p53 and breast cancer, an update

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Abstract

p53 plays a key role in mediating cell response to various stresses, mainly by inducing or repressing a number of genes involved in cell cycle arrest, senescence, apoptosis, DNA repair, and angiogenesis. According to this important function, p53 activity is controlled in a very complex manner, including several auto-regulatory loops, through the intervention of dozens of modulator proteins (the 'p53 interactome'). p53 mutations are observed in a significant minority of breast tumours. In the remaining cases, alterations of interactome components or target genes could contribute, to some extent, to reduce the ability of p53 to efficiently manage stress events. While the prognostic and predictive value of p53 is still debated, there is an increasing interest for p53-based therapies. The present paper aims to provide updated information on p53 regulation and function, with specific interest on its role in breast cancer.

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Introduction

While p53 seems to be dispensable for normal development (Donehower *et al.* 1992), it plays an important role in regulating cell fate in response to various stresses, either genotoxic (DNA alterations induced by irradiation, UV, carcinogens, cytotoxic drugs) or not (hypoxia, nucleotide depletion, oncogene activation, microtubule disruption, loss of normal cell contacts). The protein may be viewed as a node for the stress signals, which are then transduced, mainly through the ability of p53 to act as a transcription factor. p53 exerts its anti-proliferative action by inducing reversible or irreversible (senescence) cell cycle arrest, or apoptosis. It may also enhance DNA repair and inhibit angiogenesis.

Many types of stresses may be encountered during tumour development. The p53 function is often altered in cancer. It has been suggested that p53 could have evolved in higher organisms specifically to prevent tumour development (see notably in Vousden & Lu 2002). It is believed that this specific action is exerted mainly through the triggering of apoptosis (see notably in Haupt *et al.* 2003, Yu & Zhang 2005). Indeed, loss of p53 activity disrupts apoptosis and accelerates the appearance of tumours in transgenic mice (Attardi & Jacks 1999).

The qualitative and quantitative activity of p53 depends on its integrity (mutation status), its amount, and its specific posttranslational modifications induced by the activation of the different stress-induced signalling pathways. This leads to variable patterns of association between p53 and a number of other co-regulatory proteins, of which some may be tissue- or cell type-specific. Despite this complexity, p53 activity has been associated with prognosis and prediction of tumour response to various therapies and deserves further investigations with the perspective of developing more targeted treatments.

Structure of p53

p53 is encoded by the *Tp53* gene. Located at 17p13, this contains 11 exons spanning 20 kb. It belongs to a family of highly conserved genes that also includes *TP63* and *TP73*, encoding p63 and p73 respectively.

Three functionally distinct regions have been identified in p53.

1. An acidic N-terminal region (codons 1–101), itself containing two major domains. (i) A transactivation acidic domain (codons 1–42). Codons 17–28 may interact with the ubiquitin ligase mouse double minute-2 homologue

(MDM2), which plays a major role in p53 degradation (see below). Codons 22–26 (LWKLL) constitute an LXXLL-type co-activator recognition motif (Savkur & Burris 2004) involved in histone acetyltransferase P300 binding. It is believed that codons 11–27 may function as a secondary nuclear export signal (NES) and that DNA damage-induced phosphorylation may inhibit this activity. (ii) A proline-rich domain (codons 63–97) required for interaction with various proteins involved in the induction of apoptosis. It contains five PXXP motifs (PRMP at 64–67; PVAP at 72–75; PAAP at 77–80; PAAP at 82–85; PSWP at 89–92) that are involved in p53 interaction with P300 (Dornan *et al.* 2003). Interestingly, a polymorphism has been demonstrated at codon 72, where the proline is frequently replaced by an arginine. Both forms are morphologically wild-type and do not differ in their ability to bind to DNA in a sequence-specific manner. However, there are a number of differences between these p53 variants in their abilities to bind components of the transcriptional machinery, to activate transcription, to induce apoptosis, and to repress the transformation of primary cells (Thomas *et al.* 1999).

2. A central DNA-binding core region (codons 102–292). It recognizes a promoter consensus motif made of two 10 bp segments (RRRCWWGYYY) separated by 0–13 bp. This region is highly conserved throughout evolution. It is also the most homologous region among p53 family members (P63, P73).

3. A basic C-terminal region (codons 293–393), involved in tetramerization and regulation of p53 activity. It notably contains: (i) three nuclear localization signals (codons 305–322, 369–375, 379–384) recognized by a hetero-dimeric complex composed of importin alpha and beta that allows the p53 nuclear import (Fabbro & Henderson 2003); (ii) a tetramerization domain (codons 323–356), itself containing a primary NES (codons 339–352) recognized by the export receptor CRM1/exportin (Fabbro & Henderson 2003). p53 is active as a transcription factor only in the homotetrameric form. Tetramerization of p53 masks the primary NES and prevents export from the nucleus; (iii) a negative regulatory region (codons 363–393). By binding short non-specific DNA sequences, this region may prevent specific DNA binding to the core region (Weinberg *et al.* 2004).

Genomic and non-genomic actions of p53

In normal cells not exposed to stress, the level and activity of p53 are very low. Upon stress, p53 is activated through a series of post-translational modifications and becomes able to bind to specific DNA sequences. The p53 recognition sequence is very loose and has been found in several hundred genes that are differentially modulated (induced or repressed) depending on the cell type, the nature of stress and the extent of damage. At low cellular levels, p53 modulates only a subset of the genes regulated at higher levels. The kinetics of target gene modulation may also vary.

In a study with a micro-array carrying 6000 capture sequences, 107 genes were found to be induced and 54 genes were repressed by p53 (Zhao *et al.* 2000). This result extrapolates to at least 500 up-regulated and 260 down-regulated p53 target genes.

Table 1, based on several papers (Yu *et al.* 1999, Vousden & Lu 2002, Liang & Pardee 2003, Nakamura 2004, Miled *et al.* 2005) lists a non-exhaustive series of p53-target genes that have been found to be altered by various stresses in many cell types.

Modulation of cell cycle-related genes by activated p53 may mediate arrest of cells at one of two major cell-cycle checkpoints, in G_1 near the border of S-phase (key role played by $P21^{WAF1/CIP1}$) or in G_2 before mitosis (important roles for GADD45 and 14-3-3σ). The transcriptional program responsible for p53-mediated apoptosis is much less clearly defined. However, the observation, for instance, that mice lacking the $P21^{WAF1/CIP1}$ gene (*CDKN1A*), unlike p53-null mice, do not develop tumours indicates that it is this apoptotic program that plays an essential role in p53 tumour suppression. p53 may modulate the expression of genes associated with either the extrinsic or the intrinsic apoptotic pathways. The extrinsic pathway (in which genes such as *TNFRSF10A*, *TNFRSF10B*, *FAS*, *PERP*, *LRDD* are implied) involves engagement of particular 'death' receptors. The intrinsic pathway is triggered in response to DNA damage and is associated with mitochondrial depolarization and release of cytochrome c from the mitochondrial inter-membrane space into the cytoplasm. Some genes associated with this pathway are *APAF1*, *BAK1*, *BAX*, *BCL2* (repressed), *FDXR*, *PMAIP1*,

Table 1 A non-exhaustive series of p53-target genes that have been found altered by various stresses in many cell types.

Function	Regulated genes
Cell cycle	14-3-3σ (*SFN*), ATF3 (*ATF3*), BTG2 (*BTG2*), CYCLIN A2 (*CCNA2*), CYCLIN B1 (*CCNB1*), CYCLIN G1 (*CCNG1*), DDA3 (*DDA3*), DSCP1 (*DSCP1*), GADD45 (*GADD45A*), P21$^{WAF1/CIP1}$ (*CDKN1A*), RAI3 (*GPCR5A*), REPRIMO (*RPRM*), TERT (*TERT*)
Apoptosis and survival	AMID/PRG3/AIF (*PRG3*), APAF-1 (*APAF1*), BAK1 (*BAK1*), BAX (*BAX*), BCL2 (*BCL2*), BCL2-like 14 (*BCL2L14*), BID (*BID*), BNIP3L/NIX (*BNIP3L*), BOK (*BOK*), DAPK1 (*DAPK1*), DR4 (*TNFRSF10A*), DR5/Killer (*TNFRSF10B*), FAS (*FAS*), FDXR (*FDXR*), IGFBP3 (*IGFBP3*), MAP4K4 (*MAP4K4*), MNSOD (*SOD2*), MYC (*MYC*), NDRG (*NDRG1*), NOXA (*PMAIP1*), P53AIP1 (*P53AIP1*), P53CSV (*HSPC132*), P53DINP1 (*TP53INP1*), P73 (*TP73*), PAC1 (*DUSP2*), PEG3/PW1 (*PEG3*), PERP (*PERP*), PIDD (*LRDD*), PIG3 (*TP53I3*), PIG6 (*PRODH*), PIG8 (*EI24*), PIG11 (*TP53I11*), PIR121 (*CYFIP2*), PTEN (*PTEN*), PUMA (*BBC3*), SURVIVIN (*BIRC5*), WIG-1/PAG608 (*WIG1*), WIP1 (*PPM1D*)
DNA repair	53BP2 (*TP53BP2*), DDB2 (*DDB2*), P53R2 (*RRM2B*), RECQ4 (*RECQL4*), XPC (*XPC*)
Angiogenesis	BAI1 (*BAI1*), ENDOSTATIN (*COL18A1*), KAI1/CD82 (*KAI1*), MASPIN (*SERPINB5*), MMP2 (*MMP2*), TSP1 (*THBS1*), VEGF (*VEGF*)
Others	BRCA1 (*BRCA1*), CHK1 (*CHEK1*), CHK2 (*CHEK2*), COP1 (*COP1*), CSPG2 (*CSPG2*), MDM2 (*MDM2*), PCAF (*PCAF*), PIRH2/ZNF363 (*RCHY1*), PML (*PML*)

and *BBC3*. Both pathways lead to a cascade of activation of caspases, ultimately causing apoptosis. p53 could promote the convergence of the extrinsic and intrinsic pathways through *BID* regulation.

Besides the regulation of apoptosis-related genes, p53 also appears to be able to act directly at the mitochondria. It can interact with BCL2 family members, such as the anti-apoptotic BCL2 itself and BCL-XL, and the pro-apoptotic BAK, thereby triggering mitochondrial outer membrane permeabilization and apoptosis (Schuler & Green 2005).

The quantitative, or even qualitative contribution of the direct, transcription-independent action to the global apoptotic activity of p53 has been debated. Observations such as the radio-resistant phenotype of the PUMA (*BBC3*)- and NOXA (*PMAIP1*)-knockout mice have been used as arguments against the general importance of transcription-independent mechanisms *in vivo* (Yu & Zhang 2005). It has also been observed that, in various cell lines, DNA damage induced by either ionizing radiation (IR) or topoisomerase inhibitors triggered a robust translocation of a fraction of p53 to mitochondria to a similar extent. Nevertheless, the cells succumbed to apoptosis only in response to topoisomerase inhibitors, but remained resistant to apoptosis induced by IR, suggesting that mitochondrial translocation of p53 does not *per se* lead to cell death (Essmann *et al.* 2005). Other investigators, by examining 179 mutant p53s, found no significant correlation between their apoptotic property and their ability to activate transcription of six

p53-responsive genes (*CDKN1*, *MDM2*, *SFN*, and the apoptosis-related *BAX*, *p53AIP1*, *BBC3*) (Kakudo *et al.* 2005). It is possible that rapid transactivation-independent events could modulate the extent of apoptosis, which would however depend on transactivation-dependent events. However, recent observations suggest that the inverse could be true. Indeed, it has been shown that after genotoxic stress, the major regulator of apoptosis, BCL-XL, sequestered cytoplasmic p53. Nuclear p53 caused expression of PUMA, which then displaced p53 from BCL-XL, allowing p53 to induce mitochondrial permeabilization. Mutant BCL-XL that bound p53, but not PUMA, rendered cells resistant to p53-induced apoptosis irrespective of PUMA expression. These observations thus identify PUMA as the protein coupling the nuclear and cytoplasmic pro-apoptotic functions of p53 (Chipuk *et al.* 2005).

