most hazardous effect of increasing UV light for humans is, however, increased risk of skin cancers (de Gruijl 1999, Armstrong and Kricker 2001).

### *Cellular damage caused by UV radiation*

Organic material can be harmed by absorbed UV radiation. Cells contain photosensitive molecules, chromophores, which can receive photons from the radiation and subsequently lift electrons to a higher energy state. This excitation can occur only when the energy of the photon is close to the difference between the energy states of the electron. A chromophore can pass its excited energy to another molecule and cause a chain reaction (Tyrrell 1994). The occurrence and nature of these events depend on the chemical structure of the chromophore, wavelength of radiation, and specific reaction conditions. Due to the complexity and amount of different biomolecules, UV radiation can cause various events within a cell (Figure 5). These photobiological events can occur extremely fast, but their consequences may be detectable years afterwards, e.g. in the form of cancer (Tyrrell 1994, de Gruijl 1999).

The main cellular chromophores for UV radiation are DNA and oxygen-dependent chromophores. Because of the ring structures of its bases, DNA absorbs short wavelength UV very efficiently (de Gruijl *et al*. 2001). It is the main chromophore for UVC and absorbs a significant amount of energy also in UVB wavelenghts (Ravanat *et al*. 2001). UV radiation can cause several types of damage to DNA molecules. The most apparent are cyclobutane-type pyrimidine dimers (CPDs) and (6-4)-photoproducts (6-4PPs), which crosslink adjacent DNA bases (Ravanat *et al*. 2001). These UV-induced DNA bulges halt RNA polymerase elongation along the DNA, thus inhibiting gene expression (Tornaletti and Hanawalt 1999). Oxidative events become increasingly important in the longer wavelengths and are the main damage caused by UVA (Kielbassa *et al*. 1997) (Figure 5). Oxygen-requiring events provoked by UV radiation are divided into two types. The first involves an excited photosensitive molecule joining to oxygen to form a reactive radical. In the second, an excited molecule transfers energy or an electron to oxygen, forming

either a singlet oxygen or a superoxide anion radical. Via these reactive oxygen species (ROS), UV radiation induces oxidative stress in cells causing e.g. lipid peroxidation and additional types of DNA damage. Longer wavelength UV radiation can induce relatively small amounts of DNA breaks and DNA-protein crosslinks. UV radiation can also harm lipids and proteins, but the reproducibility of these cellular molecules makes them biologically less significant chromophores. Large amounts of UV radiation can, however, induce degeneration of the inner and outer cellular membranes, inhibition of macromolecular synthesis, and a chaotic state in cellular metabolism (Tyrrell 1994).

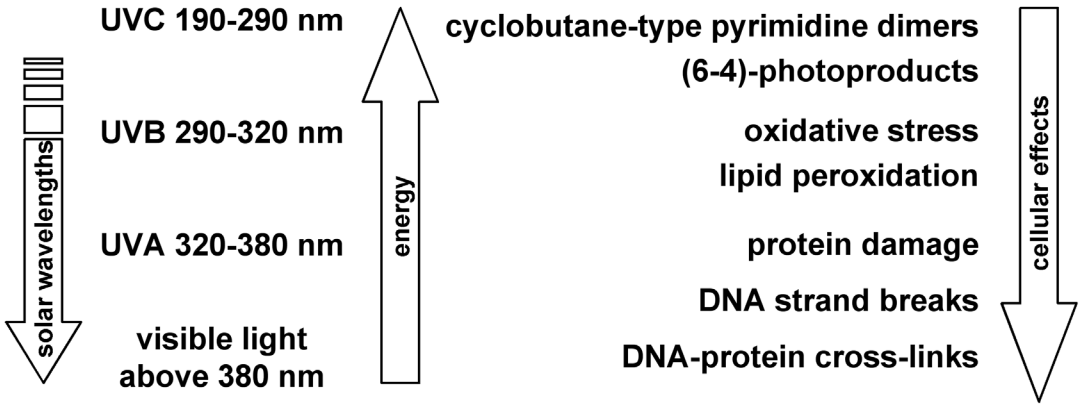



**Figure 5.** UV radiation and the types of cellular damage it causes. UV light is divided to three wavelength areas (UVA, UVB, UVC). Solar UVC is mostly restrained in the stratospheric ozone layer. The damage which UV radiation inflicts on cellular chromophores depends on its wavelength, which is inversely correlated to its energy content.

### *Cellular adaptation to UV radiation-induced damage*

Both oxidative and DNA damage caused by UV radiation provoke adaptive cellular responses by inducing repair events, signaling cascades, and changes in transcription (de Gruijl *et al*. 2001, Bender *et al*. 1997). Repair of UV-induced DNA lesions is launched immediately after a UV insult. At the same time, both a cellular response, either a replication arrest or apoptosis, and paracrine events are induced (Kulms and Schwarz 2000, Decraene *et al*. 2001). The latter involves changes in the ECM and signaling to initiate inflammation and the following photoprotective responses in skin as an organ (Clydesdale *et al*. 2001, Sturm 1998). These events are mediated by several cellular signaling cascades targeting pre-existing pools of transcription factors, which then change the gene expression pattern of the cell to achieve an adaptive transcriptional response (Bender *et al*. 1997).

*Cellular response pathways induced by UV radiation*

The DNA damage induced by UV radiation triggers several signaling cascades. Activated ATR leads to phosphorylation of Chk1 kinase, immediate cell cycle arrest through degradation of Cdc25, and activation of tumor suppressor p53-dependent prolonged cell cycle arrest. If the UV damage is successfully repaired, the cells are eventually allowed to re-enter the cell cycle by yet unidentified mechanisms (Bartek and Lukas 2001a). DNA damage is thought to be the main trigger of UV-induced cell cycle arrest and apoptosis, but UV radiation has also several other means to provoke a cellular response (Kulms and Schwarz 2000). It induces clustering and internalization of several cell surface growth factor and cytokine receptors independently of their ligands (Rosette and Karin 1996). This results in activation of the respective downstream signaling cascades, the major signal transducers being mitogen-activated protein (MAP) kinases JNK (*j*un *N*-terminal *k*inase) and p38 (Bode and Dong 2003). Intracellular increase in ROS activates MAP kinases through activation of Raf and MEKK1 (Rittie and Fisher 2002). UV radiation induces also GTP-binding protein family members Ras, Rac, and Cdc42, which act upstream of MAP kinases Erk, JNK, and p38 (Takai *et al*. 2001). MAP kinases are induced dependently on the dose, time, and wavelength of UV radiation, subsequently acting in the regulation of cell growth control, survival, chromatin remodeling, and apoptosis (Bode and Dong 2003).

Several transcription factors take part in the UV response, of which AP-1 (*a*ctivating *p*rotein *1*), NFκB (*n*uclear *f*actor *κB*), and p53 are the most recognized and well characterized ones (Bender *et al*. 1997). AP-1 is a transcription factor family inducing different sets of target genes and responses depending on the activating stimuli. After UV radiation, AP-1 seems to mostly work in an antiapoptotic fashion (Schreiber *et al*. 1995, Wisdom *et al*. 1999) and regulate several ECM proteins (e.g. matrix metalloproteinases, type I procollagen) (Rittie and Fisher 2002). NFκB is activated both by UV radiation-induced DNA damage and plasma membrane-originated events (Legrand-Poels *et al*. 1998). DNA damage provokes NFκB-dependent production of immunoregulatory cytokines involved in contact hypersensitivity, inflammation, and immune suppression. NFκB is thought to act mainly in an antiapoptotic manner, but it has both anti- and proapoptotic properties (Bours *et al*. 2000). The response resulting from NFκB activation is determined in collaboration with other stress-responsive pathways, especially that of p53 (Ryan *et al*. 2000).

*Nucleotide excision repair*

Helix-distorting DNA bulges, such as those induced by UV radiation, are repaired by NER. It involves 20-30 proteins each with specific functions (de Boer and Hoeijmakers 2000). Most proteins in NER recognition and incision steps have been identified and named according to seven complementation groups of Xeroderma pigmentosum (XP), a photosensitivity disorder resulting from deficient NER (XPA to XPG) (Hanawalt 1998). When the NER repair complex has access to a UV lesion, DNA is opened around the injury. The damaged strand is incised at approximately 15 nucleotides from both sides of the bulge, and the lesion-containing piece is removed. Finally, new DNA is synthetisized

and ligated to fill the place of the removed sequence (de Laat *et al*. 1999, de Boer and Hoeijmakers 2000). UV damage causes local inhibition of transcription around the damaged areas in the nucleus through deprivation of TFIIH, which is needed for both NER and transcription initiation (Moné *et al*. 2001).

There are two mechanistically different subtypes of NER, transcription-coupled repair (TCR) and global genomic repair (GGR) (Hanawalt 2002). TCR occurs relatively rapidly and only repairs the template strand of transcriptionally active DNA. GGR, on the other hand, occurs slower repairing also the non-template strand and the non-transcribed areas. Cell survival depends more on TCR than GGR (Ljungman and Zhang 1996), but, eventually, genomic integrity is influenced more by GGR (Hanawalt 2001). As GGR is launched by XPC-hHR23B protein complex recognizing UV-type of DNA damage (Sugasawa *et al*. 1998), TCR is probably triggered by RNAPII complexes halting at UV-induced DNA bulges (Tornaletti and Hanawalt 1999). In TCR, when repair enzymes are recruited, they must remove the polymerase to access the lesion. This occurs either by ubiquitination and the following degradation of RNAPII (Ratner *et al*. 1998), or by some other, still unresolved mechanism (Hanawalt 1998). In addition, TCR requires CS-A and CS-B proteins not needed for GGR, the deficiency of which results in UV radiation hypersensitivity disorder Cockayne syndrome (Venema *et al*. 1990).

### *Role of UV radiation in skin carcinogenesis*

The damage induced by solar UV radiation contributes to all skin cancer types, including basal cell carcinoma, squamous cell carcinoma, and cutaneous melanoma. All these cancer types show correlation to sun exposure, either the total sun exposure in long term or episodes of sunburn (de Gruijl *et al*. 2001). Importance of NER in suppressing UV-induced tumorigenesis is highlighted in the extremely tumor-prone phenotype of most XP complementation groups (Kraemer *et al*. 1994). Deficiency in DNA repair of a UV-lesion may result in a mutation within the next two rounds of cell division, since the damaged bases are often misinterpreted during DNA replication. Conventional DNA polymerases cannot bypass a UV lesion, but a specific DNA polymerase (η) can, although only by inserting an adenine opposite to the lesion (de Gruijl *et al*. 2001). Even though this activity is controlled by a proofreading exonuclease (Bebenek *et al*. 2001), mutations frequently occur, leading almost exclusively to C to T transitions (de Gruijl *et al*. 2001). UV acts as an initiator of carcinogenesis through the DNA damage it causes, but also promotes tumorigenesis by inducing local and systemic immunosuppression (Clydesdale *et al*. 2001).

## TUMOR SUPPRESSOR p53

p53 is a central, damage-responsive transcription factor which governs the adaptive and protective cellular stress responses (Levine 1997). It is activated following several types of exo- and endogenous cellular damage, such as DNA damage, hypoxia, nucleotide imbalance, oxidative damage, and spindle damage (Figure 6). It also reacts to alterations in cell proliferation induced by activation of oncogenes or viral transformation. Upon activation, p53 determines the fate of the cell based on the severity of the damage. It can halt progression of the cell cycle and direct damage repair. On the other hand, if a particular cell has suffered extensive and unrepairable damage, p53 induces apoptosis (Prives and Hall 1999, Vousden and Lu 2002). p53 elicits its normal functions mainly by acting as a transcription factor, and it regulates genes contributing to the cell cycle, DNA repair, and apoptosis. Even though p53 was found over 20 years ago and has been vigorously studied ever since (May and May 1999, Prives and Hall 1999), it is not yet clear what determines whether a transient protective response or a terminal apoptotic response is induced by p53 following a certain insult.

As p53 protein is potentially very harmful to cells, it must be kept well controlled under normal cell growth conditions. Yet, it has to react to a variety of stress signals rather quickly and to function in a complex manner upon activation. Thus, it is not unexpected that p53 is subject to several regulating mechanisms and can interact with dozens of other proteins (Prives and Hall 1999). Distinct activation pathways exist for p53, and they show specificity for e.g. stress stimulus, cell type, and growth phase. Some general mechanisms are common for these pathways: p53 protein level is constantly balanced between synthesis and degradation, and this most often includes regulation by the inhibitory factor Murine double minute 2 (Mdm2). The intrinsic activity of p53 protein can also be modulated, as well as its localization and interactions with other proteins. Altered posttranslational modifications of p53 are often included in these different modes of regulation (Meek 1999, Appella and Anderson 2001). The net effect of all these events contribute to the outcome of a cellular response driven by p53 activation.

Through its actions to prevent cellular functions from becoming abnormal, or to even destroy aberrant cells, p53 acts as an essential tumor suppressor. Over 50% of human tumors harbor *TP53* mutation, which renders the p53 protein functionally impaired. This makes *TP53* the most commonly mutated gene in human cancers. It is also speculated that the rest of human tumors have dysfunctional p53, through, for example, disturbed regulation or protein-protein interactions, and that functional impairment of p53 is required for malignant tumor development (Vousden and Lu 2002). Loss of p53 function does not seem to be connected with the first steps of tumorigenesis, but with the transformation of a benign tumor to a malignant cancer.

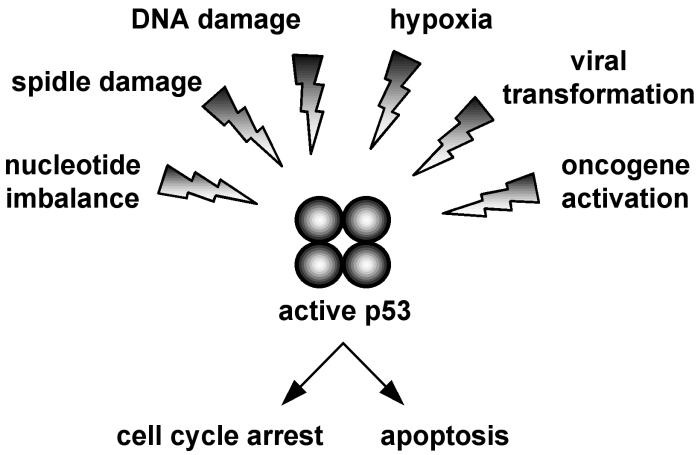



**Figure 6.** Tumor suppressor p53 is activated by several forms of cellular stress, which induce either p53-mediated cell cycle arrest or apoptosis.

### *Structure of p53*

The *TP53* gene is located in the short arm of chromosome 17 (17p13) and belongs to a family with three identified members in mammalian cells to date. The gene spans about 20 kb and contains 11 exons, of which the first exon is non-coding and localized 8-10 kb away from exons 2-11 (Benchimol *et al*. 1985, Isobe *et al*. 1986, McBride *et al*. 1986, Miller *et al*. 1986). *TP53* codes for a protein which is relatively well conserved in evolution (Soussi *et al*. 1987) especially in five regions (amino acids 13-23, 117-142, 171-181, 234-250, 270-286; designated as I-V, Figure 7) (Soussi *et al*. 1990). Human p53 is 393 amino acids long and has a molecular mass of 53 kDa. As most transcription factors, p53 has several distinct, but inter-dependent, functional domains with specific properties (Prives and Hall 1999) (Figure 7). Transactivation domain (TAD; amino acids 1-42) is located in the amino-terminal (N-terminal) part of the protein next to a proline-rich area (63-97). Sequence-specific DNA binding is mediated through the central core of p53 (102-292). Carboxy-terminal (C-terminal) part of p53 composes of a flexible linker region (300-318), oligomerization domain (323-356), and a basic, regulatory C-terminal domain (CTD; 363-393) (May and May 1999, Prives and Hall 1999).

The TAD is acidic allowing p53 to recruit basal transcription machinery when bound to DNA. The TAD can directly interact with TBP and TBP-associated factors (TAFs) of TFIID (Lu and Levine 1995, Thut *et al*. 1995). The N terminus of p53 can also interact with replication protein A (RP-A) and p62 subunit of TFIIH (Dutta *et al*. 1993, He *et al*. 1993, Li and Botchan 1993, Xiao *et al*. 1994). Amino acids 22-23 in the conserved region I have a key role in p53 transcriptional activity (Lin *et al*. 1994). Many p53 inhibitory proteins, like Mdm2 and several viral proteins, bind to this region blocking the transcriptional activity of p53 (Prives and Hall 1999). The Pro-rich area of p53 has a series of repeated proline residues typical for interactions with SH3-binding domain-containing signal molecules (Gorina and Pavletich 1996). The exact function of this regulatory domain remains unclear, but it is needed for p53 to induce apoptosis in some systems

(Sakamuro *et al*. 1997) and to mediate tumor cell growth suppression (Walker and Levine 1996). Deletion of the Pro-rich domain does not influence transcriptional activity of p53 directly, but alters p53-mediated transrepression, as well as the stability and DNA binding of the protein (Müller-Tiemann *et al*. 1998, Venot *et al*. 1998, Berger *et al*. 2001, Dumaz *et al*. 2001).