The central core region of p53 is of key importance in regulating apoptotic function, either transcription-dependent or -independent, as supported by the number of mutations affecting this region in apoptosis-deficient p53 cells. In addition to inducing genes that drive apoptosis, p53 can also activate the expression of genes that inhibit survival signalling (such as *PTEN*) or inhibit inhibitors of apoptosis (such as *BIRC5*) (Vousden & Lu 2002, Haupt *et al.* 2003, Meek 2004, Nakamura 2004, Lu 2005, Yu & Zhang 2005). Besides the central core, the proline-rich domain has been specifically associated with the apoptotic activity of p53 (Walker & Levine 1996). Deletion of this region

Table 2 An overview of the p53 biochemical modifications that have been described to date.

Target residue or doublet	Type of modification	Modifying protein
Ser6	Phosphorylation	CHK1
Ser9	Phosphorylation	CHK1, CK1 (if Ser6 is phosphorylated)
Ser15	Phosphorylation	ATM, ATR, DNA-PK, CHK1, P38, RSK2
Thr18	Phosphorylation	CHK1, CK1 (if Ser15 is phosphorylated)
Ser20	Phosphorylation	CHK1, CHK2
Ser33	Phosphorylation	P38, CAK, JNK
Ser33-Pro34	*Cis-trans* isomerisation	PIN1
Ser37	Phosphorylation	ATR, DNA-PK
Ser46	Phosphorylation	P38, HIPK2
Ser46-Pro47	*Cis-trans* isomerisation	PIN1
Thr55	Phosphorylation	TAF$_{II}$250, ERK2
Thr81	Phosphorylation	JNK
Thr81-Pro82	*Cis-trans* isomerisation	PIN1
Ser127-Pro128	*Cis-trans* isomerisation (potential)	PIN1
Thr150-Pro151	*Cis-trans* isomerisation (potential)	PIN1
Thr155	Phosphorylation	CK2, CSN-associated kinases
Ser215	Phosphorylation	STK15
Lys305	Acetylation	P300
Ser313	Phosphorylation	CHK1, CHK2
Ser314	Phosphorylation	CHK1, CHK2
Ser315	Phosphorylation	STK15, CDKs, CDC2
Ser315-Pro316	*Cis-trans* isomerisation (potential)	PIN1
Lys320	Acetylation	PCAF
Ser366	Phosphorylation	CHK2
Lys370	Ubiquitination	MDM2 + other ubiquitin ligases?
	Acetylation	P300/CBP
	Neddylation	MDM2
Ser371	Phosphorylation	PKC
Lys372	Methylation	SET9
	Ubiquitination	MDM2 + other ubiquitin ligases?
	Acetylation	P300/CBP
	Neddylation	MDM2
Lys373	Ubiquitination	MDM2 + other ubiquitin ligases?
	Acetylation	P300/CBP
	Neddylation	MDM2
Ser376	Phosphorylation	PKC, CAK
Thr377	Phosphorylation	CHK1, CHK2
Ser378	Phosphorylation	PKC, CAK, CHK1, CHK2
Lys381	Ubiquitination	MDM2 + other ubiquitin ligases?
	Acetylation	P300/CBP
Lys382	Ubiquitination	MDM2 + other ubiquitin ligases?
	Acetylation	P300/CBP
Lys386	Ubiquitination	MDM2 + other ubiquitin ligases?
	Sumoylation	PIAS proteins
Thr387	Phosphorylation	CHK1
Ser392	Phosphorylation	PKR, FACT (complexed to CK2), P38

ATM, ataxia-telangiectasia mutated (gene *ATM*); ATR, ataxia-telangiectasia and Rad3-related (*ATR*); CAK, CDK activating kinase; CBP, CREB-binding protein (*CREBBP*); CDC2, cell division cycle 2 kinase (*CDC2*); CDKs, cyclin-dependent kinases (multiple members); CHK1, cell cycle checkpoint kinase 1 (*CHEK1*); CHK2, cell cycle checkpoint kinase 2 (*CHEK2*); CK1, casein kinase 1 (multiple isoforms); CK2, casein kinase 2 (multiple isoforms); CSN, COP9 signalosome (protein complex); DNA-PK, DNA-dependent protein kinase (*PRKDC*); ERK2, p42 mitogen activated protein kinase (*MAPK1*); FACT, facilitating chromatin-mediated transcription; HIPK2, homeodomain-interacting protein kinase 2 (*HIPK2*); JNK, Jun N-terminal kinase (*MAPK8*); MDM2, mouse double minute 2 homologue; P38, p38 mitogen activated protein kinase (*MAPK14*); P300, E1A-binding protein, 300-kDa (*EP300*); PCAF, P300/CBP-associated factor (*PCAF*); PKC, protein kinase C (multiple isoforms); PKR, double stranded RNA-dependent protein kinase (*PRKR*); PIAS, protein inhibitor of activated STAT (multiple isoforms); PIN1, peptidyl-prolyl-cis-trans isomerase 1 (*PIN1*); RSK2, ribosomal S6 kinase 2 (*RPS6KA3*); SET9, SET-domain containing protein 9 (*SET7*); STK15, serine/threonine protein kinase 15 (*STK15*); TAF$_{II}$250, TATA-binding protein associated factor 250-kD (*TAF1*).

leads to a complete loss of the apoptotic activity of p53. It could constitute an auxiliary protein-binding site and could be necessary for cellular cofactors specifically involved in the apoptotic activity of p53.

The p53-regulated genes that bring about senescence are less well characterized. However, *CSPG2* has been strongly associated with senescence in prostate cancer cells (Schwarze *et al*. 2005).

Biochemical modifications of p53

Posttranslational modification is a major mechanism regulating protein function. p53 may be phosphorylated, cis/trans isomerized, acetylated, ubiquitinated, methylated, sumoylated, neddylated, glycosylated at multiple sites, reflecting its biological importance. This multisite modification, which exhibits a cell and tissue specificity and depends on the position in the cell cycle, is a complex regulatory program that fluctuates in response to cellular signalling triggered by DNA damage, proliferation and senescence, and thus appears as a dynamic 'molecular barcodes' (Yang 2005).

An overview of the p53 modifications that have been described to date is provided in Table 2. It is based on papers used for Table 1 and additional reports (Appella & Anderson 2001, Meek 2002, Bode & Dong 2004, Ou *et al*. 2005).

Since it is impossible to give a detailed description of all p53 modifications, only the most widely observed and well-known alterations will be discussed briefly here.

Phosphorylation

p53 phosphorylation has been widely investigated. In most cases, it is associated with protein stabilization.

Three N-terminal sites, Ser15, Thr18, and Ser20, are particularly interesting because when phosphorylated, the interaction between p53 and its major negative regulator, MDM2, is diminished, while the binding of the acetyltransferase P300 is promoted, thereby increasing the level and stability of p53. Notably, Ser15 may be phosphorylated by IR (*via* ataxia-telangiectasia mutated; ATM) or UV (*via* ataxia-telangiectasia and Rad3-related; ATR). These stresses also lead to Ser20 phosphorylation, through the action of cell cycle checkpoint kinase 2 (CHK2) and CHK1 respectively. In fact, besides IR and UV, almost all stresses have been shown to induce Ser15 phosphorylation, which is

thought to nucleate a series of subsequent p53 post-translational modifications (Meek 2004).

In some cases, p53 phosphorylation events are sequential. For instance, phosphorylation of Ser9 and Thr18 by CK1 is dependent of Ser6 and Ser15 phosphorylation respectively.

Another crucial N-terminal residue is Ser46. Its phosphorylation selectively promotes a p53 apoptotic response. Various kinases may be involved in this event, reflecting the activation of different stress pathways. For instance, HIPK2 mediates Ser46 phosphorylation in response to UV irradiation, although it seems that this alone is not sufficient to induce apoptosis. It has also been proposed that P38 can mediate the phosphorylation of Ser46 in response to UV. Neither P38 nor HIPK2 are involved in the Ser46 phosphorylation in response to IR, which requires both ATM and the p53-inducible gene, *Tp53INP1*, coding for p53DINP1. ATM does not directly phosphorylate p53, but it is likely to induce a kinase that might be co-activated by p53DINP1 to facilitate Ser46 phosphorylation (apoptosis-selective auto-regulatory loop) (Vousden & Lu 2002).

One important apoptosis-related protein, p53AIP1, is induced only when Ser46 is phosphorylated. Studies with the drug, etoposide, have confirmed that phosphorylation of p53 at Ser46 determines promoter selection and whether apoptosis is attenuated or amplified. High dose chemotherapy induced the phosphorylation of p53 on Ser46, whereas low dose chemotherapy did not. While Ser46-phosphorylated p53 targeted the promoter of the tumour suppressor *PTEN* in preference to *MDM2* (thus abrogating the auto-regulatory loop that contributes to keeping the p53 level low), the inverse was observed in the absence of Ser46 phosphorylation. Accordingly, only high dose chemotherapy led to *p53AIP1* induction, caspase 3 activation, and cell death (Mayo *et al*. 2005).

In addition to a common polymorphism at codon 72 (see below), p53 tumour also exhibits a rare single nucleotide polymorphism at residue 47. Wild-type p53 encodes proline at this residue, but in <5% of African Americans, this amino acid is serine. Notably, phosphorylation of the adjacent Ser46 by the proline-directed kinase P38 is known to greatly enhance the ability of p53 to induce apoptosis. The Ser47 polymorphic variant, which replaces the proline residue necessary for recognition by proline-directed kinases, is a markedly poorer substrate for phosphorylation on Ser46 by P38. Consistent with this finding, the Ser47 variant has an up to five-fold

decreased ability to induce apoptosis compared with wild-type p53. This variant has a decreased ability to transactivate two p53 apoptotic target genes, *p53AIP1* and *BBC3*, but not other p53 response genes; thus, the codon 47 polymorphism of p53 is functionally significant and may play a role in cancer risk, progression, and the efficacy of therapy (Li *et al.* 2005).

Experiments using p53 mutants with substitutions at Ser33, Ser46 or Thr81 have shown that phosphorylation of these sites (by P38 or Jun N-terminal kinase (JNK)) may independently lead to p53 stabilization, notably after exposure to UV (Appella & Anderson 2001).

In contrast to Ser315, Ser392 is phosphorylated only poorly after exposure of cells to IR, while it is strongly modified in response to UV (Appella & Anderson 2001).

In the C-terminal region of p53, phosphorylation of Ser315, Ser371, Ser376, Ser378, and Ser392 is well known. More recently, it has been shown that additional sites were also phosphorylated: Ser313, Ser314, Thr377, Ser378 (by both CHK1 and CHK2), Ser366 (by CHK2 only) and Thr387 (by CHK1 only). These events may alter the pattern of acetylation at Lys373 and Lys382, but not at Lys320, thus distinguishing between P300/CREB-binding protein (CBP) and P300/CBP-associated factor (PCAF) activity (see below) (Ou *et al.* 2005).

While most p53 phosphorylation events result in an increase in stability/activity of the protein, the phosphorylation of some sites (Thr55, Thr155, Ser215, Ser376) has been associated with enhanced p53 degradation. For instance, Thr55 can be phosphorylated by TAF$_{II}$250, the largest subunit of the general transcription factor TFIID, and this event enhances p53 degradation. Exposure of cells to UV decreases phosphorylation at Thr55 (Appella & Anderson 2001).