The DNA binding domain of p53 interacts sequence-specifically with double-stranded DNA (Kern *et al*. 1991, Bargonetti *et al*. 1991). Its central core is composed of a large β-sandwich and three loop-based elements (Cho *et al*. 1994) (Figure 7). Loop 1 (L1) contacts DNA within the major groove and loop 2 (L2) within the minor groove. Loop 3 (L3) packs against L1 and stabilizes it. L2 and L3 are connected by a zinc-atom ($Zn^{2+}$) tetrahedrally coordinated on amino acids Cys176, His179, Cys238 and Cys242 in their reduced state, which stabilizes the loops. The loops overlap with four conserved regions which contain the major mutational hot spots of p53 (Pavletich *et al*. 1993, Bargonetti *et al*. 1993, Wang *et al*. 1993), underlining the importance of DNA binding for tumor suppressive function of p53. The most frequent cancer-associated p53 mutations occur on Arg248 and Arg273, residues directly contacting DNA. The second most frequent type of cancer-related p53 mutations target residues which are structurally stabilizing. In solution, p53 is predominantly as tetramers, which are the most effective oligomer forms in terms of DNA binding (Kraiss *et al*. 1988, Clore *et al*. 1995). Tetramerization occurs through the oligomerization domain of p53, which has a β-sheet-turn-α-helix-motif governing the formation of a dimer of dimers (Waterman *et al*. 1995).

The C terminus of p53 recognizes DNA and possesses autonomous DNA binding and strand reassociation ability, but no sequence-specificity in itself (Prives and Hall 1999). The CTD is predominantly composed of basic residues, and it can be heavily modified posttranscriptionally. The CTD regulates sequence-specific DNA binding of p53, but the mechanism is not entirely resolved (Hupp 1999, Ahn and Proves 2001, Yakovleva *et al*. 2002; see later). The CTD can also bind RNA and is postulated to recognize DNA damage as it binds DNA ends and single-stranded DNA (Bakalkin *et al*. 1995), mismatches (Lee *et al*. 1995), Holliday junctions (Lee *et al*. 1997), and DNA irradiated with IR (Reed *et al*. 1995).

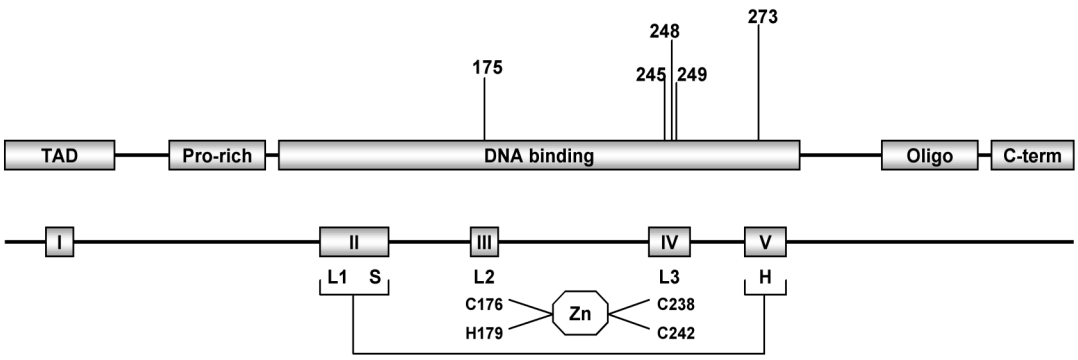



**Figure 7.** Structure of p53 protein. See text for details.

### *Regulation of p53 stability by degradation through the proteasome*

During normal metabolism in eukaryotic cells, the 26S proteasome degrades most proteins outside the lysosomal pathway (Kierszenbaum 2000). It recognizes its targets by ubiquitin, a small, 8.5-kDa protein which can be covalently linked to lysine residues of proteins (Weissman 2001). Targeting proteins to degradation via the proteasome pathway is the most important function known for ubiquitin ligation so far (Hochstrasser 1996). Target proteins can be either monoubiquitinated with single ubiquitin molecules or polyubiquitinated with ubiquitin chains (Weissman 2001). However, only polyubiquitination promotes degradation (Thrower *et al*. 2000). At least three types of enzymes are needed to mediate ubiquitination of a protein. First, an E1 ubiquitin-activating enzyme sequesters a ubiquitin molecule, transfers it through an E2 ubiquitin-conjugating enzyme to an E3 ubiquitin ligase, which, finally, conjugates the ubiquitin on the target protein. Usually, E3 ligases recognize substrates and determine the specificity of the ubiquitination process (Weissman 2001). An additional enzyme, E4, is involved in the assembly of the branched ubiquitin polymers in the polyubiquitination process (Koegl *et al*. 1999).

p53 is expressed constantly, but its levels are kept low by continuous protein degradation (Prives and Hall 1999). Under normal cellular growth conditions, p53 has a half-life of less than 20-30 min. Upon stress, p53 is accumulated to cells. This occurs by blocking its degradation, which leads to increased protein half-life, rather than by upregulating gene expression or mRNA stability. p53 is degraded through the 26S proteasome; a process which is driven by the ubiquitination of p53 to several C-terminal lysine residues (Rodriguez *et al*. 2000). The major E3 ubiquitin ligase for p53 is Mdm2, the most important regulator of p53 (Momand *et al*. 2000, Alarcon-Vargas and Ronai 2002). Mdm2 inhibits the transcriptional activity of p53 by binding to the p53 TAD (Juven-Gershon and Oren 1999, Momand *et al*. 2000) and ubiquitinating p53 and itself, leading to degradation of the proteins (Honda *et al*. 1997, Haupt *et al*. 1997, Kubbutat *et al*. 1997). However, Mdm2 can only monoubiquitinate p53 (Lai *et al*. 2001), and it relies on its ability to bind p300 to mediate p53 degradation (Grossman *et al*. 1998, Zhu *et al*. 2001). This was recently shown to be due to an intrinsic ubiquitin ligase activity of p300, by which it performs p53 polyubiquitination (Grossman *et al*. 2003).

Even though it is believed that Mdm2 shuttles p53 from nucleus to cytoplasm (Roth *et al*. 1998), nuclear export is unessential for p53 degradation (Yu *et al*. 2000, Lohrum *et al*. 2001, Xirodimas *et al*. 2001), and p53 can be degraded by both cytoplasmic and nuclear proteasomes (Shirangi *et al*. 2002). After stress, p53 accumulation occurs through weakening of p53-Mdm2 interaction, which can be achieved by two mechanisms: inhibition of Mdm2 by interacting proteins, or changes in posttranslational modifications of p53 and/or Mdm2 (Figure 8). The best characterized Mdm2-interacting protein capable of inhibiting p53 degradation is the alternative reading frame protein (ARF), which is induced by e.g. oncogene activation (Sherr and Weber 2000) (Figure 8; see below). HIF-1α (*h*ypoxia *i*nducible *f*actor *1α*) also interacts with Mdm2 and thereby inhibits p53 degradation (Chen *et al*. 2003). Mdm2 is induced by p53, creating an autoregulatory loop to downregulate p53 at the end of a stress response (Juven-Gershon and Oren 1999, Momand *et al*. 2000). Recently, another E3 ligase for p53 was identified: Pirh2 can ubiquitinate p53 *in vitro* and promote p53 degradation *in vivo* independently of Mdm2 (Leng *et al*. 2003). Interestingly, Pirh2 is a p53 target gene, possibly forming a feedback regulatory loop (Leng *et al*. 2003).

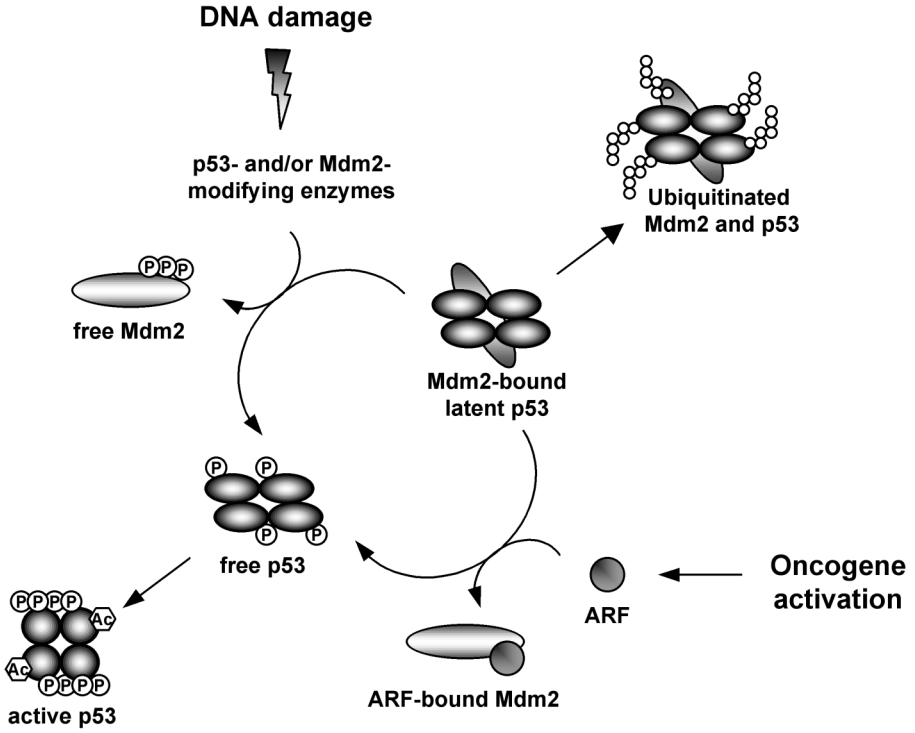



**Figure 8.** Regulation of p53 protein. See text for details.

### *Mdm2*

Mdm2 (in human cells also referred to as Hdm2) is an oncogene due to its inhibitory effects on p53 (Deb 2002). It has also p53-independent properties relevant to tumor suppression, presumably through its interactions with the cell cycle regulators Rb and E2F1 (Momand *et al*. 2000, Daujat *et al*. 2001). *HDM2* gene is located on chromosome 12q13-14, a region often amplified in human cancers. As an alternative to p53 mutation, overexpression of Mdm2 protein is a tumor-associated mechanism to inactivate p53, and amplification of *HDM2* occurs in 7% of human cancers (Momand *et al*. 2000). Nuclear exclusion represents an additional, Mdm2-related mechanism to abrogate p53 function (Liang and Clarke 2001, Stommel *et al*. 1999). Knock-out (KO) of *Mdm2* in mice is embryonic lethal, a phenotype which can be rescued in a *p53*-null background, underlining the importance of Mdm2 in inhibiting p53 activity (Montes de Oca Luna *et al*. 1995, Jones *et al*. 1995).

The major Mdm2 transcript in human cells codes for a 90-kDa protein with 491 amino acids and a RING finger motif on its C terminus, responsible for the E3 ubiquitin ligase activity (Momand *et al*. 2000). Several regulatory phosphorylation sites on Mdm2 have been identified both in its N terminus, which binds p53, and in its central acidic domain mediating p53 ubiquitination (Hay and Meek 2000). Mdm2 is phosphorylated by Akt kinase, which promotes nuclear translocation of Mdm2 and its ability to ubiquitinate

p53 (Mayo and Donner 2001, Zhou *et al*. 2001, Ashcroft *et al*. 2002, Lin *et al*. 2002). Other Mdm2 modifications which may influence Mdm2 ability to promote p53 degradation are phosphorylation on Ser395 by ATM (Maya *et al*. 2001), on Ser267 by casein kinase 2 (CK2) (Hjerrild *et al*. 2001), on Thr218 by cyclin A-containing CDK complexes (identified from Thr216 in murine Mdm2 by Zhang and Prives 2001), and on Tyr394 by c-Abl (Goldberg *et al*. 2002). After IR, dephosphorylation of several residues in the central domain of Mdm2 decreases Mdm2 ability to promote p53 degradation (Blattner *et al*. 2002).

A recently cloned Mdm2 homologue, MdmX, is also capable of inhibiting p53-mediated transactivation (Stad *et al*. 2000). MdmX does not promote degradation of p53, but can inhibit Mdm2 doing so, thus stabilizing both p53 and Mdm2 (Jackson and Berberich 2000, Stad *et al*. 2001). The significance of MdmX-mediated regulation of p53 in DNA damage responses is unclear. Levels of MdmX do not change after DNA damage, but it might be regulated by translocation from cytoplasm to nucleus (Shvarts *et al*. 1996, Li *et al*. 2002a). MdmX is overexpressed in some tumors, and its gene *MDM4* amplified, correlating with elevated levels of p53 (Ramos *et al*. 2001). Loss of the *Mdm4* gene in mice is embryonic lethal and can be rescued with loss of *p53*. This suggests that Mdm2 and MdmX work in non-overlapping pathways to regulate p53 (Parant *et al*. 2001).

### *ARF*

ARF ($p14^{ARF}$ in human, $p19^{ARF}$ in mice) is produced from an alternative reading frame of the INK4a locus which also codes for the CKI protein $p16^{Ink4a}$ (Quelle *et al*. 1995), thus linking the p53 and Rb pathways important for suppression of tumorigenesis. ARF is a small, basic protein which interacts with the C terminus of Mdm2, abrogating the ability of Mdm2 to promote p53 degradation (Kamijo *et al*. 1998, Pomerantz *et al*. 1998, Stott *et al*. 1998, Zhang *et al*. 1998, Honda and Yasuda 1999). ARF is able to translocate Mdm2 to the nucleoli (Weber *et al*. 1999), but this is not required for inhibition of Mdm2 activity (Llanos *et al*. 2001). ARF is also capable of interacting with MdmX and sequestering it to the nucleoli, resulting in an increase in the transcriptional activity of p53 (Jackson *et al*. 2001). ARF responds to abnormal cell proliferation resulting from oncogenic stimulus and viral transformation, and it can be induced by at least Myc (Zindy *et al*. 1998), Ras (Palmero *et al*. 1998), E2F1 (Bates *et al*. 1998), and adenoviral E1A (de Stanchina *et al*. 1998). Induction of ARF causes growth arrest and represses transformation through induction of p53 (Quelle *et al*. 1995, Kamijo *et al*. 1997, Pomerantz *et al*. 1998). In addition, ARF-null cells immortalized in cell culture often retain functional p53, suggesting that suppression of cellular transformation by p53 is mediated by ARF (Kamijo *et al*. 1997). Interestingly, *ARF*, *Mdm2,* and *p53* triple-null mice develop tumors with a greater frequency than *p53*/*Mdm2* double-null or p53 KO mice, suggesting that ARF has other tumor-suppressive functions in addition to regulation of p53-Mdm2 pathway (Weber *et al*. 2000). These may be due to the ability of ARF to induce antiproliferative genes and to inhibit ribosomal RNA processing independently of p53 (Kuo *et al*. 2003, Sugimoto *et al*. 2003). ARF is not essential for the DNA damage response of p53 (Kamijo *et al*. 1997). p53 negatively regulates ARF expression, thus returning the ability of Mdm2 to downregulate p53 (Stott *et al*. 1998).

### *Other p53-interacting proteins*

In addition to Mdm2, p53 is found to interact with over 100 proteins which regulate or contribute to multiple p53 functions (Prives and Hall 1999, Vousden and Lu 2002). p53 is regulated by several enzymes modifying it posttranslationally, most of them being kinases (described in more detail below). As a typical transcription factor, p53 interacts with several transcription-related proteins. Interaction with HATs, such as p300/CBP, and histone deacetylases (HDACs) affect both chromatin structure and acetylation status of p53. Interactions with basal transcription factors TBP, TFIIH, and TAFs, and other transcription factors like Sp1 and Oct-1, promote formation of an active RNAPII initiation complex (Levine 1997, Prives and Hall 1999).

Examples of other proteins affecting p53 functions by protein-protein interactions are WT-1 (*W*ilms *t*umor-*1*), which interacts with p53 stabilizing it and inhibiting p53-mediated apoptosis (Maheswaran *et al*. 1995), redox/repair protein Ref-1 (*r*edox *f*actor *1*), which is a potent activator of p53 DNA binding and transactivation (Jayaraman *et al*. 1997), and p33ING1 (*in*hibitor of *g*rowth *1*) (Garkavtsev *et al*. 1998). ASPP1 and ASPP2 (*a*poptosis *s*timulating *p*roteins of *p*53 *1* and *2*) are proapoptotic, p53-interacting proteins, which regulate the activity of p53 towards a subset of target genes (Vousden and Lu 2002). The central region of p53 binds 53BP1 (p*53 b*inding *p*rotein *1*), which is a DSB-responsive checkpoint protein able to stimulate p53-dependent transactivation (Abraham 2002). p53 interacts with e.g. NER proteins XPB and XPD (Wang and Prives 1995), DNA replication factor RP-A (*r*eplication *p*rotein *A*), and DNA repair protein Rad51 (Buchhop *et al*. 1997) to promote DNA repair. In addition, p53 interacts with other stress-responsive proteins, such as PML (*p*ro*m*yelocytic *l*eukemia protein) and heat shok factors Hsp40, Hsp70, and Hsp90 (Prives and Hall 1999). The significances of these interactions are not entirely clear, however.

As p53 can sense abnormal proliferation and its activity can lead to destruction of the cells, several viruses have evolved a mechanism to inactivate it. p53 was originally identified as the cellular factor targeted by the large T antigen of Simian virus 40, which binds to p53 and inhibits its transcriptional activity (Levine 1997). Adenoviral E1B-55kDa and hepatitis B virus X protein also bind to p53 and inhibit its activity. E6 of human papilloma virus (HPV) inhibits p53 by serving as an E3 ubiquitin ligase, thereby targeting p53 to degradation (Prives and Hall 1999).

## *Posttranslational modifications of p53*

In addition to ubiquitination, p53 protein can be posttranslationally modified to several amino acid residues in its N- and C-terminal domains by phosphorylation, acetylation, sumoylation, and glycosylation (Figure 9) (Meek 1999, Appella and Anderson 2001). Modifications can occur either basally or inducibly, after for example a genotoxic insult, and affect p53 activity, its interactions with other proteins, and its localization. Significant interplay exists between the different p53 modifications (Meek 1999, Appella and Anderson 2001).