The COP9 signalosome (CSN) is an eight-subunit heteromeric complex that has homologies with the 26S proteasome bid complex. CSN has been reported to modulate ubiquitin ligase activity, as it directly interacts with cullin-domain ubiquitin ligases, catalyses deneddylation of these ligases, and is required for their proper function. Interestingly, CUL4A, a CSN-associated cullin-domain ubiquitin ligase has been shown to induce p53 degradation (see below). The CSN-associated kinases, CK2 and protein kinase D, are able to phosphorylate p53, and CK2 does so on Thr155. This dedicates p53 to rapid degradation by the ubiquitin–proteasome system. The importance of

Thr155 is underlined by the fact that mutation of this residue is sufficient to stabilize p53 against human papilloma virus E6 oncoprotein-dependent degradation, which is mediated by E6AP, a ubiquitin ligase different from CUL4A. E6 is believed to play a major role in carcinoma of the cervix, where p53 mutations are rare.

Phosphorylation of Ser215 by the mitotic kinase serine/threonine protein kinase 15 (STK15) (also known as Aurora A) abrogates p53 DNA binding and transactivation activity (Liu *et al.* 2004*b*).

Ser376 (and Ser378) are constitutively phosphorylated by protein kinase C (PKC), which can contribute to p53 degradation (Chernov *et al.* 2001).

Not only the qualitative and quantitative pattern, but also the timing of p53 phosphorylation may vary depending on the stress. For instance, in response to IR increased phosphorylation of Ser6, Ser9, and Ser15 has been observed as early as 30 min after treatment, while exposure to UV induced a less-rapid, but more long-lived increase in the phosphorylation of these sites. This reflects the fact that ATR is more slowly activated than ATM (Appella & Anderson 2001).

Dephosphorylation

In vitro dephosphorylation of p53 by the phosphatases PP1, PP2A, PP5, PPM1D and CDC14 has been shown. These may have different specificities, as shown, for instance, by the fact that PP1, but not PP2A, can dephosphorylate phospho-Ser15 (Haneda *et al.* 2004). PPM1D is of high interest, as it is induced by p53 and may dephosphorylate both p53 (at Ser15) and CHK1 (which may phosphorylate p53 at various sites) (Lu *et al.* 2006). Amplification of the *PPM1D* gene has been observed in breast cancer and seems to be associated with high aggressiveness (Rauta *et al.* 2006). Dephosphorylation of Ser376 by an ATM-regulated phosphatase allows 14-3-3σ binding to phosphorylated Ser378, thereby contributing to p53 stabilization with consequent effects on site-specific DNA binding.

Cis/trans isomerization

p53 activation involves a conformational change, brought about by *cis/trans* isomerization of certain proline residues by peptidyl-prolyl-cis-trans isomerase 1 (PIN1). PIN1 binds protein sites consisting of a phosphorylated serine or threonine followed by a proline; it then catalyses the isomerization of proline residues, which changes the conformation

of p53. There are four Ser–Pro (Ser33–Pro34, Ser46–Pro47, Ser127–Pro128, Ser315–Pro316) and two Thr–Pro (Thr81–Pro82 and Thr150–Pro151) motifs on human p53 protein. Single mutations on these Ser–Pro or Thr–Pro sites do not lead to marked reduction of the p53-PIN1 interaction. However, a double point mutant (Ser33Ala, Ser315Ala) shows less binding to PIN1, and the triple point mutant (Ser33Ala, Ser315Ala, Thr81Ala) exhibits further reduced binding activity for PIN1, suggesting that these three sites are important for the p53-PIN1 interaction. It is possible that the Ser46–Pro47 site could also be involved in the process of *cis/trans* isomerization, considering the importance of Ser46 phosphorylation in p53 function. Whether the Ser127–Pro128 and Thr150–Pro151 motifs may be effectively targeted by PIN1 remains unknown at this time. The precise conformational changes induced by p53 due to different stress responses at different Ser–Pro or Thr–Pro sites are not yet clear. PIN1-induced conformational change in p53 inhibits the binding and/or stimulates the detachment of MDM2, leading to p53 stabilization. In addition, the conformational change may enhance the ability of P300 to acetylate p53 C-terminal lysines, and it may promote the binding of the p53 core domain to its specific promoter cognate sites, particularly those promoting apoptosis (Kohn & Pommier 2005).

Pro82 is essential for p53 interaction with CHK2 and consequent phosphorylation of Ser20 in response to DNA damage. These physical and functional interactions are regulated by PIN1. A sequence of events may thus be identified, in which phosphorylation of Thr81 allows PIN1 to isomerize p53, which further leads to p53-CHK2 interaction and phosphorylation of Ser20 (Berger *et al.* 2005).

Acetylation

Acetylation has been shown to augment p53 DNA binding and to stimulate p53-mediated transactivation of target genes through the recruitment of co-activators. Acetylation is also thought to contribute to p53 stabilization by impairing ubiquitination of the acetylated residues. Intriguingly, while all evidence so far indicates that acetylation positively regulates p53 function (Brooks & Gu 2003), this modification seems also to regulate p53 subcellular localization, at least in part by activating its nuclear export (Kawaguchi *et al.* 2006).

P300, CBP and PCAF are ubiquitous transcriptional co-activators. They act as histone acetyltransferases, but may also acetylate various transcription factors, including p53. According to current data, P300/CBP may compete with MDM2 for binding to N-terminus of p53, so that a decrease in MDM2-p53 interaction associated with phosphorylation of N-terminal (especially Ser15) sites may favour P300/CBP binding and acetylation of Lys373 and Lys382. On the other hand, Ser15 phosphorylation is not absolutely required for p53 acetylation, as shown, for instance, by actinomycin D, which does not induce Ser15 phosphorylation but is a powerful agent in triggering p53 acetylation (Appella & Anderson 2001). Other p53 residues acetylated by P300/CBP are Lys370, Lys372 and Lys381. PCAF may acetylate Lys320.

It has been shown that upon non-apoptotic DNA damage such as that induced by cytostatic doses of cisplatin, PCAF acting in cooperation with homeodomain-interacting protein kinase 2 (HIPK2) may acetylate p53. This HIPK2 action is independent of the Ser46 phosphorylation performed by the kinase upon severe genotoxic damage. Co-action of PCAF and HIPK2 selectively induce p53 transcriptional activity towards the *CDKN1A* promoter while depletion of either HIPK2 or PCAF abolishes this function. So, PCAF participates in the complex mechanisms allowing p53 to make a choice between growth arrest and apoptosis (Di Stefano *et al.* 2005). Interestingly, *PCAF* is a p53-induced gene (growth arrest-selective auto-regulatory loop) (Watts *et al.* 2004), while it is targeted for degradation (ubiquitinated) by MDM2 (Jin *et al.* 2004).

Experiments with histone deacetylase inhibitors on prostate cancer cells suggest that the acetylation of p53 at Lys373 is required for the p53-mediated induction of cell cycle arrest and apoptosis, while acetylation of p53 at Lys382 induces only cell cycle arrest (Roy *et al.* 2005).

The activation of p53 by P300/CBP can be achieved in a cooperative manner through the p53-binding proteins PRMT1 and CARM1 (co-activator-associated arginine methyltransferases). Whether p53 is a direct substrate for these two proteins is presently unknown.

Deacetylation

It is likely that deacetylation provides a quick acting mechanism to stop p53 function once transcriptional activation of target genes is no longer needed. Deacetylation of p53 may be performed by multiple histone deacetylases (HDACs), at least by HDAC 1-3. The deacetylase sirtuin 1 (SIRT1) shows an *in vitro* activity on p53 peptides and it

seems that cellular p53 is a major *in vivo* substrate of SIRT1 but not of the other six known SIRT proteins (SIRT 2-7) (Michishita *et al.* 2005). In fact, both HDAC1 and SIRT1 could be critical for p53-dependent stress response (Gu *et al.* 2004).

MTA2 (metastasis-associated protein 2)/PID (p53 target protein in the deacetylase complexes) specifically interacts with p53 both *in vitro* and *in vivo*, and its expression reduces significantly the steady-state levels of acetylated p53 by recruiting the HDAC1 complex. MTA2/PID expression strongly represses p53-dependent transcriptional activation, and, notably, it modulates p53-mediated cell growth arrest and apoptosis (Luo *et al.* 2000).

Numerous proteins modulating p53 activity have been shown to interfere with acetylation/deacetylation processes (not shown here).

Ubiquitination

In normal cells, degradation is the only mechanism that abrogates all functions of p53, and this appears to be accomplished, in part, by the ubiquitin-26S proteasome system (the other way is ubiquitin-independent). The highly conserved protein, ubiquitin, targets substrate proteins for degradation by the 26S proteasome to peptides. Ubiquitin ligases realise the last step of ubiquitination. These enzymes exhibit a high level of target specificity.

In normal cells, the RING domain MDM2 is considered as the main ubiquitin ligase regulating the amount of p53. MDM2 binds to the N-terminal region and represses p53 activity via two mechanisms: by promoting p53 export to the cytoplasm and its consequent degradation and by blocking p53 transcriptional activation. The export of p53 requires an intact p53 NES. Several lysine residues located at the C-terminus of p53 may be MDM2-ubiquitinated: Lys370, Lys372, Lys373, Lys381, Lys382, Lys386 (Rodriguez *et al.* 2000). The ubiquitination of these lysine residues in the p53 C-terminus, including Lys305, is required to expose the NES even when p53 is bundled as a tetramer.

MDM2 is up-regulated by activated p53 and this generates a p53-MDM2 auto regulatory loop.

According to a current view, DNA damage leads to destabilization and accelerated degradation of MDM2. This limits MDM2 binding to p53 during the stress response and enables p53 to accumulate and remain active, even as p53 transcriptionally activates more MDM2. Thus, the induction of MDM2 RNA by activated p53 may create a reserve of MDM2 that can inactivate p53 once the DNA damage stimulus has abated and MDM2 is re-stabilized.

The physiological relevance of the p53-MDM2 loop is supported by various observations: (1) MDM2-knockout mice have an embryonic lethal phenotype (which can be abolished by the simultaneous inactivation of p53; (2) disruption of the p53-MDM2 interaction with synthetic competitive inhibitors is sufficient to induce a p53 response in cultured cells; (3) blocking MDM2 degradation via proteasome inhibition prevents p53 transactivation in DNA-damaged cells; (4) the activity of MDM2 is controlled by numerous factors and the p53-MDM2 loop is the focal point of the many different stresses that activate the p53 pathway (see below)

As many tumours inactivate wild-type p53 through MDM2 over-expression, exploiting the pathways that trigger MDM2 auto-degradation may be an important new strategy for chemotherapeutic intervention (Stommel & Wahl 2005).

COP1 (constitutive photomorphogenesis protein 1) is a RING domain ubiquitin ligase that inhibits p53-dependent transcription. Depletion of COP1 by short interfering RNA (siRNA) stabilizes p53 and arrests cells in the G_1 phase of the cell cycle. Over-expression of COP1 correlates with a striking decrease in steady state p53 protein levels and attenuation of the downstream target gene, *CDKN1A*, in cancers that retain a wild-type p53 gene status. Moreover, like *MDM2*, *COP1* is a p53-inducible gene (Dornan *et al.* 2004).

The cytosolic chaperone-associated U-box domain ubiquitin ligase CHIP (C-terminus of hsc70-interacting protein) may induce the proteasomal degradation of p53. CHIP is thought to act in the quality control of protein folding, specifically ubiquitinating unfolded proteins associated with the molecular chaperones. CHIP-induced degradation has been observed for mutant p53, which was previously shown to associate with the chaperones Hsc70 and Hsp90, and for the wild-type form of the protein. Thus, mutant and wild-type p53 transiently associate with molecular chaperones and can be diverted onto a degradation pathway through this association (Esser *et al.* 2005).