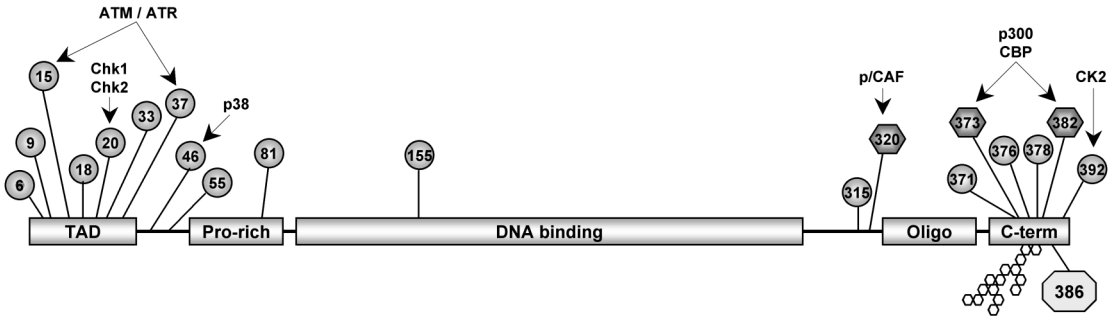



**Figure 9.** Posttranslational modifications of p53 protein. Amino acid residues available for modification by phosphorylation (circles), acetylation (hexagons), and sumoylation (octagon), and area of glycosylation are shown, as well as the most recognized enzymes responsible for certain modifications.

### *Phosphorylation*

Intracellular signaling is largely mediated by kinases and phosphatases. In addition, transcription factors are often regulated by changes in their phosphorylation patterns (Holmberg *et al*. 2002). p53 makes no exception to this rule by containing both in its N- and C-terminal regions several amino acid residues which can be phosphorylated. Most of them are modified in response to p53-activating stress signals, but some during normal cell growth conditions. Phosphorylating kinases for most of these sites have been identified, and several phosphoacceptor residues can be modified by more than one kinase (Meek 1999, Appella and Anderson 2001, Alarcon-Vargas and Ronai 2002).

N-terminal half of p53 contains seven serine and two threonine residues which are phosphorylated at least upon IR and UV radiation (Appella and Anderson 2001). Ser15, Ser20, Ser33, and Ser37 can be phosphorylated by PI-3-like kinases DNA-PK (*DNA*-dependent *p*rotein *k*inase), ATM, and ATR (Prives and Hall 1999, Appella and Anderson 2001). In addition, the checkpoint kinases Chk1 and Chk2 phosphorylate Ser20 (Shieh *et al*. 2000). Ser9 and Thr18 are phosphorylated by CK1, an event which is facilitated by prior phosphorylations on Ser 6 and Ser15, respectively (Dumaz *et al*. 1999, Higashimoto *et al*. 2000). Ser33 is phosphorylated by CAK (*C*DK *a*ctivating *k*inase) (Ko *et al*. 1997), which is a component of TFIIH, or p38 (Bulavin *et al*. 1999) *in vitro*. Based on experiments on murine p53, Ser33 could also be phosphorylated by JNK (Milne *et al*.

1995, Hu *et al*. 1997). The most N-terminal phosphorylation sites are located in the TAD and apparently take part in the regulation of transcriptional activity of p53 (Meek 1999, Appella and Anderson 2001, Alarcon-Vargas and Ronai 2002). Several of these phosphorylations have been suggested to inhibit the binding of Mdm2 to p53 and thus increase p53 stabilization. Phosphorylations on Ser15 and Ser37 block Mdm2 binding *in vitro* (Pise-Masison *et al*. 1998, Shieh *et al*. 1997, Siliciano *et al*. 1997), but contrasting reports also exist (Mayo *et al*. 1997, Dumaz and Meek 1999). Thr18 and Ser20 phosphorylations have reported to weaken Mdm2 interaction (Bottger *et al*. 1999, Craig *et al*. 1999, Unger *et al*. 1999). Mutation of Ser20 to Ala promotes p53 degradation and abrogates p53 stabilization by DNA damage (Chehab *et al*. 1999, Dumaz *et al*. 2001). Phosphorylation of p53 by DNA-PK can inhibit MdmX interaction (Jackson and Berberich 1999). The N-terminal phosphorylations may also directly stimulate p53 activity. Phosphorylation on Ser15 and Ser20 promote p53 binding to p300/CBP (Lambert *et al*. 1998, Dumaz and Meek 1999, Dornan *et al*. 2003), and combinatorial phosphorylations on Ser15 and Ser37 stimulate p53 interaction with TFIID (Pise-Masison *et al*. 1998).

Ser46 of p53 is phosphorylated by p38 *in vitro* (Bulavin *et al*. 1999) and by HIPK2 (*h*omeodomain-*i*nteracting *p*rotein *k*inase-*2*) after UV radiation (D'Orazi *et al*. 2002). Phosphorylation on Ser46 promotes UV-induced apoptosis (Bulavin *et al*. 1999) at least by increasing p53-induced transcription of apoptosis-promoting p53AIP1 (*p53*-regulated *a*poptosis-*i*nducing *p*rotein *1*) (Oda *et al*. 2000a). Thr55 is basally phosphorylated in unstressed cells (Gatti *et al*. 2000). Thr81 in the p53 Pro-rich domain is phosphorylated by JNK, contributing to stabilization and activation of p53 in response to UV radiation (Buschmann *et al*. 2001). Thr155 is phosphorylated in unstressed cells, targeting p53 to degradation (Bech-Otschir *et al*. 2001).

One phosphorylation site has been identified in the oligomerization domain of p53, namely Ser315, which can be phosphorylated by S and G2/M phase CDKs to selectively stimulate p53 binding to a subset of p53 binding sites (Wang and Prives 1995). The N-terminal domain of p53 is basally phosphorylated on Ser376 and Ser378 in unstimulated cells. Ser376 is rapidly dephosphorylated after IR (Waterman *et al*. 1998), which allows binding of 14-3-3 protein to p53 and increases p53 transcriptional activity. These residues lie within the epitope of antibody PAb421 (residues 371-380), an area which is phosphorylated by PKC (*p*rotein *k*inase *C*) (Meek 1999). PKA (*p*rotein *k*inase *A*) (Waterman *et al*. 1998) and CAK (Lu *et al*. 1997) also phosphorylate Pab421 epitope at least *in vitro*.

The most C-terminal phosphorylation site, Ser392, activates specific DNA binding and transcriptional activity of p53, presumably by stabilizing p53 tetramers (Hupp *et al*. 1992, Sakaguchi *et al*. 1997). Ser392 can be modified by CK2 (Meek *et al*. 1990) and dsRNA activated protein kinase PKR (Cuddihy *et al*. 1999). Phosphorylation on Ser392 may selectively regulate p53-mediated transrepression (Hall *et al*. 1996), and it occurs upon UV radiation, but not in response to IR (Appella and Anderson 2001).

The significance of p53 phosphorylations has been questioned by experiments showing that mutations on key phosphorylation sites on N or C terminus have no effect on p53 stabilization by UV radiation or IR (Blattner *et al*. 1999, Ashcroft *et al*. 1999), the transcriptional activity of p53 (Fiscella *et al*. 1994, Fuchs *et al*. 1995, Ashcroft *et al*. 1999), or its interaction with Mdm2 (Ashcroft *et al*. 1999). There are also differences between modifications on murine and human p53 (Meek 1999). In addition, lack of tumor-associated mutations on the modification sites of p53 argue that phosphorylations are dispensable for p53 function (Prives and Hall 1999, Vousden 2002).

*Acetylation*

Acetylation of lysine residues by HATs is a common means for regulating activity of transcription factors (Workman and Kingston 1998, Chen *et al*. 2001). p53 is acetylated on several C-terminal residues, which increases sequence-specific DNA binding of p53 and thus promotes its transcriptional activity (Gu and Roeder 1997). Protein acetylation and ubiquitination often compete for the same lysine residues (Weissman 2001), and acetylation of p53 was recently shown to inhibit its ubiquitination (Li *et al*. 2002b). Lys320 is acetylated by p/CAF, and Lys373 and Lys382 by p300/CBP, in response to both IR and UV radiation (Appella and Anderson 2001). The N-terminal phosphorylation and C-terminal acetylation of p53 are linked. Phosphorylations on Ser15 (Lambert *et al*. 1998) and/or Ser33 and Ser37 (Sakaguchi *et al*. 1998) promote recruitment of HATs and thus enhance p53 acetylation. Ser20 phosphorylation stabilizes p53 binding to p300 (Dornan *et al*. 2003). Mdm2 and MdmX can inhibit p300-mediated acetylation of p53 (Kobet *et al*. 2000, Sabbatini and McCormick 2002). Indeed, the N-terminal modifications tend to occur before the C-terminal ones (Chernov *et al*. 1998), suggesting that stabilization and activation of p53 by posttranslational modifications are separate, but co-operative, events.

*Sumoylation*

Modification by covalent attachment of SUMO (*s*mall *u*biquitin-related *mo*difier; also called sentrin) is a recently identified means to regulate proteins (Müller *et al*. 2001). SUMO is a small, 11-kDa polypeptide closely related to ubiquitin (Verger *et al*. 2003). It can be attached to target protein lysine residues within a consensus motif Ψ-Lys-X-Glu (where Ψ represents a large, hydrophobic amino acid, most commonly Ile or Val, and X any amino acid) in a process analogous to ubiquitination (Melchior 2000, Verger *et al*. 2003). Mammalian cells express three different forms of SUMO (SUMO-1, SUMO-2, SUMO-3), of which only SUMO-2 and SUMO-3 can form polymeric chains (Verger *et al*. 2003). SUMO conjugation targets include several transcription-related proteins like c-Jun, HSF2 (*h*eat *s*hock *f*actor *2*), IκB (*i*nhibitor of NF*κB*) and p300 (Melchior 2000, Girdwood *et al*. 2003). In addition, PML and several other proteins located to the PML nuclear bodies are sumoylated (Seeler and Dejean 2001). The exact function of sumoylation has remained unclear, but the modification may be involved in regulating protein stability by competing of the same lysine residues as ubiquitin and acetylation (Freiman and Tjian 2003). Most studies suggest that SUMO alters interaction properties of its targets, often affecting their subcellular localization (Seeler and Dejean 2001, Verger *et al*. 2003). p53 can be sumoylated on Lys386 (Müller *et al*. 2000) in a process where PIAS1 and PIASxβ (*p*rotein *i*nhibitors of *a*ctivated *S*TAT) act as specific E3 sumoylation ligases (Kahyo *et al*. 2001, Schmidt and Müller 2002). SUMO-1 is suggested to increase the transcriptional activity of p53 (Gostissa *et al*. 1999, Rodriguez *et al*. 1999), although contrasting reports exist (Kwek *et al*. 2001, Schmidt and Müller 2002). Controversial is also whether SUMO directs p53 localization to PML nuclear bodies (Fogal *et al*. 2000, Kwek *et al*. 2001).

*Glycosylation*

p53 can be O-glycosylated in the C terminus in a cell type-specific manner (Shaw *et al*. 1996). Little is known about the occurrence and significance of this modification, but evidence from myocytes suggests that glycosylation promotes activation of p53, and that hyperglycemia may induce p53-induced cell death (Fiordaliso *et al*. 2001).

### *Intracellular localization of p53*

Cell nucleus is highly organized and is structured by filamentous nuclear matrix. Transcription by RNAPII takes place at discrete sites in the nucleus (Szentirmay and Sawadogo 2000). Altering localization of transcription factors both inside the nucleus and between nucleus and cytoplasm is a powerful means to regulate transcription. p53 is predominantly located in the nucleus and excluded from the nucleoli. It exists both in free form and bound to nuclear matrix colocalizing with active transcription sites (Rubbi and Milner 2000). Interaction of p53 with nuclear matrix is increased after DNA damage (Jiang *et al*. 2001). This is mediated at least in part by p53 binding to F-actin, which is induced by phosphorylation on Ser392 of p53 (Okorokov *et al*. 2002). In addition, microtubules and the dynein motor protein participate in the transport of p53 and facilitate its accumulation in the nucleus after DNA damage (Giannakakou *et al*. 2000).

Nuclear localization of proteins is often controlled by nuclear localization (NLS) and export (NES) signals. Basic NLS sequences bind importins α and β to mediate docking to the nuclear pore complex, thereby initiating nuclear import (Liang and Clarke 2001). p53 C-terminal region has three nuclear localization sequences (NLS1, NLS2, NLS3). Mutations in NLS1, which is located in the C-terminal linker region (residues 316-325), render p53 completely cytoplasmic. Mutations in NLS2 (369-375) and NLS3 (379-384) in the C-terminal basic region result in distribution of p53 in both nucleus and cytoplasm (Dang and Lee 1989, Shaulsky *et al*. 1990). Nuclear export of p53-Mdm2 complexes is thought to be driven mainly by a NES in Mdm2, even though the C terminus of p53 contributes to this process (Lohrum *et al*. 2001). p53 contains two NESs. The first is located in the N-terminal Mdm2 binding region (residues 11-27). Phosphorylation on Ser15 within this region has been suggested to inhibit nuclear export of p53 driven by this NES (Zhang and Xiong 2001). The second NES is located in the oligomerization domain. It is masked in p53 tetramers, but is capable of exporting monomeric p53 to cytoplasm (Stommel *et al*. 1999, Liang and Clarke 2001). As for most proteins containing a leucine-rich NES, p53 export is mediated by exportin 1 (also called CRM1) (Liang and Clarke 2001).

Less conventional places for p53 are the nucleolus, where a fraction of p53 colocalizes with ribosomal RNA transcription sites (Rubbi and Milner 2000), and mitochondria, where p53 can directly contribute to apoptosis (see below) (Marchenko *et al*. 2000, Mihara *et al*. 2003). p53 can also localize to the PML nuclear bodies (Gottifredi and Prives 2001, Vousden 2002). Although the specific role of PML bodies is not known, they may serve as centers for transcription factor modification and thus contribute to transcriptional regulation (Borden 2002). Indeed, the interaction of p53 with PML and the subsequent localization of p53 to the PML bodies enhance the transcriptional activity of p53 and its ability to induce apoptosis and cellular senescence (Vousden 2002).

### *Regulation of transcriptional activity of p53*

Stabilization of p53 is insufficient to induce p53-dependent growth arrest and apoptosis, for which the transcriptional activity of p53 has to be upregulated (Yakovleva *et al*. 2002). On the other hand, stabilization of p53 is not required for its transcriptional activity (Hupp 1999), indicating that stabilization and activation of p53 are at least partly separate events. This has led to a model in which p53 is thought to exist in a latent state in nonstressed cells and to become active upon a stress signal (Hupp 1999). Regulation of transcriptional activity occurs at the levels of p53 ability to bind DNA and to affect formation of an active RNAPII complex, which depend on posttranslational modifications of p53 and its interactions with other proteins (Hupp 1999, Vousden 2002).

Transcriptional activation of a target gene by p53 occurs via sequence-specific binding of the p53 core region to a response element (RE) on the target gene promoter. A consensus composition of the p53 RE has been defined (El-Deiry *et al*. 1992), and it composes of tandem repeats of the 10 bp motif 5'-PuPuPuC(A/T)(A/T)GPyPyPy-3' separated by 0-13 bp (Pu, purine; Py, pyrimidine). Each half-site binds a dimer of p53, explaining why p53 tetramers are the most efficient forms in DNA binding (Hupp 1999, Vousden 2002). The spacing distance between the two decamers is an important determinant of p53 affinity. Binding is optimal when the decamers are contiguous or separated in a manner that the two half sites are orientated on the same helical face of DNA (Wang *et al*. 1995a). p53 affinity for the REs is further regulated by the local spacial structures of DNA, such as chromosomal looping and the degree of superhelicity (Hupp 1999). In addition, the redox state of p53 affects its DNA binding ability; reduction leads to increased binding, as oxidation abolishes it due to a loss of $Zn^{2+}$ coordination by Cys residues in the core region (Hainaut and Milner 1993, Rainwater *et al*. 1995). The Pro-rich region also affects the transcriptional activity of p53, as its deletion activates sequence-specific DNA binding of p53 (Müller-Tiemann *et al*. 1998).

p53 contains two DNA-binding sites (Yakovleva *et al*. 2002). The CTD of p53 binds DNA without sequence-specificity, but is capable of regulating the sequence-specific DNA binding of the p53 core region (Ahn and Prives 2001). Phosphorylation or deletion of the C terminus, as well as binding of antibodies or small peptides to it, stimulate DNA binding of p53 *in vitro* (Hupp *et al*. 1992, 1995), indicating that the C terminus allosterically regulates transition between latent and active forms of p53. This model is, however, challenged by studies showing that p53 conformation is identical with or without the CTD (Ayed *et al*. 2001). Recent studies also indicate that p53 is bound on DNA *in vivo* even in unstressed cells (Kaeser and Iggo 2002), and that modifications of the CTD are not required for DNA binding and promoter activation (Espinosa and Emerson 2001, Göhler *et al*. 2002). In addition, neither mutation of Ser392 nor all of the C-terminal phosphorylation sites have a significant effect on p53 transcriptional activity (Fiscella *et al*. 1994, Fuchs *et al*. 1995, Ashcroft *et al*. 1999). The allosterical CTD regulation model may be due to usage of short oligomers in binding assays (Ahn and Prives 2001), as full-length p53 does not bind to REs in short DNA fragments unless the CTD is deleted or modulated by phosphorylation, acetylation, or binding by PAb421 antibody (Yakovleva *et al*. 2002).

It is clear, however, that the CTD does regulate sequence-specific DNA binding of p53 *in vivo* (Ahn and Prives 2001). The CTD could compete for binding to DNA with the core domain (Anderson *et al*. 1997). This is supported by experiments showing that long,

nonspecific DNA molecules inhibit sequence-specific DNA binding of full-length, but not of the CTD-deleted, p53 (Anderson *et al*. 1997). Alternatively, the CTD may govern p53 binding to DNA, after which p53 slides along DNA until it finds a RE to bind sequence-specifically to. p53 has been shown to constantly associate with and disassociate from DNA, and it is able to slide along it (Jiao *et al*. 2001). In yet another model, CTD is suggested to regulate p53 binding to only a subset of REs depending on their three-dimensional conformation. C-terminally unmodified p53 prefers binding to palidromic REs, which form cruciform structures (REs in head-to-head orientation; Yakovleva *et al*. 2002). This may be caused by the CTD, which can also bind four-way Holliday junctions (Lee *et al*. 1997). Initiation of transcription could be limited to cruciform targets in this unstressed situation. After stress, modifications of the CTD could be required for p53 to bind the target sites that are in head-to-tail orientation and do not form cruciform structures (Yakovleva *et al*. 2002).

Several hundred genes are known to be regulated, most of them induced, by p53 (El-Deiry 1998, Vousden and Lu 2002, Zhao *et al*. 2000). Over 4000 putative p53 target sequences have been identified in the human genome (Wang *et al*. 2001b). In addition, not all p53 REs are similar to the consensus sequence, and p53 binding to target promoters can be directed by other factors (Vousden and Lu 2002). This leaves the true number of p53 target genes to be clarified. p53 can also specifically repress several promoters, but the mechanism is poorly understood. p53-mediated transrepression occurs without sequence-specific DNA binding, and it most likely depends on interactions of p53 with other proteins, such as TBP (Seto *et al*. 1992, Ragimov *et al*. 1993, Truant *et al*. 1993) and HDACs via corepressor Sin3 (Murphy *et al*. 1999).

## *Multiple functions of p53*

p53 is a very efficient inhibitor of cell growth. Non-stressed cells overexpressing p53 undergo G1 arrest (Diller *et al*. 1990, Lin *et al*. 1992) or apoptosis (Yonish-Rouach *et al*. 1991, Shaw *et al*. 1992). These can be separate events, but many aspects of the responses are in common (Vousden and Lu 2002). It is not clear how the decision is made whether p53 induces cell cycle arrest or apoptosis. p53 has a direct role in determining which response pathways are activated, but p53-interacting factors are bound to influence the outcome (Vousden 2000). The decision is affected by survival factors and cross-talk with other pathways controlling cell proliferation and survival, such as the Rb and NFκB pathways (Hickman 2002, Vousden and Lu 2002). In addition, the intracellular levels of p53 affect the outcome, as low levels preferably activate cell cycle arrest and high p53 doses rather induce apoptosis (Chen *et al*. 1996). Low levels of p53 may in fact protect cells from apoptosis (Lassus *et al*. 1996) at least partly due to p53-mediated cell cycle arrest (Vousden and Lu 2002). p53 mainly excerts its functions by acting as a transcription factor, but it has other activities as well, such as 3'-5' exonuclease activity, and ability to bind DNA lesions and single stranded DNA (Bakalkin *et al*. 1995, Lee *et al*. 1995, Janus *et al*. 1999).

p53 is not essential for normal development of mice *in utero* (Donehower 1996). During embryogenesis, transcriptional activity of p53 is present mainly in the developing nervous system (Komarova *et al*. 1997). After DNA damage by IR, p53 can induce apoptotic and antiproliferative responses *in vivo* cell type-specifically (Clarke *et al*. 1994, Midgley *et al*. 1995). p53 activity is induced by IR mainly in rapidly proliferating tissues, e.g. epithelia of the small intestine, and in spleen and thymus of the tissues with low proliferation rate (Gottlieb *et al*. 1997, Komarova *et al*. 1997). Interestingly, haplo-insufficiency for p53 function has been detected in mouse models (Gottlieb *et al*. 1997).

### *Cell cycle arrest*

p53 can arrest the cell cycle in G1 and G2 phases. The most important transcriptional target for p53-induced G1 arrest is the CKI protein $p21^{Cip1}$, which is a potent inhibitor of several CDK complexes (El-Deiry 1993, May and May 1999). In S phase cells, $p21^{Cip1}$ may act also by binding to PCNA (*p*roliferating *c*ell *n*uclear *a*ntigen) and blocking the elongation step of DNA replication (Waga *et al*. 1994). $p21^{Cip1}$ may also take part in G2 arrest, as it can inhibit cyclin A- and cyclin B-containing CDK complexes (Li *et al*. 1994), but it is not essential for the immediate G2 checkpoint response (Levedakou *et al*. 1995). p53-induced G2 arrest is enforced by 14-3-3σ which sequesters and inhibits the mitotic Cdc25C phosphatase (Hermeking *et al*. 1997). Antiproliferative p53 effects may also be mediated by induction of BTG2 (Rouault *et al*. 1996) and repression of Cdk2 (Zhao *et al*. 2000). p53 also takes part in the spindle checkpoint by preventing mitosis when sister chromatids have failed to segregate properly (Meek 2000).

*DNA repair*

p53 participates in several processes of DNA repair and DNA recombination by regulating transcription, but also by interacting with components of repair and recombination machineries. In addition, p53 promotes DNA repair by binding DNA lesions and functioning as a 3'-5' exonuclease (Janus *et al*. 1999). The first p53 target identified, Gadd45 (*g*rowth *a*rrest- and *D*NA *d*amage-inducible *45*), may promote DNA repair by its association with PCNA (Janus *et al*. 1999). p53 also induces transcription of a ribonucleotide reductase gene p53R2, thereby supplying building blocks for DNA repair after a genotoxic insult (Tanaka *et al*. 2000, Nakano *et al*. 2000).

p53 participates in nucleotide excision repair, even though it is not essential for it *in vitro* (Hanawalt 2002). Abrogation of p53 function by mutation (Ford and Hanawalt 1995) or targeting by viral proteins (Ford *et al*. 1998) renders cells defective in GGR. Other studies have argued that p53 contributes also to TCR (Wang *et al*. 1995b, Mirzayans *et al*. 1996, Therrien *et al*. 1999). p53 is essential for efficient GGR of UV-induced CPDs in human fibroblasts, and it regulates efficiency of GGR repairing both CPDs and 6-4PPs after UV radiation (Hanawalt 2002). NER-deficient cells accumulate p53 by lower doses of UV than normal cells, and they have a prolonged p53 response which correlates to amount of unrepaired CPDs (Dumaz *et al*. 1997).

The mechanisms how p53 takes part in NER are still unclear (Hanawalt 2002). Transactivation of $p21^{Cip1}$ is not required for p53 to promote GGR, arguing that the induction of cell cycle arrest is dispensable for the effect (Adimoolam *et al*. 2001, Smith *et al*. 2000). The main transcriptional target of p53 for NER is the repair factor p48, but Gadd45 may be important as well (Hanawalt 2002). p53 also upregulates the NER factor XPC upon UV radiation (Adimoolam and Ford 2002) and interacts with several TFIIH-associated NER factors (Wang *et al*. 1995b). Recently, p53 was suggested to contribute to UV-induced GGR by recruiting HAT complexes to the sites of DNA damage, thus promoting chromatin relaxation needed for repair factors to access the DNA lesions (Rubbi and Milner 2003).

p53 can bind both double- and single-stranded DNA non-sequence-specifically. It also binds to DSB ends, Holliday junctions, and DNA mismatches (Janus C*et al*. 1999). Binding of p53 to damaged DNA is mediated by the CTD. This has led to a model in which p53 could sense DNA damage via its C terminus, leading to activation of the sequence-specific DNA binding and transactivation (Jayaraman and Prives 1995). 3'-5' exonuclease activity of the p53 core domain is regulated in a manner opposite to the DNA-binding activity. This biochemical function is suggested to take part in the DNA repair processes when p53 is in a non-induced state (Janus *et al*. 1999). p53 associates with RP-A needed for DNA replication, homologous recombination, and NER (de Laat *et al*. 1999). Disruption of p53-RP-A complexes upon UV radiation occur when DNA repair starts (Abramova *et al*. 1997). It has been suggested that in its latent state p53 promotes repair of replication errors by its exonuclease activities while bound to RP-A. Upon UV radiation, p53 is activated to perform its transcriptional function, as RP-A is released to take part in NER (Janus *et al*. 1999).

*Apoptosis*

Ability of p53 to induce apoptosis when the damage inflicted on the cell is irreparable may be the most important of its tumor suppressive functions (Gottlieb and Oren 1998, Vousden 2000). p53 can induce several genes which contribute to both death receptor (Fas/APO1, KILLER/DR5, PERP) and mitochondrial (e.g. Apaf-1, Bax, NOXA, p53AIP1, PUMA) apoptotic pathways (Benchimol 2001, Pietenpol and Stewart 2002, Vousden and Lu 2002) (Figure 10). Several p53 target genes have a yet unidentified mechanism of promoting apoptosis (PIG8, PIDD) (Benchimol 2001). In addition, p53 promotes apoptosis by activating genes which suppress survival signaling (e.g. IGF-BP3; for *i*nsulin-like *g*rowth *f*actor-*b*inding *p*rotein *3*) and repressing expression of survival-promoting or anti-apoptotic genes (e.g. IGF-receptor 1, Bcl-2 and survivin) (El-Deiry 1998, Hoffman *et al*. 2002, Pietenpol and Stewart 2002). Depending on the p53-inducing signal and cell type, not all apoptotic target genes are necessarily expressed at the same time (Bouvard *et al*. 2000). The ability to engage various apoptotic routes is likely to be important for tumor suppression by p53 (Vousden and Lu 2002). The Pro-rich domain is thought to be important for the apoptotic functions of p53 (Sakamuro *et al*. 1997). Sin3a interacts with the Pro-rich domain contributing to the p53-mediated transrepression, which might explain the importance of the Pro-rich domain to apoptosis (Zilfou *et al*. 2001).

p53 may launch apoptosis also independently of its ability to regulate transcription by yet unidentified mechanisms (Vousden 2000), although according to *in vivo* evidence some transactivation-deficient p53 mutants are defective in inducing apoptosis (Jimenez *et al*. 2000, Chao *et al*. 2000). p53 contributes to shuttling of death receptors to the cell surface (Bennett *et al*. 1998) and activates caspase-8 (Ding *et al*. 2000) by unknown mechanisms independent of transactivation. Mitochondria and redox regulation may also be involved in p53-dependent apoptosis (Polyak *et al*. 1997). A fraction of p53 is localized in the mitochondria after DNA damage, where p53 can directly induce permeabilization of the outer mitochondrial membrane by forming complexes with the protective Bcl-XL and Bcl-2 proteins, resulting in cytochrome c release (Marchenko *et al*. 2000, Mihara *et al*. 2003).

There are several proteins which can modulate p53-induced apoptosis. Proteins of the ASPP family interact with p53 and enhance binding of p53 to apoptotic promoter REs (Samuels-Lev *et al*. 2001). JMY co-operates with p300 to enhance the expression of p53 apoptotic targets (Shikama *et al*. 1999). Interaction of p53 C terminus with DNA helicases, such as XPD, XPB, BLM (*Bl*oo*m* syndrome), and WRN (*Wern*er syndrome), promotes apoptosis by yet unidentified means (Robles and Harris 2001). Other proteins affecting p53-induced apoptosis include LKB1, a tumor suppressor defective in Peutz-Jeghers syndrome (Karuman *et al*. 2001), and WT-1, inactivation of which leads to pediatric kidney cancer (Maheswaran *et al*. 1995). In addition, induction of cell death by p53 seems to require the presence of at least one of the other p53 family members p63 or p73 (see below) (Flores *et al*. 2002).

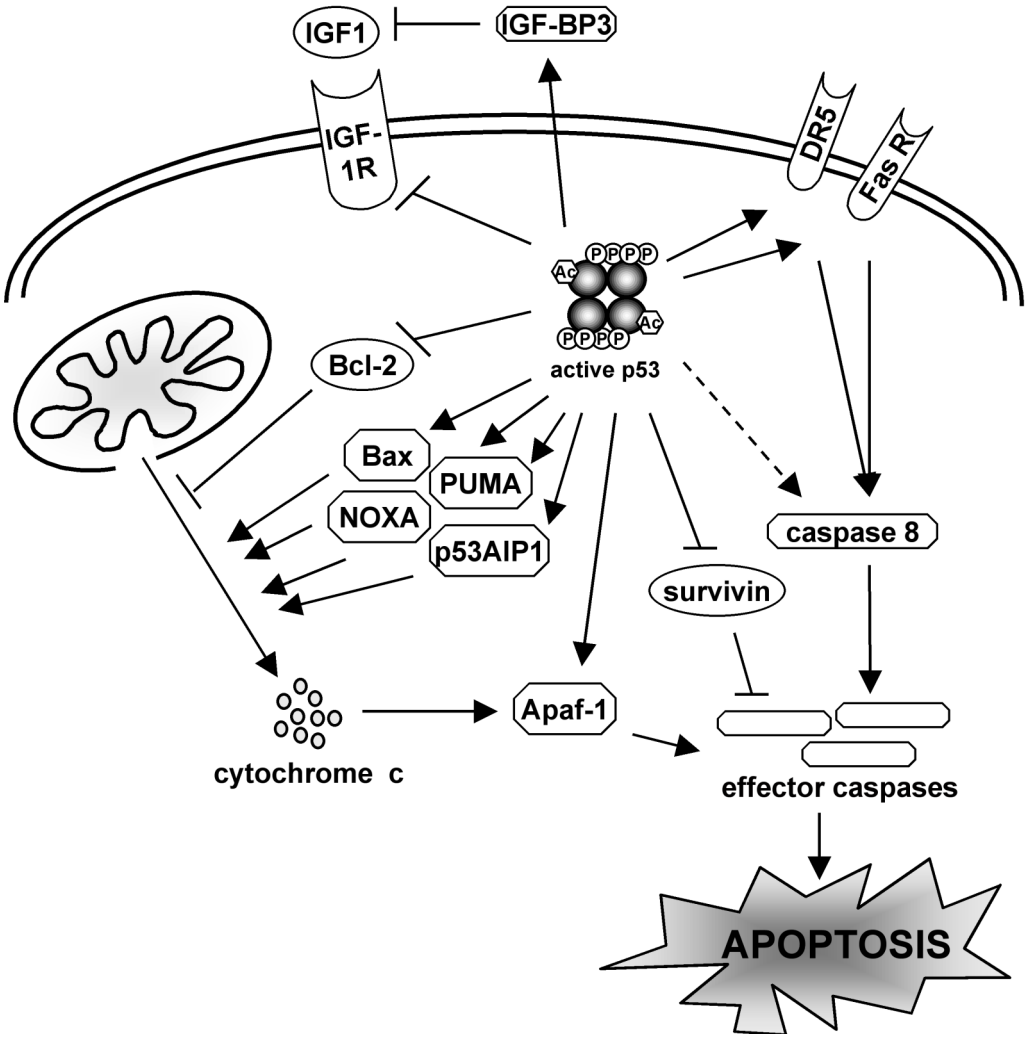



**Figure 10.** p53 promotes apoptosis by contributing to both death receptor and mitochondrial apoptotic pathways. p53 induces transcription (arrows) of death receptors Fas and DR5, inhibitor of survival signaling IGF-BP3, and apoptosis-promoting Bax, NOXA, p53AIP1, PUMA, and Apaf-1, and represses expression (blocked lines) of survival-promoting IGF-R1, and anti-apoptotic Bcl-2 and survivin. p53 also activates caspase 8 by an unknown mechanism (dashed arrow). Additional events are likely to take part in p53-mediated apoptosis. Adapted from Pietenpol and Stewart 2002.

*Other functions of p53*

In addition to Mdm2 and ARF, p53 regulates other target genes to terminate its own response. p53 induces cyclin G, which negatively regulates accumulation of p53 (Ohtsuka *et al*. 2003) apparently by recruiting PP2A (*p*rotein *p*hosphatase *2A*) to dephosphorylate Mdm2 Thr218, which thus promotes its ability to mediate p53 degradation (shown for murine Thr216; Okamoto *et al*. 2002). p53 target WIP1 (*w*ildtype p53-*i*nduced *p*hosphatase *1*) downregulates p38 kinase, leading to decreased Ser46 phosphorylation on p53 and reduced ability of p53 to transactivate the proapoptotic target p53AIP1 (Takekawa *et al*. 2000). Other p53 functions than those involved in cell cycle arrest or apoptosis may also be crucial for tumor suppressor function of p53 (Vousden and Lu 2002). For example, p53 activation accelerates degradation of β-catenin, oncogenic activity of which contributes to a variety of epithelial tumors. KAI1 (*ka*nga*i 1*), a tumor metastasis suppressor, is induced by p53. p53 stimulates angiogenesis through upregulation of thrombospondin-1, but it also induces secretion of anti-angiogenic factor GD-AIF (*g*lioma-*d*erived *a*ngiogenesis *i*nhibitory *f*actor). p53 promotes degradation of extracellular matrix by inducing MMP-2 (type IV collagenase; gelatinase A), but it also induces protease inhibitor maspin (Vousden and Lu 2002).

p53 regulates limited cell proliferation potential and cellular senescence, which is another safeguard mechanism to suppress tumorigenesis (Itahana *et al*. 2001, Donehower 2002). In late passage cultured cells, p53 helps to maintain a nonproliferative state; a function which is at least partly due to the ability of p53 to upregulate $p21^{Cip1}$. The p53-posed proliferation block has to be overcome for cells to immortalize (Itahana *et al*. 2001). For human cells, other transforming events are required also. Murine cells are more easily transformed in culture than human cells, and p53-null murine cells spontaneously immortalize and transform, as well as have a higher degree of genomic instability than their wild type (wt) counterparts (Donehower 1996). p53 may also control cell proliferation under normal circumstances as *p53*-null mouse embryonal fibroblasts (MEFs) divide more rapidly, have a shorter cell cycle time, and grow more readily in unfavorable conditions than MEFs harboring wtp53 (Harvey *et al*. 1993).