The cullin-domain ubiquitin ligase CUL4A (cullin 4a) associates with MDM2 and p53, and ubiquitinates p53. Depletion of CUL4A leads to an accumulation of p53. CUL4A fails to increase the decay of p53 in mouse embryonic fibroblasts lacking MDM2. In addition, the CUL4A-mediated rapid decay of p53 is blocked by the MDM2 negative regulator p19[ARF] (ARF for alternate reading frame).

The results provide evidence for a cooperative role of CUL4A in the MDM2-mediated proteolysis of p53 (Nag *et al*. 2004).

The E6 oncoprotein of human papilloma viruses (HPVs) that are associated with cervical cancer utilizes the HECT domain ubiquitin ligase E6AP (E6-associated protein) to target p53 for degradation. In normal cells (i.e. in the absence of E6), p53 degradation is mediated by MDM2 rather than by E6AP. In HPV-positive cancer cells, the E6-dependent pathway of p53 degradation is not only active but, moreover, is required for degradation of p53, whereas the MDM2-dependent pathway is inactive. As the p53 pathway was reported to be functional in HPV-positive cancer cells, this finding indicates clearly that the ability of the E6 oncoprotein to target p53 for degradation is required for the growth of HPV-positive cancer cells (Hengstermann *et al*. 2001).

Nuclear localization of p53 is essential for its tumour suppressor function. In contrast to most other ligases that act, or are believed to act in the nucleus, PARC (p53-associated parkin-like cytoplasmic protein), a RING domain ubiquitin ligase, directly interacts with p53 in the cytoplasm of unstressed cells. In the absence of stress, inactivation of PARC induces nuclear localization of endogenous p53 and activates p53-dependent apoptosis. Over-expression of PARC promotes cytoplasmic sequestration of ectopic p53. This suggests that PARC is a critical regulator in controlling p53 subcellular localization and subsequent function (Nikolaev *et al*. 2003).

PIRH2 (p53-induced protein, RING-H2 domain-containing) is a RING domain ubiquitin ligase that promotes p53 ubiquitination independently of MDM2. Expression of PIRH2 decreases the level of p53 protein and abrogation of endogenous PIRH2 expression increases the level of p53. Furthermore, PIRH2 represses p53 functions including p53-dependent trans-activation and growth inhibition. PIRH2, like MDM2 and COP1, participates in an auto-regulatory feedback loop that controls p53 function (Leng *et al*. 2003).

Using an osteosarcoma cell line, it was shown that TOPORS (topoisomerase I-binding arginine-serine-rich protein) could act on p53 as a RING finger-containing ubiquitin ligase. Over-expression of TOPORS was shown to result in a decrease in p53 protein expression (Rajendra *et al*. 2004). However, the exact role of TOPORS remains unclear, as it has also been shown to sumoylate p53, thereby abrogating its transcription activity.

TOPORS was shown to associate with and stabilize p53, and to enhance the p53-dependent transcriptional activities of *CDKN1A*, *MDM2* and *BAX* promoters. Over-expression of TOPORS consequently resulted in the suppression of cell growth by cell cycle arrest and/or by the induction of apoptosis (Lin *et al*. 2005).

Although P300 is known as an acetyltransferase, it has been suggested that it could cooperate with MDM2 to induce p53 polyubiquitination. In the presence of MDM2, P300 could poly-ubiquitinate the p53 residues mono-ubiquitinated by MDM2, thus contributing to p53 degradation; in the absence of MDM2, P300 might only act as a p53 acetyltransferase and therefore stimulates the transcriptional activity of p53 (Kohn & Pommier 2005).

Apparently, multiple degradation pathways are employed to ensure proper destruction of p53. How can one explain the apparent redundancy of ubiquitin ligases? A possibility is that ubiquitin ligases are expressed or act optimally in different cell or tissue types. It is also possible that one or more of these ubiquitin ligases are involved in the maintenance of p53 levels in the non-stressed or basal state, while others act only after a stress-induced p53 is produced. It appears likely that each of these ubiquitin ligases form protein complexes in the cell and the associated proteins may well differ for each of these ligases, connecting them to different regulatory circuits.

Deubiquitination

USP7 (ubiquitin-specific protease 7, also known as HAUSP) has been shown to interact with p53, which can lead to p53 deubiquitination and stabilization. Its activity and global effect on p53 activity is, however, complex (see below).

Ubiquitin-independent p53 degradation

The proteasomal degradation of p53 is regulated by both (poly) ubiquitination, targeting p53 for degradation by the 26S proteasome and by a MDM2- and ubiquitin-independent process. This appears to be mediated by the core 20S catalytic chamber of the 26S proteasome and is regulated by NAD(P)H quinone oxidoreductase 1 (NQO1). NQO1 physically interacts with p53 in an NADH-dependent manner and protects it from 20S proteasomal degradation. Remarkably, the vast majority of NQO1 in cells is found in physical association with the 20S proteasomes, suggesting that NQO1

functions as a gatekeeper for these 20S proteasomes. By competing with NADH, NQO1 inhibitors including dicoumarol and various other coumarins and flavones induce ubiquitin-independent proteasomal p53 degradation and thus inhibit p53-induced apoptosis.

The NQO1 pathway plays a role in p53 accumulation in response to IR, as co-expression of NQO1-specific siRNA with p53 prevented the accumulation of the latter following IR. Escaping MDM2-mediated degradation is probably not sufficient for efficient p53 stabilization following IR, because p53 is still susceptible to 20S proteasomal degradation. In order to achieve efficient p53 accumulation following irradiation, NQO1-p53 interaction could be increased to eliminate p53 degradation by the 20S proteasomes. NQO1 might notably play a role in p53 accumulation under oxidative stress. Reactive oxygen species (ROS) are known to induce NQO1, which, in turn, reduces ROS. The ability of NQO1 to support p53 accumulation following oxidative stress may contribute to cellular defence mechanisms against ROS.

The core 20S proteasomes are abundant and ubiquitously present in the cells. They have been widely regarded as being incapable of degrading folded proteins and are therefore considered to be latent proteasomes. Degradation studies with natively unfolded proteins suggest that unstructured proteins might have an intrinsic capacity to enter the pore of the 20S proteasome. Furthermore, the unstructured protein even when flanked with well-structured regions is still susceptible to 20S proteasomal degradation. Therefore, a common feature of ubiquitin-independent and 20S proteasomal degraded proteins could be the presence of an unstructured protein region. Indeed, both the N- and the C-terminal regions of p53 have been identified as unstructured regions and could facilitate p53 degradation by the 20S proteasomes. p53 could be inherently unstable and degraded 'by default' by the 20S proteasome, unless stabilized by a molecule like NQO1. p53, when engaged in a large functional complex could be protected from 20S proteasomal degradation as a consequence of the masking of its unstructured regions. (For a review on NQO1 in p53 degradation, see Asher & Shaul 2005.)

The tumour suppressor p19[ARF], which inhibits the ability of MDM2 to target p53 for degradation (see below), also inhibits dicoumarol-induced p53 degradation. Therefore, p19[ARF] exhibits a double lock activity that inhibits p53 degradation by both the MDM2-dependent and the NQO1-regulated pathway, ensuring maximal p53 accumulation under certain physiological conditions.

Sumoylation

The p53 residue Lys386 may be sumoylated. SUMO (small ubiquitin-related modifier) is a ubiquitin-related protein that covalently binds to other proteins using a mechanism analogous to, but distinct from, ubiquitin. Protein inhibitor of activated STAT (PIAS)-1, PIASxα, PIASxβ, PIASy function as SUMO ligases for p53. In contrast to ubiquitination, sumoylation is not involved in protein degradation. Sumoylation affects target protein function by altering sub-cellular localization of the protein or by antagonizing other modifications (for example ubiquitination at the same acceptor site). Sumoylation most frequently correlates with decreased transcriptional activity and thus repression of target genes. PIAS proteins exert a strong repressive effect on p53-dependent trans-activation (Schmidt & Muller 2002). It is thought that the physical association of MDM2 with p53 is important for the enhancement of SUMO conjugation to p53. However, mutant p53 that does not associate with MDM2 is still sumoylated, albeit at a reduced level.

Methylation

The p53 residue Lys372 may be methylated by the SET9 (SET domain-containing protein 9) methyltransferase. Methylated p53 is restricted to the nucleus and the modification positively affects its stability. SET9 regulates the expression of p53 target genes in a manner dependent on the p53-methylation site (Chuikov *et al.* 2004).

Neddylation

Unexpectedly, MDM2 was recently assigned a new role as neddylation ligase for p53. NEDD8 (neuronal precursor cell-expressed developmentally down-regulated protein 8) is a small ubiquitin-like protein. MDM2-dependent NEDD8 modification of p53 was shown to inhibit its transcriptional activity (Xirodimas *et al.* 2004).

ADP-ribosylation

Poly(ADP-ribosyl)ation is a reversible post-translational protein modification implicated in the regulation of a number of biological functions. It is

catalysed mainly by the enzyme poly(ADP-ribose) polymerase 1 (PARP-1). PARP-1 is rapidly activated by DNA strand breaks, which finally leads to the modulation of multiple protein activities in DNA replication, DNA repair and checkpoint control. PARP-1 may be involved in homologous recombination, and poly(ADP-ribosyl)ation of p53 represents one possible mechanism that activates p53 as a recombination surveillance factor (Wesierska-Gadek *et al*. 1996).

O-glycosylation

Addition of bulky residues such as sugar groups could disrupt p53 intramolecular interactions involving the basic region, thus activating DNA binding by p53 (Shaw *et al*. 1996).

Modulators of p53 activity

Besides those 'biochemical modificators' of p53 mentioned above, a considerable number of other proteins have been shown to interact with p53, thus underlining its crucial role in controlling cell fate. An extensive description of all these proteins (more than 100 have been identified) cannot be envisaged here, yet some of them will be discussed. Indeed, they illustrate how the activity of p53 may be quantitatively or qualitatively regulated, closely or remotely, by mechanisms allowing the finely tuned integration of various signals.

The key role of MDM2

As MDM2 is a major regulator of p53 level, it is not surprising that numerous proteins can modulate its own activity. This allows the integration of various stress signals.

Ribosomes, MDM2 and p53

The ribosomal proteins L5, L11 and L23 (RPL5, RPL11, and RPL23) lower MDM2 activity, thus preventing p53 ubiquitination and increasing its transcriptional activity. This suggests an important link between ribosomal biogenesis and p53 activity, perhaps highlighting a pathway that integrates the p53 response with protein synthesis (Coutts & La Thangue 2005). This link is also supported by the recent observation that another ribosomal protein, RPL26, is able preferentially to bind to the $5'$ untranslated region of p53 mRNA after DNA damage and to enhance association of p53 mRNA

with heavier polysomes, which increases the rate of p53 translation, induces G_1 cell-cycle arrest, and augments irradiation-induced apoptosis.

Growth factors, MDM2 and p53

AKT (v-akt murine thymoma viral oncogene homologue, also known as PKB/protein kinase B) is a serine/threonine kinase, which in mammals comprises three highly homologous members (AKT1-AKT3). AKT is activated in cells exposed to diverse stimuli such as hormones, growth factors (epidermal growth factor (EGF), insulin-like growth factor-I (IGF-I)...), and extracellular matrix components. The activation mechanism occurs downstream of phosphoinositide 3-kinase (PI-3K), which is itself activated by phosphatidyl inositol triphosphate (PIP3). AKT signalling is believed to promote proliferation and increase cell survival by inhibiting apoptosis, thereby contributing to cancer progression. In agreement with this, phosphorylation of MDM2 at Ser166 and Ser188 by activated AKT results in inhibition of MDM2 self-ubiquitination and in its translocation into the nucleus where it reduces p53 activity (Milne *et al*. 2004). PTEN (phosphatase and tensin homologue), a dual specificity PIP3 phosphatase that antagonizes AKT signalling, is capable of blocking MDM2 nuclear translocation, thus preventing the negative effects of growth factors on p53 activity.

PTEN may be viewed as a tumour suppressor. In addition, PTEN appears to modulate *MDM2* transcription by negatively regulating its P1 promoter in a p53-independent manner (Chang *et al*. 2004). Indeed, the induction of MDM2 gene transcription by p53 requires the P2 promoter (Kohn & Pommier 2005). The induction of PTEN has been shown to be essential for p53-mediated apoptosis in mouse cells, underscoring the importance of the AKT survival signalling in determining the final outcome of the p53 response.

Oncogenes, MDM2 and p53

In the frequency of its disruption in human cancer, the *CDKN2A* (also known as *INK4A/ARF*) gene, located at 9p21, is second only to *Tp53* (Haber 1997). In fact, this locus encodes two proteins translated in alternate reading frames: P16[INK4A], a tumour suppressor, is a cyclin-dependent kinase inhibitor that acts upstream of retinoblastoma (RB) protein to promote cell-cycle arrest; P19[ARF] is more related to p53 activity. P16[INK4A] and

P19[ARF] are often co-deleted in tumour cells, as notably observed in the widely used, wild-type p53 MCF-7 breast cancer cell line (see Craig *et al.* 1998), but mice lacking P19[ARF] alone are highly susceptible to breast cancer (Haber 1997), thus underlining its importance.

P19[ARF] activates p53 by sequestering MDM2 into the nucleolus, thus preventing it degrading p53. The P19[ARF]-p53 axis is critical for eliminating potential tumour cells containing deregulated oncogene expression. The adenoviral proteins E1A and MYC, when over-expressed, may promote apoptosis through p53 activation. By the same pathway, V-Ha-ras Harvey rat sarcoma viral oncogene homologue (HRAS) may induce cell senescence. It has been shown that P19[ARF] is strictly required to mediate these effects on p53 (Lowe 1999). P19[ARF] may also mediate the positive effects of beta-catenin on p53 activity (Harris & Levine 2005).

Interestingly, P19[ARF] also inhibits dicoumarol-induced p53 degradation. For instance, E1A, which stabilizes p53 by inducing P19[ARF], also inhibits dicoumarol-induced p53 degradation, which is mediated by NQO1. Therefore P19[ARF] exhibits a double lock activity that inhibits p53 degradation by both the MDM2-dependent and the NQO1-regulated pathway, ensuring maximal p53 accumulation under certain physiological conditions.

ABL, MDM2 and p53

ABL (v-abl Abelson murine leukemia viral oncogene homologue) is a ubiquitously expressed non-receptor tyrosine kinase and a critical factor that under physiological conditions is required for the maximal and efficient accumulation of active p53 in response to DNA damage. Mice that lack both p53 and ABL are not viable. ABL protects p53 by antagonizing the inhibitory effect of MDM2, an action that requires a direct MDM2 phosphorylation at Tyr394 by ABL, observed *in vivo* as well as *in vitro*. In addition, ABL has been shown to directly interact with p53 and could protect the latter from ubiquitination by other inhibitors of p53, such as the E6/E6AP complex that inhibits and degrades p53 in HPV-infected cells (Levav-Cohen *et al.* 2005).

MDM4 (MDMX), MDM2 and p53

MDM4 (mouse double minute 4, also known as MDMX) is a structural homologue of MDM2 that can bind to p53 and inhibit its transcription function. Knockout of MDM4 in mice results in embryonic lethality due to hyper-activation of p53. Thus, MDM4 is an essential regulator of p53 during embryonic development, which is not the case for MDM2. The current thought is that MDM4 inhibits p53 activity both directly and indirectly by facilitating the p53-MDM2 feedback loop. MDM4 alone does not promote p53 ubiquitination or degradation *in vivo*. However, formation of the MDM2-MDM4 heterodimer stimulates the ubiquitin ligase activity of MDM2 for itself and for p53, suggesting that MDM4 may serve as a regulator or cofactor of MDM2. Although the role of MDM4 in DNA damage-mediated control of p53 activity remains unclear, MDM2 is believed to target MDM4 for degradation after DNA damage, thereby increasing p53 activity (Coutts & La Thangue 2005, Pan & Chen 2005).

MDM4 also possesses the ability to inhibit 53-dependent transcription in an MDM2-independent manner. This could be a consequence of inhibition of P300/CBP-mediated acetylation of p53 (reviewed in Marine & Jochemsen 2005).

MDM4 over-expression can lead to transformation in cell culture; MDM4 gene amplification and over-expression have been observed in 5% of primary breast tumours, all of which retained wild-type p53. MDM4 is notably amplified and highly expressed in the widely used MCF-7, a breast cancer cell line harbouring wild-type p53, and siRNA-mediated reduction of MDM4 markedly inhibits the growth potential of these cells in a p53-dependent manner. Together, these results make MDM4 a putative drug target for cancer therapy (Danovi *et al.* 2004).

USP7 has been shown to interact with p53, which can lead to p53 deubiquitination and stabilization. However, it appears that total ablation of USP7 is indeed accompanied by an increase in p53 levels. In fact, USP7 may indirectly affect p53 activity and stability by associating with MDM2, leading to MDM2 stabilization. Furthermore, USP7 may also bind to MDM4, leading to its deubiquitination and stabilization. Of interest, the deubiquitination activity of USP7 towards MDM2 and MDM4 is impaired after DNA damage. Indeed, MDM2 and MDM4 phosphorylation by the DNA damage-activated ATM lowers their affinity for USP7, providing a possible mechanism for the instability of MDM2 and MDM4 after DNA damage. This example shows that USP7, MDM2, MDM4, and p53 entertain complex interactions (Meulmeester *et al.* 2005*a*,*b*).

The adenoviral protein E1A may stabilize p53 tumour suppressor through the activation of P19^{ARF} (see above). E1A may also bind to MDM4 and form a complex with p53 in the presence of MDM4, resulting in the stabilization of p53 in a P19^{ARF}-independent manner. Although it has no effect on the p53-MDM2 interaction, E1A facilitates MDM4 binding to p53 and inhibits MDM2 binding to MDM4, resulting in decreased nuclear exportation of p53 (Li *et al.* 2004).

Gankyrin, MDM2 and p53

Gankyrin, also known as PSMD10 (proteasome 26S subunit, non-ATPase, 10), is an ankyrin repeat oncoprotein commonly over-expressed in certain carcinomas. Gankyrin has an anti-apoptotic activity in cells exposed to DNA damaging agents. Down-regulation of gankyrin induces apoptosis in cells with wild-type p53. Gankyrin binds to MDM2, facilitating p53-MDM2 binding, and increases ubiquitination and degradation of p53. Gankyrin also enhances MDM2 auto-ubiquitination in the absence of p53. Down-regulation of gankyrin reduced amounts of MDM2 and p53 associated with the 26S proteasome. Thus, gankyrin is a co-factor that increases the activities of MDM2 on p53 (Higashitsuji *et al.* 2005).

KAP1, MDM2 and p53

By interacting with MDM2, the nuclear co-repressor KAP1 (KRAB-associated protein 1, also known as TRIM28/tripartite motif-containing protein 28) inhibits p53 acetylation and promotes p53 ubiquitination and degradation. P19^{ARF} competes with KAP1 in MDM2 binding and oncogene induction of P19^{ARF} expression reduces MDM2-KAP1 interaction (Wang *et al.* 2005*a*).

RB1, MDM2 and p53

The RB1 (retinoblastoma 1) protein can be found in cells in a complex with MDM2 and p53, resulting in high p53 activity and enhanced apoptotic activity. RB1 is generally associated with the transcription factors E2Fs. By complexing to RB1, MDM2 allows the liberation of E2Fs. Both MDM2 and RB1 may be phosphorylated and inhibited by the cyclin E-cdk2 complex. Following DNA damage, activated p53 stimulates the synthesis of P21$^{WAF1/CIP1}$, the product of the *CDKN1A* gene. P21$^{WAF1/CIP1}$ inhibits the cyclin E-cdk2 complex,

and this, in turn, acts positively upon the RB1-MDM2 complex that promotes p53 activity and apoptosis (apoptosis-selective auto-regulatory loop associated with RB1) (Yamasaki 2003, Harris & Levine 2005).

Of note, E2Fs not bound to RB1 contribute to p53 stabilization, notably by increasing transcription of P19^{ARF}, ATM and CHK2, and switches the p53 response from G_1 arrest to apoptosis, notably by up-regulating the expression of ASPP1, ASPP2, JMY and Tp53INP1, four pro-apoptotic cofactors of p53 (see below) (Hershko *et al.* 2005).

Interactions between p53 and p63/p73

p63 and p73 are highly related to p53. In contrast to p53, their genes are rarely affected by inactivating mutations. On the other hand, their targeted deletion causes severe developmental defects, in contrast to a deletion of *Tp53*. Hence, p63 and p73 appear responsible for biological effects that cannot be elicited by p53 alone. It has been speculated that, during the course of evolution, p63 and p73 have first pursued a broader range of activities, whereas p53 later specialized on genome maintenance (Blandino & Dobbelstein 2004).

A role of p73 in resistance to various drugs has been suggested (Melino *et al.* 2002). A complex network of interactions between p53, p63 and p73 has been demonstrated. p63 and p73 may exist as isoforms. Long isoforms (TAp63, TAp73) are able to transactivate the same target genes as p53, while short isoforms (DeltaN-p63, DeltaN-p73) have an opposite activity via dominant negative mechanisms. While common genes may be activated by p53 and p73, recent microarray analysis has, however, suggested that the cellular response induced by p73 during adriamycin treatment could involve specific genes, as suggested by microarray analysis (Vayssade *et al.* 2005)

Of interest, p53 has been shown to induce the expression of DeltaN-p73, at both the mRNA and protein levels, through a specific p53-responsive promoter element. This induction of DeltaN-p73 expression establishes an auto-regulatory feedback loop that keeps the trigger of cell death under tight control (Kartasheva *et al.* 2002).

Mechanisms of p53 apoptosis vs growth arrest – p53 apoptotic co-regulators

Apoptosis appears as the critical function of p53 in tumour suppression (Haupt *et al.* 2003, Yu &

Zhang 2005). The choice between growth arrest and apoptosis likely involves the complex interplay of numerous factors.

1. According to a quantitative model, genes involved in growth arrest contain high-affinity p53 binding sites in their promoter, while low-affinity sites are present in the promoter of apoptosis-related genes (Chen *et al.* 1996). This is in line with observations that increased levels or activity of p53 can lead to the onset of apoptosis, presumably by achieving a certain threshold level. Moreover, p53 mutants with marginally altered conformations retain sufficient activity to induce growth arrest but not apoptosis, presumably because they can still interact only with high-affinity sites. However, despite the degenerative nature of p53 binding sequences, the apoptotic targets of p53 do not necessarily contain low-affinity promoters. For example, chromatin immuno-precipitation experiments have revealed that the apoptotic gene *BBC3* contains high-affinity p53 binding sites (Kaeser & Iggo 2002). The quantitative model is thus not sufficient.