Limited replication potential of human cells is at least partly due to shortening of telomeres on chromosome ends during each round of DNA replication. It is not clear how shortening of telomeres under critical length is recognized by the cells, but it has been suggested to serve as a DNA damage-like signal inducing p53-mediated damage response (Itahana *et al*. 2001). hTERT (*h*uman *te*lomerase *r*everse *t*ranscriptase), the catalytic subunit of human telomerase, can prevent shortening of telomeres by synthetisizing telomere DNA, and thus contribute to increased replication potential of cells (Itahana *et al*. 2001). p53 downregulates telomerase activity by repressing hTERT expression (Xu *et al*. 2000) and, possibly, via interaction with hTEP1 (*h*uman *te*lomerase-associated *p*rotein *1*) (Li *et al*. 1999).

## *Deficiency of p53 function*

### *Sporadic human cancers*

Most human cancers seem to have lost p53 function in one way or the other (Vousden and Lu 2002). Mutations of *TP53* itself are found in over half of human tumors (Hollstein *et al*. 1991). *TP53* is mutated most often in cancers of the lung (70%), the colon (60%), the ovary (60%), the bladder (60%), and those of the head and neck (60%), but mutated relatively rarely in leukemia, melanoma and prostate cancers (Soussi 2000). Inactivation of p53 can also occur by lesions which prevent activation of p53, or by mutations in downstream mediators of p53 functions (Vousden and Lu 2002). Overexpression of Mdm2 occurs via amplification of *HDM2* in 7% of human cancers, with the highest frequency found in soft tissue sarcomas (20%) (Momand *et al*. 2000). Mdm2-mediated nuclear exclusion can be found from tumors of the breast and the colon, and neuroblastoma (Liang and Clarke 2001, Stommel *et al*. 1999). In addition, MdmX overexpression may occur in some tumors (Ramos *et al*. 2001). Polymorphism of *TP53* can also contribute to tumorigenesis. Polymorphism at codon 72 results in either a Pro or Arg residue. Arg replaces one of the five PXXP motifs in the Pro-rich domain and is more susceptible to degradation mediated by HPV protein E6. Clinically, patients carrying Arg72 p53 are more susceptible to HPV-associated tumorigenesis (Storey *et al*. 1998). Interestingly, Arg72-coding alleles are preferentially mutated and retained in squamous cell tumors arising from germline heterozygotes coding for both Arg72 and Pro72 p53 (Marin *et al*. 2000).

Over 90% of tumor-associated mutations of *TP53* are point mutations which result in single amino acid substitutions (Vousden and Lu 2002). Many tumor cells retain their ability to express mutant p53, and the mutants are often more stable than wtp53. In addition, loss of heterozygosity occurs frequently to eliminate the wt allele (Greenblatt *et al*. 1994). Some of the tumor-associated mutants, e.g. Arg175His, may acquire new transforming functions which contribute to tumor development (gain-of-function). There are also p53 mutants which work in a dominant-negative fashion (Sigal and Rotter 2000). Only 5% of p53 mutations have been found in the regulatory domains (N- and C-terminal regions), whereas 95% of the mutations occur in the DNA binding core region of p53 (May and May 1999, Prives and Hall 1999, Vousden and Lu 2002). Mutations are most often located in the conserved regions II-V disturbing DNA contacts or conformation of the whole domain, and thus resulting in loss of DNA binding ability of p53. Some p53 mutants selectively lose their ability to induce apoptotic targets (Vousden and Lu 2002). Additionally, different classes of p53 mutants have differential effects on the resistance of tumor cells to chemotherapy (Blandino *et al*. 1999).

*TP53* mutations can be found in 50% of all skin cancers and in up to 90% of tumors in DNA repair-deficient XP patients (Giglia-Mari and Sarasin 2003). *TP53* mutations in non-melanoma skin cancers often show a "UV pattern", i.e. they are mostly C to T transitions likely to have originated from UV radiation-induced DNA lesions. Sunburn cells in human skin often harbour p53 mutations (Ziegler *et al*. 1994). As sunburn is associated with apoptosis, loss of p53 provides a survival advantage to UV-damaged cells. Clusters of cells with mutant p53 often found in healthy human skin suggest that mutant p53 can "wait" for other mutated genes to start the tumorigenic process, as it may itself have a more profound role later in the tumorigenic process (de Gruijl *et al*. 2001). Importance of p53 as a target in UV-induced skin cancers has been verified in mouse models (de Gruijl *et al*. 2001).

*Li-Fraumeni syndrome*

Germline mutations resulting in p53 deficiencies cause a rare hereditary cancer predisposition called the Li-Fraumeni syndrome. The patients have 50% likelihood of developing cancer by the age 30, in contrast to 1% in the general population (Donehower 1996). The tumor spectrum includes soft tissue sarcomas and cancers of the bone, the breast, the brain, and the genito-urinary tract (Malkin 1993). Most Li-Fraumeni-associated p53 mutations reside in the core domain, but some are found in the oligomerization domain (Lomax *et al*. 1997). Li-Fraumeni syndrome-like symptoms can also be caused by germline mutations on *Chk2* (Bell *et al*. 1999).

*Mouse models*

Although p53 is unessential for normal development of mice *in utero*, approximately 20% of female *p53*-null embryos die during embryogenesis due to a neural tube closure defect resulting from overgrowth of neural tissue. Fertility is also reduced particularly in females of the *p53* KO mice (Donehower 1996). The most prominent effect of p53 deficiency in mice is severely enhanced tumorigenesis (Donehower *et al*. 1992, Jacks *et al*. 1994). The *p53*-/- mice develop tumors within 4.5 months on average and their heterozygous littermates by 18 months, as their wt littermates do not develop tumors before 18 months. Less than 25% of the wt mice have tumors by the age of two years, but all *p53*-null mice have tumors by 10 months of age (Donehower 1996).

The tumor spectrum of *p53*-null mice include lymphomas (60%), soft tissue sarcomas (over 20%), and brain and lung tumors, but malignant carcinomas are found rarely (Donehower 1996, Parant and Lozano 2003). In heterozygotes, the tumors observed are more varied, including lymphomas, soft tissue sarcomas, and osteosarcomas, and the incidence of carcinomas is increased (Donehower 1996). The genetic background of the mice affects the tumor spectrum (Parant and Lozano 2003). *p53* co-operates with other cancer-associated genes in mouse models, as mice deficient for both *p53* and *RB* show accelerated tumorigenesis, and also novel tumor types compared to the parental mice deficient for either gene (Williams *et al*. 1994). *p53* mutations are found in tumors at a rather late stage of the malignant development. Interestingly, loss of *p53* can inhibit the initial steps of tumorigenesis, as mice which lack *p53* develop fewer papillomas in response to carcinogen exposure than wt mice (Kemp *et al*. 1993).

Even though heterozygous *p53* mice seem to offer a good model for the Li-Fraumeni syndrome, the tumors in the *p53* KO mice have been speculated not to correspond to the situation in human cancers particularly well (Donehower 1996). The deletions introduced to the KO mice do not resemble the point mutations observed in human patients. In attempts to better mimic the situation in sporadic human cancers, transgenic mice with *p53* point mutants have been generated. For example, transgenic mice with oncogenic codon 135 point mutation had accelerated tumorigenesis, but only in the presence of one or both wild type alleles, suggesting that it functions through inactivating wtp53 (Harvey *et al*. 1995). Mice heterozygous for Arg172His (corresponds to Arg175His in human) differed from *p53*+/- mice in their tumor spectrum with a higher incidence of carcinomas and metastasizing tumors (Liu *et al*. 2000).

### *p53 family of proteins*

After 15 years of solitude, p53 was found to have two family members, p63 and p73 (Kaghad *et al*. 1997, Yang *et al*. 1998). These proteins are strikingly conserved, and all members have an N-terminal TAD, a DNA binding domain, and an oligomerization domain (Kaghad *et al*. 1997, Yang *et al*. 1998) (Figure 11). p63 appears to be the evolutionary ancestor, and p63 and p73 resemble each other more than p53. p63 and p73 have a C-terminal sterile α-motif (SAM) domain involved in protein-protein interactions, and a post-SAM domain, which has an inhibitory effect on the transactivation properties of the proteins. Both p63 and p73 have two promoters, 30-40 kb apart and with unique regulatory sites, driving transcription of sequences coding for proteins either with or without the N-terminal TAD (designated as ΔNp63/ΔNp73). In addition, alternative splicing creates variation in the C termini, resulting in at least six major transcripts of both genes (Yang *et al*. 2002).

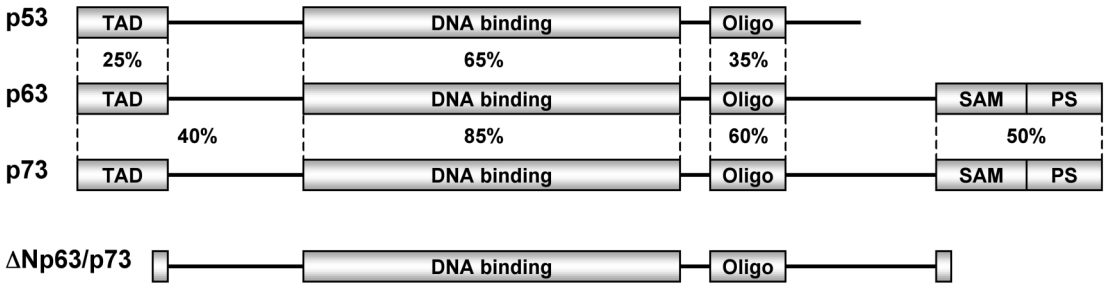



**Figure 11.** Structure and homology of p53 family of proteins. Upper percentages represent homology between domains of p53 and other family members, lower persentages homology between p63 and p73. Modified from Yang *et al*. 2002.

Full length p63 and p73 can bind to many p53 binding sites, transactivate certain p53 target genes, including $p21^{Cip1}$, Mdm2, Gadd45, and Bax, and induce cell cycle arrest or apoptosis (Yang *et al*. 2002, Benard *et al*. 2003). p63 and p73 may inhibit transcriptional activity of p53 by competing of the same binding sites on DNA, but also co-operate in target gene activation. For example, in MEFs p63 and p73 are needed for p53-induced apoptosis through their ability to stabilize p53 binding to the apoptotic promoters (Flores *et al*. 2002). Interaction of p63 and p73 by mutant p53 impairs their sequence-specific DNA binding, thereby inhibiting their transcriptional activity (Strano *et al*. 2002). ΔNp63 or ΔNp73 may inhibit p53 in a dominant-negative fashion through forming heterocomplexes with p53 (Yang *et al*. 2002). p53 induces ΔNp73 both on mRNA and protein levels, forming a possible feedback loop to terminate p53-mediated stress response (Kartasheva *et al*. 2002). This may be supported by the ability of ΔNp73 to inhibit Rb, and thus promote cell proliferation (Stiewe *et al*. 2003).

Both Mdm2 and MdmX can bind p63 or p73 forms containing the TAD, inhibit their transcriptional activities, but not induce their degradation (Irwin and Kaelin 2001, Kadakia *et al*. 2001, Calabro *et al*. 2002). Mdm2 can also induce alterations in the subcellular localization of p63 and p73, MdmX at least in the localization of p73 (Wang *et al*. 2001a, Kadakia *et al*. 2001). p73 is stabilized by Mdm2 and MdmX (Irwin and Kaelin 2001), but the stabilization of p63 remains controversial (Kadakia *et al*. 2001, Calabro *et al*. 2002). Instead, viral proteins do not bind and inactivate p63 or p73 as they do for p53 (Irwin and Kaelin 2001).

Even though members of the p53 family of proteins are structurally and functionally related, p63 and p73 seem to have at least partly distinct roles from p53. As p53 is essential for cellular stress responses and tumor suppression, p63 and p73 seem mostly to have cell type-specific developmental roles (Irwin and Kaelin 2001, Yang *et al*. 2002, Benard *et al*. 2003). p63 is constitutively present in the stem cell compartment of many epithelial tissues, almost entirely as the transcriptionally inactive ΔN form (Yang *et al*. 2002). *p63*-null mice are born without limbs and many epithelial structures, including skin, prostate, breast, and urithelial tissue (Mills *et al*. 1999, Yang *et al*. 1999). p63 is needed for sustaining viable pool of epithelial stem cells, and *p63*-null basal cells undergo terminal differentiation (Yang *et al*. 2002). *p63* is not mutated in human tumors, but is in fact located in a region (3q27) amplified, rather than lost, in various cancers (Benard *et al*. 2003). ΔNp63 levels are increased in some squamous cell carcinomas (Hibi *et al*. 2000), suggesting a possible role for the inhibition of transcription by p63 in this particular tumor type.

Even though *p73* lies in 1p36, a region with frequent LOH in human cancers, it seems that p73 is not a tumor suppressor (Benard *et al*. 2003). No evidence exists of inactivating p73 mutations in tumors, and *p73*-null mice do not show increased rate of spontaneous tumorigenesis (Yang *et al*. 2002). Instead, they do show neurological, pheromonal and inflammatory defects. p73 can protect symphatetic neurons from p53-induced apoptosis during development, as these cells show decreased survival in *p73*-null mice (Pozniak *et al*. 2000). The clinical impact of p73 has been proved so far only for neuroblastoma, where ΔNp73 expression correlates with poor prognosis (Casciano *et al*. 2002). Oncogenes, such as E2F1, c-Myc, and E1A, can activate p73 to induce apoptosis independently of p53 (Irwin and Kaelin 2001). A role for p73 is indicated in E2F1-induced apoptosis in thymocytes, and p73 could be involved in radiation-induced murine T-cell lymphomas (Irwin and Kaelin 2001, Benard *et al*. 2003). p73 is DNA damage-responsive, as it is phosphorylated by c-Abl and stabilized upon IR (Irwin and Kaelin 2001). DNA damage-dependent acetylation of p73 by p300 selectively enhances the activation of the apoptotic target genes (Costanzo *et al*. 2002).

# Aims of the present study

As the cellular response to ionizing radiation is therapeutically relevant, studies addressing the DNA damage response of p53 have mostly concentrated on IR. Conclusions from these studies have somewhat inaccurately been applied to create a model in which p53 response to all types of DNA damage is thought to be alike. Accumulating evidence shows, however, that both the response of p53 and other cellular events induced by IR and UV radiation are substantially different, most likely due to the different DNA damage types induced by these stress factors. In addition, it has seldomly been taken into account that p53 response to UV radiation varies depending on the wavelength and the amount of UV radiation inflicted on the cells.

We wanted to study the basic mechanisms of p53 function and the transcriptional responses in cells undergoing either a transient cell cycle arrest or apoptosis upon a UV insult. More specifically, the aims of this thesis research were to:

1. compare the response of p53 to UV radiation at UVB and UVC wavelengths
2. study the dependence of these p53 responses on the magnitude of UV radiation damage
3. determine differences between UV-induced p53 and p53 passively accumulated following proteasome inhibition
4. study the effects of UV radiation on gene expression on a genomic scale
5. to identify new UV-responsive genes

# Materials and methods

## *Cell culture*

The cell lines used in this study are listed in the table below:

| Cell line | Description and reference | Used in | Culture medium |
|---|---|---|---|
| HEL299 | human lung fibroblast (CCL-137, ATCC) | I | DMEM + 10% FCS |
| NIH3T3 | mouse fibroblast (CRL-1658, ATCC) | II | DMEM + 10% NBCS |
| p53-/-, mdm2-/- MEF | mouse embryonal fibroblast (Montes de Oca Luna *et al*. 1995) | II | DMEM + 10% FCS |
| SaOS-2 | osteosarcoma, p53-null (HTB-85, ATCC) | II, III | DMEM + 15% FCS |
| WS1 | human skin fibroblast (ATCC CRL-1502) | I-IV | DMEM + 10% FCS + NAA |

DMEM, Dulbecco's modified Eagle medium; FCS, fetal calf serum; NBCS, newborn calf serum; NAA, non-essential amino acids

All cells were maintained in a humidified atmosphere containing 5% $CO_2$ at + 37°C. UV treatment of cells was carried out with Stratalinker 2400 (Stratagene, La Jolla, CA) with bulbs emitting either UVC (254 nm) or UVB (312 nm). BioBeam 8000 $^{137}$Cs γ-ray source (STS GmbH, Braunschweig, Germany) was used for IR-treatment. Proteasome inhibitors used were MG132 (Z-Leu-Leu-Leu-CHO; Affiniti Research Products Ltd) and ALLN (N-Ac-Leu-Leu-norleucinal; Calbiochem). For PI-3-kinase inhibitor treatments the cells were pre-incubated for 45 min with either LY294002 (Calbiochem) or Wortmannin (Sigma) prior applying proteasome inhibitors or UVC radiation, and the PI-3-kinase inhibitors were maintained on the cells for the rest of the incubation periods.

## *Preparation of cellular extracts*

### *Cell lysates*

All the steps were performed in + 4°C. Monolayer cells were washed with Tris-buffered saline, scraped, and pelleted with brief centrifugation. The pellet was resuspended to buffer containing 25 mM Tris-HCl pH 8.0, 120 mM NaCl, 0.5 % Nonident P-40, 4 mM NaF, 100 μM $Na_3VO_4$, 100 KIU/ml approtinin, 1 mM phenylmethylsulphonyl fluoride (PMSF), and 10 μg/ml leupeptin, incubated on ice for 20 min, and centrifuged (20 000 rcf, 5 min) to remove cellular debris. Supernatant fraction was collected and stored in - 20°C for further use.

*Nuclear extracts*

Nuclear extracts were prepared in principle as previously described (Andrews and Faller 1991). All the steps were performed in + 4°C. Monolayer cells were washed with phosphate-buffered saline, scraped, and pelleted with brief centrifugation. The pellet was resuspended to buffer containing 10 mM Hepes-KOH pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 50 mM NaF, 0.5 mM DTT, 0.2 mM PMSF, 0.1 KIU/ml approtinin, and 100 ng/ml each leupeptin, E64, and soybean trypsin inhibitor, incubated on ice for 10 min, and vortexed for 10 s. The insoluble fraction was pelleted with brief centrifugation and resuspended to buffer containing 20 mM Hepes-KOH pH 7.9, 25 % glycerol, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA pH 8.0, 50 mM NaF, 0.5 mM DTT, 0.2 mM PMSF, 0.1 KIU/ml approtinin, and 100 ng/ml each leupeptin, E64, and soybean trypsin inhibitor, incubated on ice for 20 min, and centrifuged (20 000 rcf, 5 min) to remove cellular debris. Supernatant fraction was collected and stored in - 70°C for further use.

## *Methods*

A description of the methods, and the reagents used in them, are found in the original publications (I-IV) as indicated in the table below.

| Method | Used and described in |
|---|---|
| 5-BrdUrd incorporation assay | I |
| Flow cytometric analysis | I |
| Immunofluorescence analysis | I, II |
| Immunoprecipitation | I, II |
| Immunoblotting | I-III |
| Northern analysis | I, III, IV |
| Chloramphenicol acetyl transferase (CAT) assay | II |
| Metabolic labeling | II |
| Transient transfections | II, III |
| Construction of mutant expression vectors | II, III |
| Bio-oligo pull-down assay | III |
| Microarray analysis | IV |

# Results and discussion

UV radiation has a profound role in skin cancer tumorigenesis, mainly due to its ability to cause mutations which disturb actions of normal cellular maintenance and stress responses. Functions of the tumor suppressor p53 are central to UV radiation-induced cell cycle arrest and sunburn apoptosis in skin, but the normal mode of these actions have not been elucidated in detail. Comparisons with results from UV-damaged skin have proven cell cultures as relevant models to study UV-induced cytotoxicity (Straface *et al*. 2001). In this study, the effects of UV radiation on p53 and adaptive transcriptional responses were assessed in diploid human fibroblasts. The ability of these cells to adapt to cell culture conditions with relatively few changes compared to other skin cell types have made them a commonly used model to study effects of UV radiation on human cells.

The doses of UV radiation used in this study varied between 1 to 5000 J/m$^2$ UVB and 1 to 100 J/m$^2$ UVC. Minimal erythemal dose (MED) in human skin is 200-1000 J/m$^2$ UVB depending on skin type and pigmentation (Clydesdale *et al*. 2001). Thus, the doses of UVB radiation used were physiologically relevant. The UVB radiation dose used as the low dose (750 J/m$^2$) to study transient cell cycle arrest roughly corresponds to 23 min exposure to sunlight at noon on a bright midsummer day in Finland (Kolari *et al*. 1986), or less than 3 minutes on midsummer noon in Italy (Meloni *et al*. 2000). The high dose of UVB (3500 J/m$^2$) used to study UV radiation-induced apoptosis equals to 1 h 45 min in Finland or 12,5 min in Italy under the corresponding circumstances. In skin, the effects of UV radiation are complex due to the different cell types and layers the skin contains. Most of sunlight is restrained to epidermis, which consists mostly of keratinocytes. Yet, 10-15% of UVB from sunlight penetrates to dermis, where the fibroblasts of the skin are located (Clydesdale *et al*. 2001). The low and high doses of UVC radiation used in this study (10 and 50 J/m$^2$, respectively) were chosen on the basis of their ability to provoke similar cellular responses than the corresponding doses of UVB radiation. This is in accordance with the ability of UVC to induce approximately 100 times more DNA damage adducts than UVB with the same amount of energy (Ravanat *et al*. 2001). The high doses of UV radiation are enough to induce RNAPII-inhibiting DNA adducts to every gene, taken that they are evenly distributed throughout the genome (Perdiz *et al*. 2000). UVC radiation was used as a simplified model compared to the physiologically more relevant UVB to study the effects of UV radiation-induced DNA damage on cells.

## Dose- and wavelength-dependent cellular responses to UV radiation (I,IV)

When diploid human fibroblasts (cell lines WS1 and HEL299) were inflicted with the low doses of UV radiation, a transient cell cycle arrest response was induced. DNA replication declined, as measured by the decreased incorporation of 5-Bromodeoxyuridine nucleotide to newly synthesized DNA (I, Figure 1A). A rapid arrest was immediately launched and was detectable by phosphorylation of Chk1 and downregulation of Cdc25A phosphatase (I, Figure 2), which is in accordance with previous studies (Mailand *et al*. 2000). A prolonged cell cycle arrest occurred also. This was observed through stabilization of tumor suppressor p53 followed by upregulation of the CKI inhibitor p21$^{Cip1}$ both at the mRNA and the protein level, as measured by western, northern, and microarray expression

analyses (I, Figures 2,3; IV, Table 1, Figure 6). The changes were mostly recovered by 24 h, and the cells continued cycling. There was no evident accumulation of cells in G1 and G2 phases of the cell cycle or decrease in S phase cells (before the changes were due to confluence of the cell cultures by 48 h)(I, Figure 1). Due to the nature of the UV type of DNA damage, synthesis of DNA is mechanistically inhibited *in situ*, and at least part of the lesions have to be removed before synthesis can proceed. The arrest induced by UV radiation is distinct from e.g. IR-induced cell cycle arrest, where the cells clearly accumulate to the gap phases of the cell cycle (Bartek and Lukas 2001b). Thus the term replication arrest seems more appropriate than cell cycle arrest to be used for the arrest response induced by the low doses of UV radiation.

High doses of UV radiation induced apoptosis in the diploid human fibroblasts. Phosphorylation of Chk1 and downregulation of Cdc25A were detectable, indicating that a rapid halting of cell proliferation by DNA damage checkpoints took place also after a fatal amount of UV damage (I, Figure 2). DNA replication ceased rapidly (before 3 h; I, Figure 1A), probably reflecting both the physical challenge posed by the DNA damage bulges to DNA replication machinery, and the active, damage-responsive events. p53 accumulation occurred more slowly in the apoptotic cells than in the transiently arrested cells, and the levels of p53 protein remained high throughout the follow-up period (48 h)(I, Figures 5,6), by the end of which most of the cells were apoptotic (I, Figure1). The cells underwent programmed cell death, detectable by activation of caspase activity and accumulation of cells undergoing degradation of DNA (cells with sub-G1 DNA content) (I, Figures 1,2). Interestingly, Cdc25A phosphatase levels were recovered after their initial downregulation in the apoptotic cells. This may be due to protein production being normalized, allowing Cdc25A to accumulate again when the initial activation phase by ATR kinase is over. However, protein synthesis is decreased in cells inflicted with high amounts of RNAPII blocking agents (Tornaletti and Hanawalt 1999), and it would seem uneconomical for the cells to consume energy for repairing and expressing an unnecessary gene. The effort to upregulate Cdc25A phosphatase in cells already determined to undergo apoptosis implies that it may have a later role in the apoptotic process.

## p53 response to UV radiation (I-III)

Already in 1984, p53 was found to be stabilized by UV radiation (Maltzman and Czysyk 1984). Since then, it has become apparent that p53 plays a central role in the cellular responses provoked by UV radiation, amongst other stress inducers (Prives and Hall 1999, Vousden and Lu 2002). p53 is essential for efficient protective UV responses in skin, and loss of its function promotes UV-induced skin tumorigenesis significantly (Ziegler *et al*. 1994, de Gruijl *et al*. 2001). Different aspects of p53 regulation by UV radiation have been extensively studied, but in various cell types, and with various radiation wavelengths and doses, leaving the results difficult to bring together. Thus, the exact events in p53 regulation taking place in the different cellular responses to UV radiation have remained elusive. In this work, the most relevant aspects of p53 regulation in response to UV were studied, namely the posttranslational modifications, transcriptional activity, and subcellular localization of p53, as well as its interaction with Mdm2, regulation by PI-3-related kinases, and the resulting effects to target gene expression.

### *UV dose-dependent posttranslational modifications of p53 (I)*

Stabilization of p53 by UV radiation is dose-dependent (I, Figures 2,4-6). In cells undergoing transient cell cycle arrest, p53 accumulated rapidly (accumulation was detectable already at 2 h, data not shown) and was mostly returned to basal levels by 24 h. In contrast, in the cells irradiated with the high UV dose, the accumulation occurred more slowly but eventually gained a higher magnitude than in the arrested cells, persisting up until the cells underwent apoptosis. The response was similar with corresponding doses of either UVB or UVC radiation. Even though some influence of oxidative stress cannot be ruled out, the results indicate that p53 stabilization by UVB is mostly attributable to the DNA damage inflicted by the radiation.

Further experiments showed that regulation of p53 is significantly different between the arresting and the apoptotic cellular responses to UV radiation. The N-terminal modifications of p53 have been suggested to release p53 from Mdm2-mediated degradation and thus increase stability of p53, but also to promote C-terminal acetylations increasing p53 activity (Meek 1999, Appella and Anderson 2001). By the use of modification-specific antibodies, we studied the kinetics of N-terminal phosphorylations of p53 on Ser15, Ser33 and Ser37, and C-terminal acetylation on Lys382, and the dependency of these modifications on UV radiation wavelength and dose (I, Figures 4,5). All these modifications occurred by both UVB and UVC radiation, but the kinetics, measured as the relative saturation degree of p53 molecules by these modifications, varied: Ser37 was phosphorylated more efficiently with lower doses, Ser15 and Ser33 with intermediate doses, and Lys382 was acetylated relatively more efficiently with very high doses of both UVB and UVC radiation.

Following the low dose of UV radiation, all the modifications were detectable already as p53 started to accumulate (I, Figure 5; data not shown). Ser37 phosphorylation was relatively quickly saturated, followed by Ser15 and Ser33 phosphorylations. The saturation of Ser15 phosphorylation was more transient, while those of Ser33 and Ser37 fluctuated and were persistent at 24 h as the cells had re-entered the cell cycle. Acetylation of Lys382 was modest in the transiently arrested cells. In the cells destined to undergo apoptosis, all the modifications had an initial burst at 6 h when p53 was not much accumulated yet. After this, the relative amount of modifications, as compared to the total levels of p53, decreased and were elevated again by 18 h. Lys382 acetylation was the only modification which decreased by 24 h in the cells irradiated with the high dose of UV, as the N-terminal phosphorylations persisted along with the high stabilization of p53 (I, Figure 5). These results significantly extend information gained from previous studies of p53 modifications in response to intermediate doses of UV radiation (Sakaguchi *et al*. 1998, Oda et al. 2000a, Takekawa et al. 2000,).

The results indicate that in the apoptotic response, the initial activation of p53-modifying enzymes is rapid, followed by a lag period, after which more activity is present again. ATR responds to DNA damage very quickly (Bartek and Lukas 2001a,b) and presumably promotes the rapid modifications of p53 (Prives and Hall 1999, Appella and Anderson 2001). At a later stage in the apoptotic response, either a second phase of ATR kinase activity follows due to persistent DNA damage or other activating signals, or apoptosis-associated kinases are responsible for the persistent p53 phosphorylations. Interestingly, this second phase of enzyme activity does not result in persistent C-terminal acetylation of p53, even though acetylation of Lys382 is more pronounced with higher doses of UV radiation (I, Figure 4). Possibly, the enhancement of p53 transcriptional activity by C-terminal

acetylation has already served its purpose early in the response promoting the apoptotic p53 targets, and is allowed to decrease thereafter. Alternatively, C-terminal Lys382 acetylation may not favor activity towards the late apoptotic targets, but may be needed for the fine-tuned activation of targets early in the response. Thus, the second phase of activators would primarily serve to promote p53 interactions with basal transcription machinery and other transcription-associated factors via the N-terminal phosphorylations. As reported during the course of this study, phosphorylations are not essential to p53 activation (Ashcroft *et al.* 1999, Blattner *et al.* 1999), but they still frequently occur in different types of p53 responses. They probably supply some advantages for p53 functions by, for example, ensuring efficient p53 accumulation during the UV response, as reported for the phosphorylation of Ser15 and Ser33 (Bean and Stark 2001). It would have been interesting to study whether other p53 modifications occur in the different cellular responses provoked by UV radiation, especially sumoylation of p53, as well as the phosphorylations on Ser46 and Ser392, which have been linked to p53-induced apoptosis and the UV response, respectively (Appella and Anderson 2001). Unfortunately, the necessary modification-specific antibodies were not at hand during the course of this study.

### *UV dose-dependent regulation of p53-Mdm2 interaction (I)*

Mdm2 protein levels were increased in cells transiently arrested by the low dose of UV radiation. Kinetics of Mdm2 accumulation were slower than that of p53, as expected for the feedback loop function. To estimate the fractions of p53 bound to Mdm2, immunoprecipitation analyses were performed (I, Figure 6). The amount of Mdm2-free p53 peaked at 16 h. After this, the interaction between the two proteins increased and led to a decrease in p53 levels (I, Figure 6). Of the N-terminal p53 phosphorylations studied here, Ser15 phosphorylation showed the best inverse correlation with p53-Mdm2 interaction in the arrested cells. In the cells damaged with the high dose of UV radiation, Mdm2 levels were decreased. Dose-dependent regulation of Mdm2 protein levels in response to UV radiation have been reported previously (Perry *et al.* 1993). As Mdm2 is not present during initiation of the apoptotic response, inhibition of Mdm2 binding by phosphorylating the p53 N terminus seems redundant. Eventually, however, Mdm2 levels were restored, and the increasing N-terminal p53 phosphorylations by 24h after UV radiation may serve the purpose of keeping the newly synthetisized Mdm2 unable to bind p53. Alternatively, the N-terminal phosphorylations may be needed for the enhanced p53 activity to induce the apoptotic target genes. This may occur via increased interaction of p53 with the transcriptional apparatus, or via other, yet unidentified mechanisms. In addition, the fraction of Mdm2-bound p53 was significantly increased by the high dose of UV radiation, even though Mdm2 protein levels were decreased. This indicates that the increase in p53 accessibility promotes p53-Mdm2 interaction, or that the affinity of Mdm2 towards p53 increases. It would be interesting to study if the putative affinity change is due to altered modifications on Mdm2.

### *Regulation of p53 by PI-3-kinases in the UV response (II)*

p53 accumulation by DNA damage is dependent on PI-3-kinases (II, Figure 1A; Meek 1999, Appella and Anderson 2001), which most likely reflects the effect of ATM and ATR on the stabilizing modifications of p53. To find out whether UV radiation induced posttranslational modifications of p53 in a PI-3-kinase-dependent manner, the modifications of p53 induced by UV radiation were studied in the absence or presence of chemical PI-3-

kinase inhibitors (II, Figure 4). The results show that p53 is phosphorylated on Ser15 and Ser33 in a PI-3-kinase-dependent manner after UV radiation. Acetylation of p53 to Lys382 by UV radiation was also PI-3-kinase-dependent, probably indirectly via the dependence of C-terminal acetylations on preceding N-terminal phosphorylations of p53 (Chernov *et al*. 1998, Lambert *et al*. 1998, Sakaguchi *et al*. 1998). We found no changes in the localizations of p53 and Mdm2 by inhibition of PI-3-kinases, either before or after UV radiation (II, Figure 2). Some reports have suggested that Ser15 phosphorylation of p53, expected to occur by ATR after UV insult, or growth factor-dependent phosphorylations of Mdm2 on Ser166 and Ser186 by PI-3-related kinase Akt affect intracellular localization of these proteins (Mayo and Donner 2001, Zhang and Xiong 2001, Zhou *et al*. 2001). As also conflicting results have been reported (Ashcroft *et al*. 2002), PI-3-kinases are concluded not to regulate localization of p53 or Mdm2 under normal cell growth conditions or upon UV radiation.

Even though inhibition of PI-3-kinases decreases p53 stabilization and posttranslational modifications induced by UV radiation, induction of p53 target genes was not affected, at least as measured by induction of target gene $p21^{Cip1}$ or activation of reporter gene transcription from a promoter containing multiple high-affinity p53 response elements (REs) (II, Figure 5). Earlier reports indicate that transcriptional activation of p53 by UV radiation is independent on the stabilization of the protein, suggesting that an excess of the protein exists (Lu *et al*. 1996, Haapajärvi *et al*. 1997). It is possible that in the cellular response to UV radiation p53 is saturated on target promoters, at least those with REs closely resembling the consensus p53 binding sequence. Despite the fact that inhibition of PI-3-kinases decreases p53 accumulation, there could still be enough active p53 to induce these targets. Inhibition of PI-3-kinases could affect the ability of p53 to induce transcription from targets with lower affinity REs.