2. According to a qualitative model, the selective activation of the p53 apoptotic genes is mediated through the interaction of p53 with certain transcription co-activators. Several proteins may interact with p53 and specifically modulate apoptosis. For instance, ASPP1 (apoptosis stimulating protein of p53-1, also known as PPP1R13B/protein phosphatase 1, regulatory subunit 13B) and ASPP2 can both favour the interaction of p53 with the promoters of apoptotic genes *BAX* and *Tp53I3/PIG3*, but not that of *MDM2*, *CCNG1* or *CDKN1A* (Yu & Zhang 2005). The effects of ASPP1 and ASPP2 may be counteracted by iASPP (inhibitor of ASPP), the most conserved inhibitor of p53-mediated apoptosis. Both P63 and P73 are thought to favour selective binding of p53 to apoptotic promoters *BAX*, *PMAIP1/NOXA* and *PERP* (Yu & Zhang 2005), an effect that could be mediated through their interaction with ASPP1 and ASPP2 (Bergamaschi *et al.* 2004). DAXX (death-associated protein 6) is a transcriptional repressor of *CDKN1A* (involved in cell growth arrest), but it does not affect the activation of proapoptotic genes, and therefore acts by influencing the balance between cell cycle arrest and proapoptotic p53

targets (Gostissa *et al.* 2004). STAT1 (signal transducer and activator of transcription 1) can act as a co-activator of p53 to induce expression of *BAX*, *PMAIP1/NOXA*, and *FAS* (Yu & Zhang 2005).

As mentioned above, phosphorylation of the p53 residue Ser46 plays an important role in permitting the apoptotic function of the protein. The interaction between p53DINP1 and Ser46 may allow this phosphorylation.

Additional proteins do not interact directly with p53, but have been implied in its apoptotic function. JMY (junction-mediating and regulatory protein) interacts with P300 to enhance, selectively, the ability of p53 to induce expression of apoptotic genes such as *BAX* (Yu & Zhang 2005). STRAP (serine/threonine kinase receptor associated protein) was originally identified as a JMY-interacting protein. After DNA damage, its phosphorylation by activated ATM allows its localization to the nucleus. It is believed that this prompts p53 acetylation through recruitment of P300/JMY and the subsequent enhancement of p53 apoptosis (Coutts & La Thangue 2005).

E2F transcription factors may contribute to p53 stabilization by regulating genes such as P19[ARF], ATM and CHK2. In addition, E2F1 has been shown to up-regulate the expression of four pro-apoptotic cofactors of p53 – ASPP1, ASPP2, JMY and Tp53INP1 – through a direct transcriptional mechanism (Hershko *et al.* 2005).

Other interactors modulating the p53 transcriptional activity

Proteins that modulate p53 activity may exert their positive or negative effects through various ways that will not be discussed here.

Among positive regulators of p53 are 14-3-3σ (Yang *et al.* 2003), activating transcription factor 3 (ATF3, Yan *et al.* 2005), BRCA1-associated RING domain 1 (BARD1, Wu *et al.* 2006), breast cancer 1, early-onset (BRCA1, Fabbro *et al.* 2004), CCAAT-binding transcription factor 2 (CTF2, Uramoto *et al.* 2003), hypoxia-inducible factor 1 alpha (HIF1α, Fels & Koumenis 2005), high-mobility group box 1 (HMGB1, Banerjee & Kundu 2003), members of the ING (inhibitor of growth family) (Gong *et al.* 2005), nuclear factor Y (NF-Y, Imbriano *et al.* 2005), prohibitin (PHB, Fusaro *et al.* 2003), and STAT1 (Townsend *et al.* 2005).

Among negative regulators are bone marrow kinase, X-linked (BMX, Jiang *et al.* 2004), CCAAT/ enhancer binding protein beta (C/EBPβ, Schneider-Merck *et al.* 2006), DNA methyltransferase-3a (DNMT3, Wang *et al.* 2005*c*), Kruppel-like factor 4 (KLF4, Rowland *et al.* 2005), SIN3 homologue A, transcription regulator (SIN3A, Zilfou *et al.* 2001), STAT3 (Niu *et al.* 2005), Y box-binding protein 1 (YB1, Homer *et al.* 2005), and YY1 transcription factor (YY1, Sui *et al.* 2004, Yakovleva *et al.* 2004).

Some of them may restrict p53 activity to specific promoters, for instance those of genes related to apoptosis.

The promyelocytic leukaemia (PML) protein is of specific interest. This tumour suppressor can selectively and dynamically recruit a number of proteins including p53 to form a sub-nuclear multi-protein chamber named PML-NBs, of which it is an essential component. After DNA damage, p53 is recruited into PML-NBs and modified by phosphorylations and acetylations, which in turn potentiate its transcriptional and pro-apoptotic activities. By sequestering p53, PML-NBs may regulate in a complex way its sub-nuclear distribution upon stress, thus allowing coordinate temporal patterns of p53-associated transcription (Bao-Lei *et al.* 2005, Coutts & La Thangue 2006).

Auto-regulatory loops in p53 action

The p53 pathway is intimately linked to other signal transduction pathways that may play a significant role in cancer. Most often, these pathways regulate entry of cells into the cell cycle. The coordination between p53 activity and these pathways may be ensured through a series of auto-regulatory loops. Here are some examples, notably based on the work of Harris & Levine (2005).

1. MDM2 is induced by p53. MDM2 promotes p53 degradation.
2. P19^{ARF} down-regulates the MDM2 ubiquitin ligase activity, thus increasing p53 levels. Activated p53 down-regulates P19^{ARF}.
3. Activated P38 protein kinase increases p53. Activated p53 induces PPM1D which inactivates P38 by preventing its phosphorylation by the RAS pathway.
4. Activated p53 induces COP1 and PIRH2. These ubiquitin ligases contribute to p53 degradation.
5. Activated p53 induces DeltaN-P73. DeltaN-P73 represses p53 transcriptional activation.

6. MDM2 activity may be inhibited by phosphorylation on Thr216 (by the cyclin A/cdk2 complex). Activated p53 induces cyclin G; cyclin G makes a complex with PP2A phosphatase, which removes the phosphate at Thr216 and increases MDM2 activity, thus reducing p53 level (Ohtsuka *et al.* 2004).
7. Activated p53 induces the ubiquitin ligase, seven in absentia homologue (SIAH)-1. SIAH-1 degrades BETA-CATENIN, which is known to up-regulate P19^{ARF} and, subsequently, to increase p53 levels.
8. Growth factors may activate AKT, which, in turn, phosphorylates and activates MDM2; it results in a decrease in p53 (survival pathway). p53 increases PTEN and PTEN decreases AKT activity.
9. Activated p53 induces 14-3-3σ. 14-3-3σ interacts with p53 and stabilizes it.
10. Activated p53 induces PML. PML helps to potentiate p53 activity.
11. PCAF is induced by p53. It contributes to p53 stabilization.
12. PPM1D is induced by p53. It may dephosphorylate both p53 and CHK1 (which may phosphorylate p53 at various sites), thus inactivating it (Lu *et al.* 2005).
13. BRCA1, CHK1 and CHK2 contribute to p53 activation upon stress. All three are down-regulated by activated p53 (Lohr *et al.* 2003, Matsui *et al.* 2004).

Mechanisms for loss of p53 activity in cancer

p53 is subject to tight regulation at multiple levels. In cancer cells, its function can be compromised by various mechanisms: mutations of Tp53, alteration of p53 regulators, alteration of p53 target genes.

p53 mutations

In humans, inheritance of a *Tp53* mutant allele results in a rare familial autosomal disorder, the Li–Fraumeni syndrome. It is characterized by a high incidence of multiple early cancers, including breast tumours.

However, most p53 mutations observed in breast cancer are of somatic origin. In fact, contrasting with the two p53 relatives p63 and p73 (Blandino & Dobbelstein 2004), p53 mutations are the most frequent genetic events in human cancer. They have been found in most types of tumours, with frequencies

ranging from 5% (cervix) to 50% (lung). Between 20 and 35% of breast tumours have been shown to express a mutant p53. However, most of the information on p53 mutations is derived from sequence analysis that included only exons 5–8 (residues 126–306) within *Tp53*, and examination of the whole p53 coding sequence is beginning to reveal an increasing number of mutations in the N- and C-termini of the protein (Vousden & Lu 2002). Nevertheless, the majority of p53 mutations appear to be localized in the DNA-binding domain, in the central part of p53. Notably, this domain is the binding site for ASPP1 and ASPP2, important cofactors in the transactivational activity of p53 in relation to apoptotic genes (see above). Most of the hot-spot p53 mutations render the protein unable to interact with ASPP1.

Since there is no evidence that *Tp53* lies in a hyper-mutable region of the genome, cells that have lost p53 function are likely to be selected during cancer development. In cells expressing a mutant p53, this protein is generally no longer able to control cell proliferation, which results in inefficient DNA repair and genetic instability. p53-deficient mice are developmentally normal but show a very high incidence of multiple early tumours and generally succumb before reaching the age of 1 year (Donehower *et al.* 1992). Moreover, when introduced into cells, a mutant p53 can transform and give to these cells a more aggressive phenotype.

The great majority of mutant p53s are defective in transactivation and may exert a dominant negative effect by preventing wild-type p53 from binding to the promoter of its target genes (Willis *et al.* 2004). However, it must be noted that not all p53 mutations are inactivating. For instance, some mutant p53s display only partial loss of their DNA binding activity, allowing the mutant to bind only to a subset of p53 response elements (Friedlander *et al.* 1996, Rowan *et al.* 1996). This has notably been observed with the mouse R172P mutation, equivalent to the human R175P alteration (Liu *et al.* 2004*a*), which is linked to differential transactivation ability. Biologically, such mutants have lost their apoptotic properties, but their cell cycle arrest activity remains similar to that of the wild-type protein. Along the same lines, it has been shown that p53 mutants can often trans-activate promoters containing a p53-responsive sequence like that found in *CDKN1A* (involved in growth arrest) but not like those present in *BAX* or *Tp53I3/PIG3* (involved in apoptosis) (Campomenosi *et al.* 2001). Regarding apoptosis, the hot-spot R175H p53 mutant was

shown to strongly inhibit transcription of the *FAS* pro-apoptotic gene. This inhibition of transcription required binding of the mutant protein to a different promoter site from that recognized by wild-type p53. Other mutants (resulting from alterations of residues 248 and 273) have a similar, but less pronounced, property (Zalcenstein *et al.* 2003). Thus, several mutant p53s, in addition to preventing the apoptotic activity of a normal p53 (encoded by a non-mutated allele) could also exert anti-apoptotic actions.

In contrast, at least 18 mutant p53s expressing an apoptotic activity higher that of wild-type p53 have been identified. The corresponding mutations tend to cluster at residues 121 (in the L1 loop – residues 115 to 135) or 290 to 292 (in the flanking region of the H2 helix). For instance, the S121F mutant is known as 'super' p53, due to its superior ability to induce apoptosis, as compared with wild-type p53 (Saller *et al.* 1999). The remaining 17 mutants are H214Q, K291E, K292T, Q144R, R290G, I162M, K291T, S121A, S121C, F212Y, E221Q, K291Q, S121Y, R156C, S215C, K292I and P153H. It has been shown that there was no significant correlation between their apoptotic property and their ability to activate transcription of six p53-responsive genes (*CDKN1A, MDM2, SFN,* and the apoptosis-related *BAX*, *p53AIP1*, *BBC3*). This suggests that transactivation-dependent mechanisms do not always play a major role in p53-dependent apoptosis (Kakudo *et al.* 2005). As expected, none of the super-apoptotic mutants described above is frequently observed in breast tumours.

Two alternative pathways that are either dependent or independent of the MDM2-ubiquitin-26S proteasome mediate proteasomal degradation of p53. The ubiquitin-independent pathway is regulated by NQO1 that prevents p53 degradation by the 20S proteasome. Compared with wild-type p53 and several mutants, the hot-spot p53 mutants R175H, R248H, and R273H were shown to exhibit increased binding to NQO1, and thus decreased degradation. However, they remained sensitive to MDM2-ubiquitin-mediated degradation. Thus, NQO1 has an important role in stabilizing some hot-spot p53 mutant proteins in human cancer. This could, at least in part, explain the relatively high steady state expression of these mutant proteins in cancer cells (Asher & Shaul 2005).

About 1400 p53 mutations observed in breast cancers are listed in the *Tp53* database maintained at the International Agency for Research on Cancer (IARC) (Olivier *et al.* 2004). The pattern

and codon distributions of p53 mutations in breast tumours show a very similar profile to all other cancers, including similar hot spots. Indeed, 34% of *Tp53* mutations affect only 10 residues – 175, 176, 179, 213, 220, 245, 248, 249, 273, and 282; three residues (175, 248 and 273) contribute 18% of mutations. More than 90% of all mutations affect the central core region (residues 103–292), which interacts with DNA. To date, only 2% and 5% of all mutations have been located to non-central regions 1–101 and 293–393 respectively. In breast cancer, there is an over-representation of TAC to TGC alteration at codon 163. This codon is rarely mutated in most cancers (less than 1%), but accounts for over 2% of all breast cancer mutations (Feki & Irminger-Finger 2004). The significance of this remains unknown.

Contrasting with other tumour-suppressor genes, p53 mutants are most frequently (~90%) missense ('point missense mutations'). Mutant p53 proteins generally have an increased stability and accumulate in the nucleus of neo-plastic cells. It is believed that this is a consequence of the inactive p53 mutant protein no longer driving the expression of the MDM2 protein required to target its own degradation. Immunohistochemical detection of the amount of nuclear p53 has long been used as an indicator of p53 alteration, but this parameter appears highly dependent on the type of mutation (see below).

The p53 status of numerous widely used breast cancer cell lines (Lacroix & Leclercq 2004*a*) has been determined. Some of these observations have been compiled at IARC (Olivier *et al*. 2004). Table 3 summarizes these data.

In agreement with the observations in tumours, the frequency of mutations in the central DNA-binding core is high in breast (see Table 3) and in non-breast cancer cell lines (O'Connor *et al*. 1997). Most mutations are missense. The percentage of cell lines with mutated p53 is higher than expected, based on the frequency of p53 mutations in breast tumours. It is possible that tumour cells expressing an altered p53 could be easier to establish in culture (for information about the bias in the process of cell lines isolation see Lacroix & Leclercq 2004*a*).

Alterations of p53 modulator and/or target proteins

The number of proteins able to interact with p53 or to be modulated by activated p53 is high. The qualitative and quantitative expression pattern of these proteins may vary from one tumour to another, thus composing a very complex picture that cannot be exhaustively detailed here.

The expression level of some proteins may reflect specific cell biology. It has been shown that cancer cells in oestrogen receptor (ER)-positive/low-grade/well differentiated breast tumours most often express a 'luminal-like' secretory phenotype, while those populating ER-negative/high-grade/poorly differentiated lesions express a 'basal/myoepithelial' portrait (Lacroix *et al*. 2004). Several effectors and targets of p53 have their expression restricted to one cell type and thus are found exclusively or at higher levels in the derived tumours. This is notably observed for P63, 14-3-3σ, IGF binding protein-3 (IGFBP3), and MASPIN (also known as SERPINB5), which are specific to the 'basal/myo-epithelial', ER-negative phenotype and are expressed mainly in ER-negative/high-grade/poorly differentiated tumours (Shao *et al*. 1992, Lacroix & Leclercq 2004*a*, Lacroix *et al*. 2004, Simpson *et al*. 2004, Charafe-Jauffret *et al*. 2006). Other proteins related to the 'basal/myoepithelial' portrait and/or to high-grade steroid receptor-negative tumours are PERP, BARD1, and SURVIVIN (Singh *et al*. 2004, Span *et al*. 2004, Charafe-Jauffret *et al*. 2006, Wu *et al*. 2006).

On the other hand, the expression of BCL2, MDM2, MDM4, PTEN and USP7 has been associated with the 'luminal-like' phenotype of breast cancer cells (BCC) and/or tumours (Bozzetti *et al*. 1999, Kappes *et al*. 2001, Phelps *et al*. 2003, Garcia *et al*. 2004, Lacroix *et al*. 2004, Charafe-Jauffret *et al*. 2006).

The expression of other genes or proteins has been shown to be differentially regulated in breast tumours compared with normal breast tissue: *GADD45A*, *INK4A/ARF*, *PRG3* and *RPRM* are frequently down-regulated, while *RAI3* is often up-regulated in tumour tissues (Silva *et al*. 2003, Wu *et al*. 2004, Nagahata *et al*. 2005, Takahashi *et al*. 2005, Wang *et al*. 2005*b*).

In most cases, the absence of gene expression is associated with gene promoter methylation (Silva *et al*. 2003, Khan *et al*. 2004, Lacroix *et al*. 2004, Takahashi *et al*. 2005, Wang *et al*. 2005*b*).

Of interest, the expression of the pro-apoptotic factors, ASPP1 and ASPP2, is frequently down-regulated, while that of iASPP is frequently up-regulated in breast cancer. Thus, there could be a selective advantage for tumour cells to lose the expression of ASPP1 and ASPP2 and to gain iASPP (Liu *et al*. 2005).

Table 3 p53 mutations in breast cancer cell lines.

Cell line	Exon	Codon	Type	Nucleotide change	Residue change
BRC230	8	266	Nonsense	GGA → TGA	Gly (G) → Stop
BT-20	5	132	Missense	AAG → CAG	Lys (K) → Gln (Q)
BT-474	8	285	Missense	GAG → AAG	Glu (E) → Lys (K)
BT-483	7	246	Deletion (1 bp)		
BT-549	7	249	Missense	AGG → AGC	Arg (R) → Ser (S)
CAMA-1	8	280	Missense	AGA → ACA	Arg (R) → Thr (T)
EVSA-T	6	213	Nonsense	CGA → TGA	Arg (R) → Stop
HCC38	8	273	Missense	CGT → CTT	Arg (R) → Leu (L)
HCC70	7	248	Missense	CGG → CAG	Arg (R) → Gln (Q)
HCC1007	8	281	Missense	GAC → CAC	Asp (D) → His (H)
HCC1395	5	175	Missense	CGC → CAC	Arg (R) → His (H)
HCC1569	8	294 (*)	Nonsense	GAG → TAG	Glu (E) → Stop
HCC1806	7	256	Insertion (2 bp)		
HCC1937	8	306 (*)	Nonsense	CGA → TGA	Arg (R) → Stop
HCC2218	8	283	Missense	CGC → TGC	Arg (R) → Cys (C)
HDQ-P1	6	213	Nonsense	CGA → TGA	Arg (R) → Stop
HMT-3909	7	249	Missense	AGG → GGG	Arg (R) → Gly (G)
Hs578T	5	157	Missense	GTC → TTC	Val (V) → Phe (F)
MAST	7	244	Missense	GGC → AGC	Gly (G) → Ser (S)
MDA-MB-134-VI	8	285	Missense	GAG → AAG	Glu (E) → Lys (K)
MDA-MB-157	7	261	Deletion (26 bp)		
MDA-MB-231	8	280	Missense	AGA → AAA	Arg (R) → Lys (K)
MDA-MB-361	5	166	Nonsense	GAA → TAA	Glu (E) → Stop
MDA-MB-435	8	266	Missense	GGA → GAA	Gly (G) → Glu (E)
MDA-MB-436	6	205	Insertion (7 bp)		
MDA-MB-453	11	367 (*)	Deletion (30 bp)		
MDA-MB-468	8	273	Missense	CGT → CAT	Arg (R) → His (H)
SK-BR-3	5	175	Missense	CGC → CAC	Arg (R) → His (H)
T-47D	6	194	Missense	CTT → TTT	Leu (L) → Phe (F)
UACC893	10	342 (*)	Nonsense	CGA → TGA	Arg (R) → Stop
DU4475	Wild-type				
MCF-7	Wild-type				
MDA-MB-175-VII	Wild-type				
UACC-812	Wild-type				
ZR-75-1	Wild-type				
ZR-75-30	Wild-type				

(*) Mutation outside the central DNA-binding core.

Another source of differential gene expression in breast tumours is the occurrence of amplification/deletion events. For instance, *MYC* and *PPM1D* are frequently amplified in breast tumours, with a high-level copy number gain. *PPM1D* amplifications are found in aggressive primary lesions (Rauta *et al.* 2006). Interestingly, virtually none of the tumours with *PPM1D* amplification was shown to carry a p53 mutation, consistent with the idea that over-expressed PPM1D contributes to functional inactivation of p53, rendering its mutation unnecessary (Lu *et al.* 2005). *TERT* is another target of amplification at 5p12-p14. Other regulators of p53 activity may be amplified in breast cancer:

MDM2, MDM4, COP1, CUL4A. MDM2 is amplified in ~5.7% of breast tumours (Al-Kuraya *et al.* 2004). MDM4 is amplified in ~5% of breast tumours, all of which have retained a wild-type p53 (Danovi *et al.* 2004). COP1 is over-expressed in a majority of breast tumours (~80%), of which most are negative for p53 (Dornan *et al.* 2004). CUL4A is amplified in 16% of primary breast cancers and over-expressed in 47% (Chen *et al.* 1998).

Other genes coding for important determinants of p53 activity may be deleted. This has notably been observed with *INK4A/ARF*, which is, for instance, deleted in the widely used MCF-7 BCC line (Craig

et al. 1998). *TNFRSF10A* is also frequently deleted in tumours (Naylor *et al.* 2005).

Hereditary breast cancer and p53

As mentioned above, *Tp53* mutations may be observed in the rare familial autosomal Li–Fraumeni syndrome. It is characterized by a high incidence of multiple early cancers, including breast tumours. Other hereditary breast cancers may be due to mutations in genes coding for p53 modulator proteins. A significant proportion of these cancers have been associated with mutations of *BRCA1*. BRCA1 may interact with p53 and has been viewed as a 'scaffold' for p53 response (Hohenstein & Giles 2003). Of interest, BRCA1 tumours often express *Tp53* mutations, but it remains to be established if this reflects the need for p53 inactivation for the development of BRCA1 tumours to occur, or rather if the loss of BRCA1-associated DNA repair properties may explain, at least partly, the high frequency of *Tp53* mutations (Lacroix & Leclercq 2005).

Other mutations leading to familial syndromes accompanied by a high occurrence of breast cancer may affect *BRCA2*, *ATM* (Ataxia-Telangiectasia), *CHEK2* (Li-Fraumeni-like syndrome), *STK11/LKB* (Peutz-Jeghers syndrome), or *PTEN* (Cowden syndrome) (Lacroix & Leclercq 2005). The products of two of these genes, *ATM* and *CHEK2*, are involved in p53 activation, while the product of *PTEN* increases p53 activity by antagonizing the cell survival effects mediated by the AKT-MDM2 pathway (see above).

p53 alterations, breast tumour characteristics, and prognosis

The potential relationships between p53 alterations and the expression of other tumour markers or pathological characteristics (grade) have been widely investigated. Many teams have also examined the value of p53 as a prognostic marker. The interpretation of data has, however, often been complicated by the fact that most initial studies used immunohistochemistry (IHC) to detect the amount of p53, while analysis of p53 mutations was performed by other investigators. The correlation between p53 accumulation measured by IHC and p53 mutation detected by sequencing has been estimated to be less than 75% in breast carcinomas (Norberg *et al.* 1998). Indeed, not all mutations yield a stable protein and some mutations lead to a truncated protein not detected by IHC. On the other hand, wild-type p53 may accumulate in some tumours as a result of a response to DNA damage or by binding to other cellular proteins, giving a positive IHC result.

Breast tumours expressing a high amount of p53 (as measured by IHC) are more frequently ER-negative and progesterone receptor (PgR)-negative. They are also associated with a high proliferation rate, high histological and nuclear grades, aneuploidy, and poorer survival. A high p53 level is frequently observed in tumours over-expressing ERBB2 (also known as Her-2/neu) (Feki & Irminger-Finger 2004).