### *Dose-dependent induction of p53 target genes by UV radiation (I,IV)*

To be able to compare the extent of p53 modifications and Mdm2 interaction with the cellular effects of p53, the UV-induced changes in expression of selected p53 target genes was studied by northern analysis of mRNA levels in diploid human fibroblasts. The target genes were regulated differentially in the two cellular responses, as expected. In the transiently arrested cells, $p21^{Cip1}$ mRNA was induced rapidly, correlating to the increase in its protein levels (I, Figures 2,3; IV, Table 1, Figure 6). Both Mdm2 mRNA and protein levels were induced slightly later, correlating to increased p53-Mdm2 interaction linked to the termination of p53 response (I, Figures 3,6). Gadd45 was induced rapidly and transiently in the arrested cells. The pro-apoptotic p53 target Bax was not induced, and repression of the anti-apoptotic target Bcl-2 did not take place, indicating that p53-mediated apoptosis was not induced. In contrast, in the cells undergoing apoptosis, induction of $p21^{Cip1}$ and Mdm2 was decreased. Proapoptotic Bax was upregulated, and anti-apoptotic Bcl-2 was slightly downregulated, indicating that p53-mediated apoptosis was induced. Interestingly, Gadd45 induction was high and persistent at the mRNA level in the cells destined to apoptosis (I, Figure 3; IV, Table 2, Figure 6). The function of Gadd45 is unclear, but it is thought to take part in DNA repair. The powerful induction of Gadd45 in the cells undergoing apoptosis indicates, however, a role for Gadd45 in UV-induced apoptosis. Similar results obtained from rat embryo fibroblasts have previously been reported for Mdm2, $p21^{Cip1}$ and Bax (Reinke and Lozano 1997). These results confirm that selection of p53 target genes occurs dose-dependently by UV radiation.

### *Redox-regulation of p53 activity (III)*

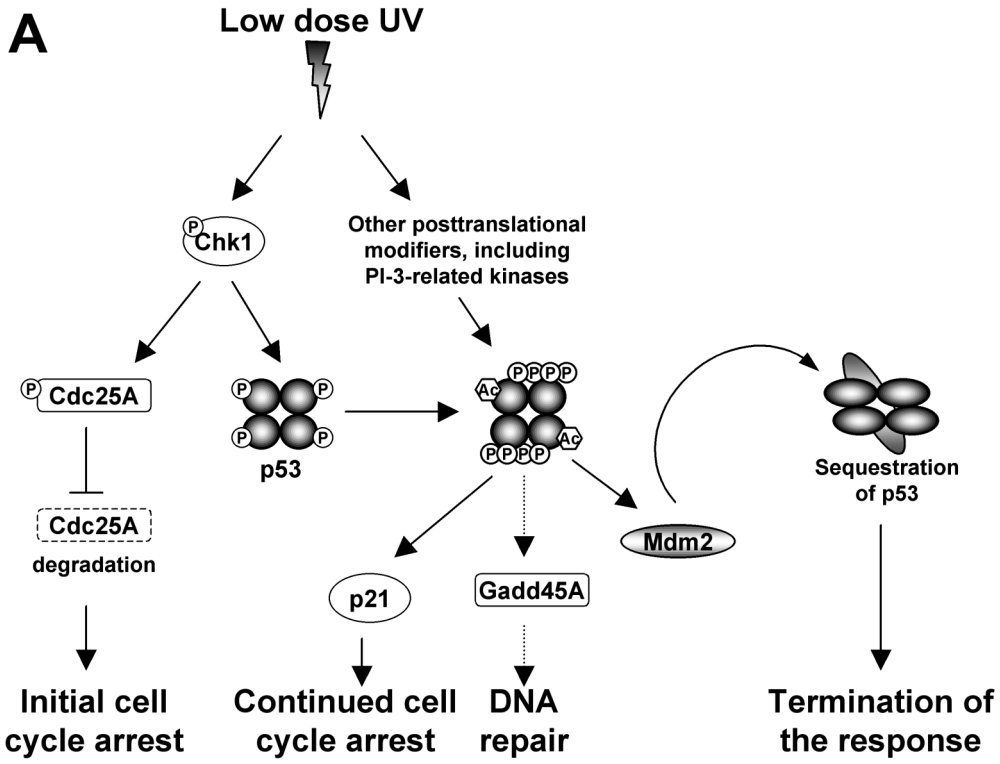
To assess possible selection amongst target gene binding sites in response to genotoxic insults, the ability of p53 to bind the REs of certain target gene promoters was studied by a DNA pull-down assay taking advantage of biotinylated oligonucleotides (III). Following DNA damage (both IR- and UVC-induced), p53 affinity towards Gadd45 RE but not $p21^{Cip1}$ RE was decreased (III, Figure 2). This effect was found to be dependent on the nucleotide at positions 3 of the RE, which directly contact the Cys277 residues of p53 tetramers (III, Figures 3,4). Further results demonstrated that if Cys277 is reduced, p53 maintains strong binding to REs containing either cytosines (C) or thymines (T) in the positions 3, as p53 with oxidized Cys277 retains high affinity only to REs containing T in these positions (III, Figure 5). This is attributable to the SH group of Cys277 forming a hydrophobic interaction with $CH_3$ group of a T, but a hydrogen bond with the $NH_2$-group of a C (Cho et al 1994). When Cys277 becomes oxidized, the hydrophobic interaction is still possible, but accepting a hydrogen bond is not. Seemingly, Cys277 of p53 becomes oxidized later in the DNA damage response, thus leaning the target gene selection towards REs rich for T in the positions 3. Oxidation of p53 is known to affect its DNA binding through disturbing $Zn^{2+}$ coordination and correct folding of the protein (Parks *et al*. 1997, Fojta *et al*. 1999). A pattern for p53 DNA binding to target REs may thus consist of three states: in an oxidized state, when coordination of $Zn^{2+}$ and proper folding of p53 is lost, no binding to target REs occurs. When p53 is in a reduced state, correctly folded and binding $Zn^{2+}$, it binds target REs without distinction of the chemical bond formed by Cys277. Finally, in a partially oxidized state, p53 retains $Zn^{2+}$ coordination but Cys277 is oxidized, and p53 prefers T-rich REs. Interestingly, *TP53* mutation database reports of 64 cases with p53 point mutations at the amino acid residue 277 (Olivier *et al*. 2002; database version R7 September 2002). The most predominating groups of cancer types harbouring this mutation were lung cancer (9 cases) and skin cancer (7 cases), which often originate from external stress capable of inducing oxidative stress (cigarette smoke and UV radiation, respectively).

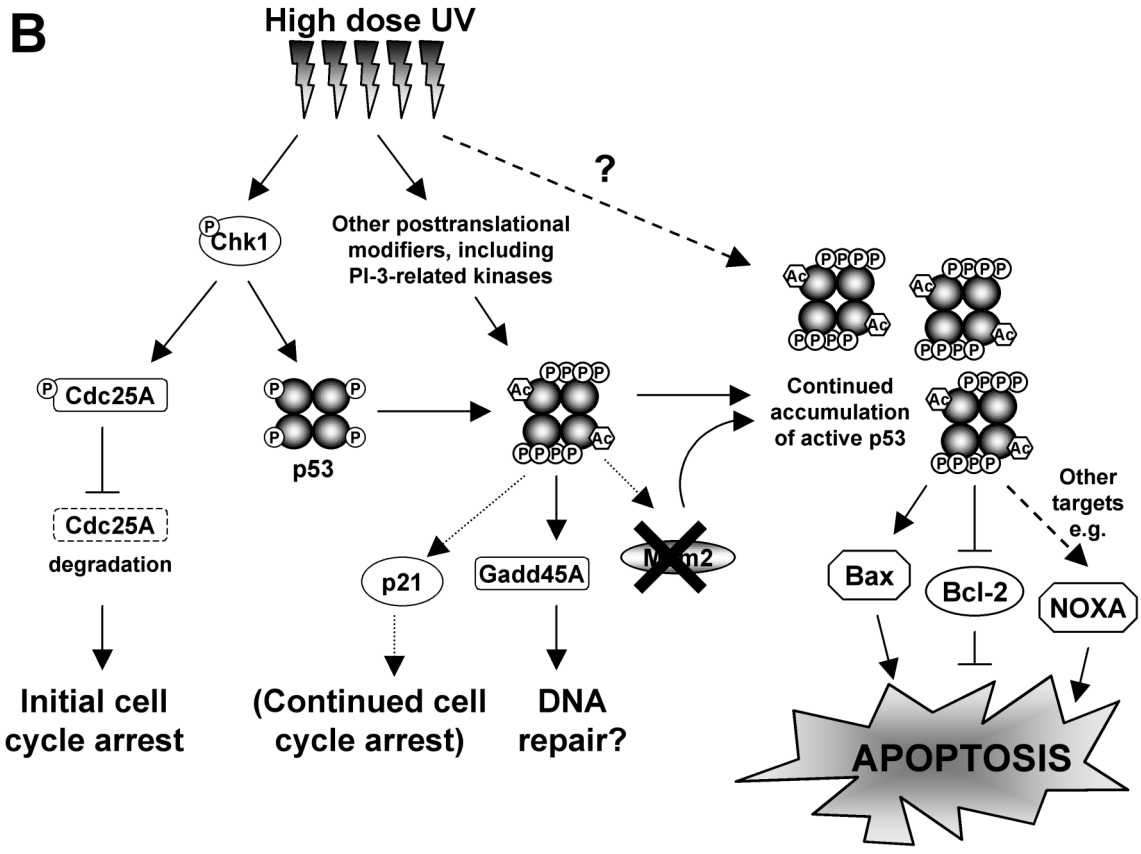
The mechanism how Cys277 oxidation occurs is not clear. The minor amounts of reactive oxygen species caused by IR and UV radiation may be enough to provoke changes in the redox state of p53. Increased intracellular ROS can cause oxidation of p53, and p53 redox regulation has earlier been shown to be biologically relevant (Meplan *et al*. 2000). However, if this is the cause of distinction between p53 REs, one would predict that a kinetic decrease in binding to Gadd45 RE would increase along with increased UV doses. As the effect of UV radiation on Gadd45 expression is quite the opposite, it is more likely that a specific, enzymatic mechanism exists to regulate p53 redox state after genotoxic insults. Potential proteins to mediate this effect are the redox-sensitive Ref-1 and HIF-1$\alpha$, which interact with p53 (Jayaraman *et al*. 1997, An *et al*. 1998). Alternatively, the sustained, high induction of Gadd45 in the apoptotic cells may be maintained by a p53-independent mechanism.

### *UV dose-dependent determination of p53-mediated cellular response*

The results so far show that regulation of p53-Mdm2 pathway is significantly different between the cells undergoing a transient arrest and apoptosis provoked by UV radiation (Figure 12). The transient arrest response showed a classical feedback loop behaviour of p53-Mdm2 regulation, while the apoptotic cells lacked accumulation of Mdm2 in response to p53 stabilization, and subsequently the p53 levels increased further. Despite the persisting N-terminal phosphorylations of p53, Mdm2 interaction with p53 was relatively increased. This suggests that the N-terminal phosphorylations on p53 may serve another purpose than blocking Mdm2 binding to p53, at least in the cells undergoing apoptosis. This could be promoting p53 interactions with different proteins of transcription machinery (Lambert *et al*. 1998, Pise-Masison *et al*. 1998, Dumaz and Meek 1999, Dornan *et al*. 2003). Yet, the impact of PI-3-kinases on p53 activity in the UV response was found to be lesser than expected. In addition, despite some kinetical differences, the N-terminal phosphorylations of p53 did not show significant distinction between the dose-dependent cellular responses to UV radiation compared to p53 levels. On the other hand, the persistence of the C-terminal acetylation of p53 during the UV response was dose-dependent. As the extent of p53 acetylation decreases late in the apoptotic cells, p53 degradation should take place as the C-terminal Lys residues are available for ubiquitination. The degradation does not take place, however, as Mdm2 is not present. It it possible that the following further accumulation of p53 is a secondary event, if p53 has already performed its transactivating functions while acetylated. Alternatively, the lack of acetylation of p53 may represent active means to regulate p53-induced apoptosis. A stepwise accumulation of p53 by UV radiation was discovered during a more detailed kinetical analysis (data not shown), and p53 accumulation has been reported to occur in an oscillating manner (Lev Bar-Or *et al*. 2000). This is thought to occur due to a cyclic nature of p53-Mdm2 feedback loop, which eventually leads to downregulation of p53 levels. As in the cells undergoing UV-induced apoptosis the fluctuations cannot be due to actions of Mdm2, deacetylation of p53 may be one of the active steps leading to further p53 stabilization. Lack of acetylation may direct p53 interactions with proteins taking part in induction of apoptotic p53 targets or transcriptional activity-independent p53 functions late in the apoptotic process. Alternatively, acetylation may directly favor p53 activity towards the early apoptotic targets, as deacetylation would make p53 transfer its activities towards the late apoptotic target genes. In addition, redox-regulation contributes to kinetical differences on p53 affinity towards different target gene REs.

**Figure 12.** (opposite page) Summary of the dose-dependent cellular response to UV radiation in diploid human fibroblasts. A low dose of UV radiation induces a transient cell cycle arrest (A). An immediate arrest is induced via Chk1 activation and subsequent degradation of cdc25A. This is followed by a continued cell cycle arrest and enhanced DNA repair through stabilization and transcriptional activation of p53, leading to induction of target genes like $p21^{Cip1}$ and Gadd45. Later in the process, p53 induces Mdm2 to downregulate its own response, allowing the cells to continue cycling. A high dose of UV radiation induces apoptosis (B). First, the initial cell cycle arrest is induced as by the low dose of UV radiation. Stabilization and transcriptional activation of p53 occur slower than in the transiently arrested cells, and are followed by induction of Gadd45 and $p21^{Cip1}$. Lack of Mdm2 induction allows p53 to accumulate further, leading to induction of the apoptotic p53 target genes and subsequent initiation of apoptosis. Dotted arrows, attenuated response compared to the other dose. Dashed arrows, connections / effectors not shown in this work.


A
Low dose UV
Chk1
Other posttranslational modifiers, including PI-3-related kinases
Cdc25A
Cdc25A
degradation
p53
p21
Gadd45A
Mdm2
Sequestration of p53
Initial cell cycle arrest
Continued cell cycle arrest
DNA repair
Termination of the response


B
High dose UV
?
Chk1
Other posttranslational modifiers, including PI-3-related kinases
Cdc25A
Cdc25A
degradation
p53
p21
Gadd45A
Continued accumulation of active p53
Other targets e.g.
Bax
Bcl-2
NOXA
APOPTOSIS
Initial cell cycle arrest
(Continued cell cycle arrest)
DNA repair?

Interestingly, the results indicate a possible affinity change on Mdm2 towards p53 in the apoptotic cells. Mdm2 may in fact try to sequester p53, but is unsuccessful due to an imbalance in the levels of the proteins. Interestingly, proness to UV-induced apoptosis in TCR-deficient cells is associated with lack of activation of Mdm2 transcription (Conforti *et al*. 2000), and loss of Mdm2 is enough to induce p53-dependent apoptosis *in vivo* (de Rozieres *et al*. 2000). Furthermore, Mdm2 can be cleaved by caspases in apoptotic cells to produce a truncated Mdm2 form deficient in the domain required for association with p53 (Chen *et al*. 1997). The truncated form works in a dominant negative fashion efficiently inhibiting the activity of full-length Mdm2, resulting in elevated levels of p53 (Alarcon-Vargas and Ronai 2002). p53 affinity for REs in the apoptotic targets seems to be lower on average than that for REs of targets inducing cell cycle arrest (Vousden and Lu 2002). This suggests that as soon as p53 gets accumulated to high enough levels, the transient p53-mediated arrest response could be overridden by an increased induction of proapoptotic targets. High accumulation of p53 by downregulation of Mdm2 may thus be a common mechanism to induce p53-dependent apoptosis, and be a more significant determinant for the UV-response than the modifications of p53 (Figure 13). Taken together, these results suggest that both p53 stabilization and activation are regulated in multistep processes to either guarantee proper termination of the UV-induced p53 response, or allow p53 to accumulate to high enough levels to initiate apoptosis.

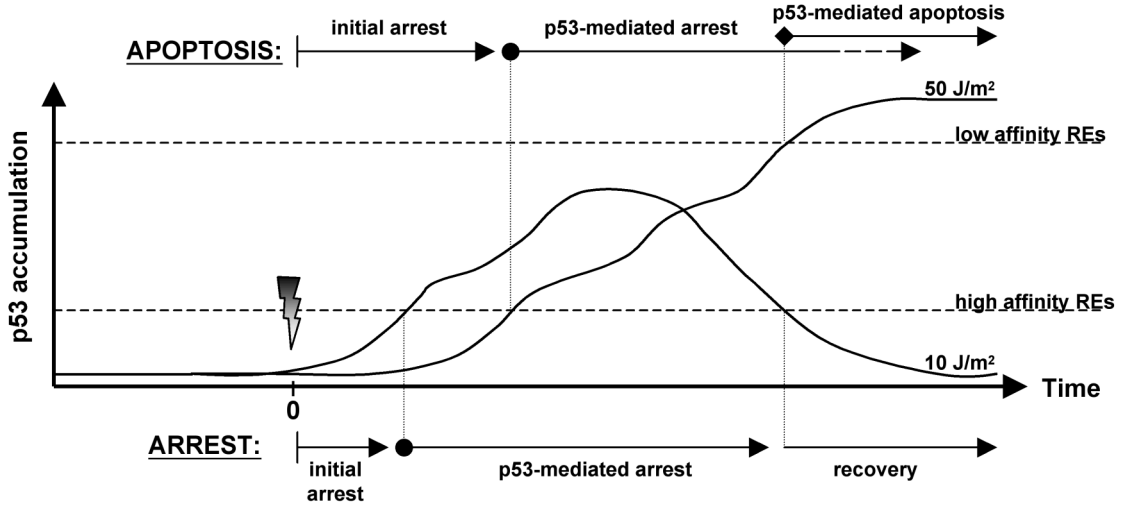



**Figure 13.** A simplified model of different p53-mediated cellular responses by UV radiation. Initial cell cycle arrest is launched despite of magnitude of the UV damage (at time 0), following a phase of p53-mediated cell cycle arrest. If damage repair is succesful, Mdm2 is allowed to accumulate and targets p53 to degradation, leading to recovery of normal cellular state. If the repair is unsuccesful, p53 is further accumulated activating also low affinity targets. This leads to initiation of apoptosis which will override the arrest response.

## Regulation of p53-Mdm2 pathway by inhibition of proteasome function (II)

As p53 and Mdm2 are continuously produced, they both accumulate when their degradation is blocked by inhibition of the proteasome (Momand *et al*. 2000). This is thought to occur in the absence of a p53-activating signal, leaving p53 in a latent state. To study the regulation of p53-Mdm2 pathway by proteasome inhibition, the posttranslational modifications of p53, the extent of interaction between p53-Mdm2, and p53 transcriptional activity were studied in the presence and absence of chemical PI-3-kinase inhibitors. Induction of target gene $p21^{Cip1}$ was deteceted by northern analysis in cells treated with proteasome inhibitors, indicating that p53 might be transcriptionally active (II, Figure 5). This is in contrast with another study reporting that $p21^{Cip1}$ is not induced by p53 accumulated by proteasome inhibition (Klibanov *et al*. 2001). The difference may be due to kinetic changes. In this study, $p21^{Cip1}$ upregulation occurred at 6 h, whereas Klibanov *et al*. reported of 16-h timepoint. p53 was confirmed to be transcriptionally active in proteasomally inhibited cells by a reporter gene assay. Accumulation and activity of p53 by proteasomal inhibition were not dependent on PI-3-kinases (II, Figures 1,5). A slight basal modification of p53 on Ser33 was detected, and, interestingly, it was decreased by inhibition of PI-3-kinases (II, Figure 4). p53 interaction with Mdm2 in cells treated with proteasome inhibitors was decreased by inhibition of PI-3-kinases, which was at least in part due to decreased protein synthesis of Mdm2 (II, Figures 1,6B). The decline in the interaction occurred also in cells deficient for p53 and in cells treated only with PI-3-kinase inhibitors (II, Figures 6A,B). Thus, PI-3-kinases regulate the basal levels of Mdm2 independently of p53.

Lys382 of p53 is not acetylated in diploid human fibroblasts treated with proteasome inhibitors (II, Figure 4; Ito *et al*. 2001), indicating that the transcriptional activity of p53 observed in the cells was not due to activating acetylation on p53 C terminus. N-terminal phosphorylations of p53, which inhibit Mdm2 interaction, are not induced in proteasome-inhibited cells (II, Figure 4; earlier reports for Ser15 phosphorylation by Shieh *et al*. 1997 and Siliciano *et al*. 1997). Yet, a significant pool of p53 free from Mdm2, as well as p53-free Mdm2, existed in these cells (II, Figure 3). Either p53 accumulation is sufficient to induce target gene induction of high affinity REs tested here, or proteasome inhibition poses a stress in itself, which induces some transcriptional activity of p53. As this would occur without activating C-terminal acetylation on p53, it might be due to other transregulatory factors promoting the transcriptional activity of p53. Interestingly, preferential T-rich RE binding of p53 later in the stress response was also detected after proteasome inhibition (data not shown), indicating active redox regulation of p53 DNA binding. Proteasome inhibition triggers heat shock response via HSF1 (*h*eat *s*hock *f*actor *1*)-mediated induction of Hsp's (Pirkkala *et al*. 2000). p53 interacts with Hsp70 and Hsp90 chaperones, which can affect conformation of p53 (Zylicz *et al*. 2001). It is tempting to speculate that heat shock-associated factors could take part in the regulation of p53 activity in response to proteasome inhibition.

Proteasome inhibition caused translocation of both p53 and Mdm2 to the nucleoli of the cells, as observed by immunofluorescence microscopy (II, Figures 2A,B). This effect could be blocked by PI-3-kinase inhibitors (II, Figures 2A,B). A small fraction of p53 has been shown to localize to the nucleoli overlapping rRNA transcription sites (Rubbi and Milner 2000), suggesting that p53 might contribute to rRNA synthesis. The nucleolar localization of p53 upon proteasome inhibition and its dependence on PI-3-kinases were, however,

independent of the ability of p53 to bind DNA (II, Figure 2D) and thus not attributable to the transactivation function of p53. ARF, although capable of translocating Mdm2 into nucleoli (Weber *et al*. 1999), was not required for Mdm2 translocation after proteasome inhibition, nor was p53, as the translocation effect was observed also in cells deficient for ARF or p53 (II, Figure 2C). Phosphorylation of Mdm2 on Ser166 and Ser186 by Akt has been described to regulate Mdm2 translocation (Mayo and Donner 2001, Zhou *et al*. 2001). Therefore, it was tested whether mutations of these sites would hinder nucleolar translocation of Mdm2 by proteasome inhibition. Mutation of these sites, or mutation of any other known or putative PI-3-kinase phosphorylation site on Mdm2, did not inhibit translocation of Mdm2 to nucleoli upon inhibition of the proteasome function (II, Figure 6C). Thus, direct modifications of Mdm2 or p53 by PI-3-kinases are not responsible for the effect. In addition to p53 and Mdm2, several proteins have been found to localize to nucleoli following proteasome inhibition, including Hsp70, PML and SUMO-1 (Mattsson *et al*. 2001). Interactions between Mdm2 and both Hsp70 and PML after proteasome inhibition have been found (Kurki S *et al*., unpublished results), suggesting that these factors may bridge the nucleolar localization of Mdm2 and possibly p53. It is also possible that nucleolar translocation of stress-responsive proteins represents a common, yet functionally unknown, response mechanism in cells which are eventually dying due to proteasome dysfunction.

## Large scale transcriptional changes in UV radiation-induced replication arrest and apoptosis (IV)

Recent microarray studies of UVB responses in keratinocytes and melanocytes (Li *et al*. 2001, Valery *et al*. 2001, Sesto *et al*. 2002, Takao *et al*. 2002) have gained interesting insights on genes responsive to low insults of UV radiation. These surveys did not assess effects of apoptosis-inducing doses of UV damage, however. To study how UV-induced arrest and apoptosis differ on transcriptional level, the study I, indicating that dose-dependent cellular responses to UV radiation are highly distinct, was expanded to analyze transcriptional changes on a large scale. As the primary interest was responses provoked by UV radiation-induced DNA damage, UVC wavelength was used as a simplified model to induce transient replication arrest or apoptosis in the fibroblasts. Levels of over 12000 transcripts were screened by microarray analysis for changes induced by UV radiation. Based on analysis of the experimental variation between the samples (IV, Figure 1), a cut-off value for the significant changes in transcript abundance was set to 3-fold (a proportion of false positives less than 5%). 460 transcripts were regulated significantly by UV radiation, of which 89% were downregulated (68 transcripts by the low and 361 transcripts by the high dose)(IV, Figure 2). Only 5% of the significantly regulated genes were common to both the transient arrest and apoptosis induced by UV radiation (10% of the induced and 4% of the repressed transcripts). In addition, transcripts undergoing significant regulation were not regulated even modestly by the other dose, indicating that the vast majority of the UV-regulated targets were highly specific for either dose (IV, Figure 4). This demonstrates that dose-dependent induction of transient replication arrest and apoptosis by UV radiation-induced DNA damage are highly distinct responses at the transcriptional level.

The significantly regulated transcripts in the cells treated with the low dose of UV radiation had transient changes in their expression patterns, followed by a recovery by 24 h

(IV, Figure 3). The cells treated with the high dose underwent slower, but more persistent, changes in transcript levels (IV, Figure 3). These results correlate well with the kinetic differences detected in the cellular responses (I). Fast and transient induction of transcripts by the low dose of UVC radiation may result from fewer damaged sites undergoing repair, enabling faster recovery from transcriptional repression. The high dose of UVC radiation represses significantly more genes from more functional groups than the low dose, indicating a more general downregulation of transcription (IV, Figure 2). This is not surprising, as the high dose is sufficient to produce RNAPII-inhibiting DNA bulks to every gene of the genome (Perdiz *et al*. 2000). The data support the idea that UV radiation-induced DNA damage induces prominent inhibition of transcription, which is mostly attributable to inhibition of RNAPII (Tornaletti and Hanawalt 1999, Ravanat 2001). Over 90% of transcripts detected in the array were, however, not significantly regulated by UVC. In addition, if the damage is assumed to distribute randomly throughout the genome, all genes repressed by the low dose should also be repressed by the high dose. This was not the case, however, indicating that in at least transiently arrested cells also target gene selective repression takes place.

Several interesting genes, which may have an impact on the UV response, were found to be regulated in the large scale assay (IV, Tables 1,2). In addition to $p21^{Cip1}$, several cell cycle-associated genes were induced and none were repressed by the low dose of UV radiation. These factors, e.g. TOB1 and the p53 target BTG2, most likely contribute to the transient proliferation arrest response, ensuring time for damage repair. Several stress response factors were also upregulated, including the major heat shock proteins Hsp70 1A and 1B. These factors work as chaperones in stress involving protein misfolding, and they are known to respond to UVB (Park *et al*. 2000, Kwon *et al*. 2002). UVC does not induce significant amounts of protein damage (Tyrrell 1994), but chaperones may be needed to keep NER proteins properly folded, as found in *E. coli* (Zou *et al*. 1998). Subunit 4 (p52) of TFIIH is directly involved in transcription and NER (Marinoni *et al*. 1997), and was identified as a novel target induced by the low dose of UVC (IV). Other NER factors which were slightly induced specifically in cells undergoing transient arrest represented GGR proteins p48/DDB2 and XPC, known to be upregulated by UV radiation (Hwang *et al*. 1999, Adimoolam and Ford 2002). The transcript of member O of the histone H2A family was induced by the low dose of UV radiation, implying that it may have a role in NER and/or histone remodeling in the UV response.

Transcriptional response in the cells undergoing apoptosis did not include prominent upregulation of proapoptotic genes. Many apoptotic initiation and effector cascades do not require *de novo* protein synthesis, but function mainly through modulation of existing pools of protein, e.g. by translocation and proteolysis (Gupta 2001, Leist and Jäättelä 2001). Thus it can be argued that transcriptional events are not the main mode of regulation in UVC radiation-induced apoptosis. An exception was NOXA, a proapoptotic p53 target gene (Oda *et al*. 2000b), which was found to be the most highly induced gene by the high UV dose (IV, Table 2). Another p53 target upregulated in the apoptotic response was ATF3, significance of which for UV-induced apoptosis remains to be elucidated. Three histones were upregulated kinetically late in the apoptotic response, indicating that they may have specific roles in apoptosis, e.g. changing the composition of nucleosomes to modify DNA accessibility to DNA degrading enzymes. Transcription was affected by the downregulation of several basal and sequence-specific transcription factors in the apoptotic response. Interestingly, these include Id-family of transcriptional repressors (*i*nhibitors of *D*NA binding; Id1, 2, 3), which negatively regulate cell cycle inhibition by downregulating transcription of CKI $p16^{INK4a}$ (Id1; Ohtani *et al*. 2001) and overriding functions of Rb (Id2;

Iavarone *et al*. 1994, Lasorella *et al*. 1996). In addition, both subunits of the NF-Y (*n*uclear transcription *f*actor Y) transcription factor were downregulated. NF-Y upregulates transcription of several damage response and cell cycle-promoting genes, such as hsp70, E2F1, cyclin A and cyclin B1 (Mantovani 1998). Downregulation of the Id-family and NF-Y may block survival-promoting transcription, and prevent the cells from entering the cell cycle erroneously in the apoptotic response. Mitosis is hampered after the high dose UV insult through downregulation of mitotic cyclins A2 and B1. Interestingly, cyclin D (D1-3) is not downregulated in the apoptotic response. This is probably attributable to the efficient G1/S arrest already induced through Cdc25A- and p53-mediated events, and downregulation of cyclin D at protein level (I, Figure 2; Bartek and Lukas 2001a, Agami and Bernards 2002).

Fibroblasts participate in UV-induced inflammation in skin (Clydesdale *et al*. 2001). The major inflammatory factors induced by both low and high doses of UV damage were IL-11 (*i*nter*l*eukin *11*) and ISG15. IL-11 is a secreted cytokine able inhibit apoptosis and proliferation, stimulate tissue fibrosis, regulate B cell functions, and to inhibit macrophage cytokine production (Leng and Elias 1997). IL-11 can reduce apoptosis in UVB-irradiated mouse skin (Scordi *et al*. 1999). ISG15 is a secreted ubiquitin-like covalent modifier protein, which has immunomodulatory characteristics (D'Cunha *et al*. 1996). Consequences of ISG15 conjugation are speculated to alter biological activities of proteins but remain unclear. Other aspects of intercellular signaling differed between the arrested and the apoptotic fibroblasts. By inducing PLAB (*pla*cental *b*one morphogenetic protein), the arrested cells may promote wound healing and inflammation (Fairlie *et al*. 1999). They may also activate integrin-mediated migration, attachment, and angiogenesis via induction of Ephrin B (Huynh-Do *et al*. 2002), and loosen cell-cell interactions by downregulation of N-cadherin (Wang *et al*. 1998). Apoptotic cells mostly suppress intercellular growth factor signaling by downregulating transcripts of the TGF-β (*t*ransforming *g*rowth *f*actor *β*) and several cytokine pathways. In addition, other events most likely able to affect their local environment are downregulation of several angiogenic transcripts and induction of proteolytic genes, such as MMP-3 which contributes to breakdown of collagen matrix in photodamaged skin (Wlaschek *et al*. 2001).

Even though changes in transcript abundance by UV radiation-induced DNA damage are thought to occur mainly due to changes in transcription, alterations in mRNA stability, resulting from both direct UV radiation-induced molecular damage and specific regulatory events, may affect the results. Eventually however, the most important determinant for the cellular response is whether the changes detected in mRNA levels are translated into altered protein levels as well. This poses a challenge for future studies. Remarkably many of the genes upregulated by UV radiation coded for proteins with unknown functions, especially in the transient arrest response. It will be interesting to determine if some (or even most) of these genes have a role in the protective DNA damage checkpoint functions, and whether their transcription is p53-dependent.

# Concluding remarks

Deprivation of the ozone layer and changes in life styles increase the amount of UV radiation we are exposed to, which, undoubtedly, will further increase the skin cancer rate in the future. Tumor suppressor p53 is an essential fighter in the battle against skin cancer, as well as against almost every other type of human cancer. Despite 20 years of determined research on p53, the exact molecular mechanisms of its functions are not clear. This thesis work posed questions about the mechanisms of tumor suppressor p53 function and transcriptional regulation in the cellular responses to UV radiation.

p53 takes actively part in two types of cellular responses induced by UV radiation: transient replication arrest and apoptosis. The choice of response is mainly determined by the amount of DNA damage induced by UVB and UVC radiation, as are the transcriptional targets regulated by p53 to enforce the cellular outcome. The extent of inhibition by Mdm2 seems to determine between the different p53-mediated cellular responses. The results also indicate a role for redox regulation and acetylation of p53 in the decision whether a cell is to die due to a UV insult. In contrast, phosphorylation and role of PI-3-kinases in the regulation of p53 may have a lesser impact than expected in the dose-dependent cellular UV response.

Proteasome inhibition is thought to induce passive accumulation of p53 and Mdm2. The results presented here suggest that some parts of p53 activity might be inducible by proteasome inhibitors, potentiating the existing ideas of using proteasome inhibitors as cytotoxic drugs in cancer treatment. In addition, by using inhibitors of PI-3-kinases, these enzymes were found to mediate basal expression of Mdm2. The combination of the drugs revealed an earlier unidentified intracellular translocation event, significance of which for normal cell function, as well as apoptosis induced by these drugs, will be determined in future experiments.

Transcriptional changes in cells undergoing either UV radiation-induced cell cycle arrest or apoptosis are clearly different. These changes provide effectors for a UV-damaged cell to either survive from the insult or die, if necessary. Transcriptional changes also provide tools for a cell to communicate with its surroundings: to influence the damage response of neighbouring cells and to receive incoming signals in return. The results implicate that fibroblasts take part in the inflammatory and angiogenic responses to UV radiation, in concert with other cell types in skin. Several functionally uncharacterized genes were identified as UV-responsive in this study. Products of some of these genes are likely to participate in the cellular defense responses to UV radiation. Future work will elucidate whether some of these genes also regulate cell cycle arrest and apoptosis, and even have the potential to contribute to tumorigenesis.

During the last 20 years – the “life” of p53 – cancer research has come a long way. We know now that cancer occurs when series of things go wrong on (mostly) genetic level, and have identified what many of these events are. The sequencing of human genome has narrowed down the group of remaining suspects, and eventually all genes contributing to tumorigenesis will be known. What remains to be achieved, is to elucidate the exact molecular mechanisms of function of these factors, before their targeting can efficiently and safely be used in cancer therapeutics.

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