The same relationships have been observed when p53 mutations were taken into account, instead of p53 accumulation. For instance, in a large (543 individuals) analysis of patients with node-negative breast cancer, p53 mutations were more frequent in breast carcinomas with amplification of the *ERBB2* gene (leading to ERBB2 over-expression). Patients with both p53 mutation and *ERBB2* amplification were associated with poor survival. The groups with p53 mutations (both with or without *ERBB2* amplification) were more likely to be ER- and PgR-negative, more likely to be grade 3 for both histological and nuclear grade, and less likely to have lobular subtype (Bull *et al.* 2004). The particularly bad prognosis associated with the coexistence of high ERBB2 and p53 alterations is supported by other studies (Rahko *et al.* 2003, Yamashita *et al.* 2004).

In a meta-analysis of more than 9000 patients, the prognostic and predictive value of high p53 expression in breast cancer, as evaluated by IHC, was found to be weak (Barbareschi 1996). On the other hand, more than 25 studies to date involving over 6000 patients have revealed the strong prognostic significance of p53 mutations (reviewed in Borresen-Dale 2003). A meta-analysis of 16 of these studies including over 3500 patients (Pharoah *et al.* 1999) confirmed that mutations in the *Tp53* confer a worse overall and disease-free survival in breast cancer cases, an effect that is independent of other risk factors. In several of the studies the presence of a *Tp53* mutation was the single most adverse prognostic indicator for both recurrence and death.

It seems that the prognostic significance of all types of mutations is not the same. Studies have shown that patients with mutations effecting or disrupting the zinc binding domains L2 and L3 (codons 163–195 and 236–251) or affecting amino acids directly involved in DNA binding, many of these

residing in the zinc binding domain, were related with the poorest prognosis (reviewed in Borresen-Dale 2003).

These findings indicate that not just p53 mutation *per se* but the full spectrum (i.e. different types, locations, and numbers) of p53 mutations needs to be examined when it is used as a prognostic marker of survival in breast cancer patients (Lai *et al*. 2004).

Recent technological advances have allowed the simultaneous evaluation of multiple RNAs (micro-arrays) or proteins (tissue arrays) in tumour samples or breast cancer cell lines. These studies have revealed that the breast tumours could be sorted into a very few classes characterized by the high level of expression of specific groups of genes/proteins. Moreover, these classes are 'stable', as most individual lesions largely maintain their 'portrait' when they evolve from *in situ* to the metastatic state (reviewed in Lacroix *et al*. 2004). The number of classes that have been defined in most micro-array-based or tissue array-based studies is three. About two-thirds of tumours express features characteristic of luminal cells. These lesions are often well differentiated, have a low grade and demonstrate relatively high levels of cytokeratins 8/18/19, ER, PgR, BCL2, CDH1 (E-cadherin), the three transcription factors GATA3, FOXA1, XBP1 (Lacroix & Leclercq 2004b), Treefoil factor (TFF)1 (pS2), TFF3, SLC39A6, P21$^{WAF1/CIP1}$, P27^{KIP1}, and cyclin D1. In contrast to the 'luminal-like' lesions, about 20% of tumours have a low level of the above cited markers, whereas they express relatively high levels of cytokeratins 5/6 and 17, CDH3 (P-cadherin), EGF receptor (EGFR), cyclin E, MIB1, MCM2, and other proliferation markers. Most of these 'basal/myoepithelial-like' tumours are poorly differentiated and have a high grade. Finally, tumours over-expressing ERBB2 as a consequence of gene amplification constitute a third class. It appears that p53 mutation is much more frequent in the 'basal/myoepithelial-like' and ERBB2 classes than in the 'luminal-like' one (82, 71 and 31% respectively, according to Sorlie *et al*. 2001). Moreover, the most well differentiated tumours have a very low level of p53 alteration (13% in Sorlie *et al*. 2001). Of note, up to 100% mutant p53 have been observed in medullary carcinoma, a specific subtype of breast cancer with a 'basal/myoepithelial-like' phenotype (de Cremoux *et al*. 1999).

The existence of breast tumour classes suggests that any tumour biology reflects to a large extent the biology of the cell of origin at the time of initiation. Tumours originating from more undifferentiated epithelial cells have a rapid growth pattern and more aggressive behaviour and outcome compared with those originating in more differentiated epithelial cells. Neoplastic progression might be p53-dependent in the tumours with a less-differentiated, 'basal/myoepithelial-like' phenotype and those over-expressing ERBB2, while it might be p53-independent in those tumours with a more differentiated, pure luminal form.

p53 alterations and response to therapy

Cell lines

Chemotherapy

O'Connor *et al*. (1997) correlated the endogenous p53 status of 58 cancer cell lines (lung, colon, breast, ovary, leukaemia, melanoma, kidney, prostate, CNS) of the National Cancer Institute (NCI) Anticancer Drug Screen with the growth-inhibitory potency of 123 anticancer agents, the majority of clinically approved cancer drugs at that time. These included: mitotic spindle poisons (microtubule inhibitors), such as paclitaxel and vincristine; anti-topoisomerase II, such as adriamycin (doxorubicin), morpholino-adriamycin and m-AMSA (amsacrine); anti-topoisomerase I, such as camptothecin; RNA antimetabolites, such as methotrexate and 5-fluorouracil; DNA antimetabolites, such as hydroxyurea and cytosine arabinoside; and alkylating agents such as carboplatin and cisplatin.

Of the 58 lines, 39 contained a mutant p53 sequence. The mutant protein was expressed at elevated basal levels in the majority of cases. In contrast to most of the wild-type p53-containing lines, cells containing a mutant p53 sequence were also deficient in γ-ray induction of P21$^{WAF1/CIP1}$, GADD45, and MDM2 mRNA and the ability to arrest in G_1 following γ-irradiation. This analysis revealed that lines with an endogenous mutant p53, while dramatically heterogeneous in their behaviour, still tended to be less sensitive than the wild-type p53 lines to most of the clinically used anticancer agents. Interestingly, however, mitotic spindle poisons were found to act independently from the p53 status.

Eight BCC lines were included in the NCI study. These were MCF-7, MCF-7/Adr (reported as MCF-7-derived cells, but their true origin remains questionable – see Lacroix & Leclercq 2004a), MDA-MB-231, Hs578T, MDA-MB-435 and its ERBB2-transfected derivative MDA-N, BT-549,

and T-47D. All cell lines except one, MCF-7, have a mutated p53. Two cell lines (MCF-7, T-47D) have a 'luminal-epithelial-like' phenotype, while the others have a more 'basal/myoepithelial-like' aspect (see Lacroix & Leclercq 2004a, de Longueville *et al*. 2005). Detailed examination of the sensitivity data (see http://www.broad.mit.edu/mpr/NCI60/GI50_RAW.txt) revealed that the most sensitive of all cell lines was MCF-7. All other BCC lines, except T-47D, were much less sensitive than MCF-7 cells. In fact, T-47D cells seemed to express an intermediary sensitivity pattern, suggesting that this feature may be only partially associated with the p53 status.

This is supported by a study in which two cell lines derived from basal epithelium (and immortalized) and two cell lines derived from luminal epithelium (MCF-7, ZR-75), all with wild-type p53, were treated with doxorubicin and 5-fluorouracil. Their transcriptional profile was thereafter analysed by microarray. While all cell lines expressed signatures of general stress response, distinct expression patterns were observed. Both luminal-like and basal-like types induced DNA damage response genes such as *CDKN1A*, but the response in the luminal cells showed higher fold changes. Luminal-like cell lines repressed a larger number of cell cycle regulated genes and other genes involved in cellular proliferation, whereas the basal-like cell lines did not. Instead, the basal-like cell lines repressed genes that were involved in differentiation. The two luminal-like cell lines showed similar response patterns to one another including the strong induction of DNA damage stress response genes, notably *CDKN1A* (Troester *et al*. 2004). Thus, despite expressing a similar p53 status, luminal epithelial-like cells seem to respond to at least two chemotherapeutic drugs to a higher qualitative and quantitative extent than basal/myoepithelial-like cells.

The role of p53 in modifying sensitivity to cytotoxic drugs has been commonly studied by creating transfection pairs of wild-type p53 parental cells and altered p53 daughter cells, or *vice versa*. Cimoli *et al*. (2004) performed a meta-analysis of 356 independent studies. Average changes of drug sensitivity after a change of p53 status were observed. These authors observed agreements between the data of O'Connor *et al*. (1997) and theirs, but the correspondence was only partial. The higher sensitivity of wild-type p53 versus mutant p53 lines to cytotoxic drugs (O'Connor *et al*. 1997) was in agreement with the finding that

transfection with a wild-type p53 tends to increase sensitivity (Cimoli *et al*. 2004). However, unexpectedly, the reciprocal seemed not to be true, as transfection with a mutated p53 did little to change the drug sensitivity of most wild-type p53 cancer lines. Rather interestingly, cells transfected with a wild-type p53 and treated with mitotic spindle poisons did not follow the general trend of an increased sensitivity. In addition, in the opposite model (from a wild-type to an altered p53), mitotic spindle poisons tended to induce a modest (about 1.7 times) but statistically significant relative sensitization with respect to the remaining drugs. This is only in partial agreement with the NCI analysis, where mitotic spindle poisons seemed essentially p53-status insensitive. A crucial indication of these findings is that the role of p53 alone in determining sensitivity/resistance to cytotoxic drugs is limited: the individual molecular pathology and differentiation of a given cancer line prevail over any average trend, and are causal to a broad spreading of the data.

Radiotherapy

There are few studies examining and comparing the radio-sensitivity of breast cancer cell lines. We thus tested the viability of six of these cell lines after exposure to γ-rays. Table 4 describes the effect of a single 8 Gy dose on their viability, as assessed 96 h post-irradiation by the Crystal violet staining test.

Among these lines, the first three (MCF-7, ZR-75-1 and T-47D) are ER-positive and express a 'luminal-like' phenotype. The three others are ER-negative. The BT-20 cells have an amplified *EGFR* gene, while *ERBB2* is amplified in SK-BR-3 cells (Lacroix & Leclercq 2004a).

While the two wild-type p53 cell lines were sensitive to irradiation, as expected *a priori*, the T-47D cells also expressed a high sensitivity. This

Table 4 Effect of a single 8 Gy γ-rays dose on the viability of six breast cancer cell lines, as assessed 96 h post-irradiation by the Crystal violet staining test.

Cell lines	p53 status	% cells, as compared with control
MCF-7	Wild-type	<50%
ZR-75-1	Wild-type	<50%
T-47D	Mutant	<50%
MDA-MB-231	Mutant	~80%
BT-20	Mutant	~80%
SKBR-3	Mutant	~80%

suggests that p53-independent parameters, including cell origin, could be partly responsible for the observed effects.

It has been shown that activation of the PI-3K/AKT pathway ('growth factor pathway', see above) prevented radiation-induced apoptosis in breast cancer cells. BT-474 BCC, which over-express ERBB2 and have mutated p53 were resistant to G_1 arrest and apoptosis caused by irradiation. However, apoptosis following irradiation was significantly increased in these cells after treatment with the PI-3K inhibitor, wortmannin. On the other hand, pre-treatment of MCF-7, which have normal expression of ERBB2, with the ERBB2 ligand and PI-3K/AKT activator, heregulin-beta1, decreased apoptosis compared with the untreated controls. Furthermore, transfection of MCF-7 cells with constitutively active AKT made the cells more resistant against apoptosis. Thus, the PI-3K/AKT signalling pathway is involved in resistance to radiation-induced apoptosis in breast cancer cells in which this signalling pathway is over-stimulated (Soderlund *et al.* 2005). This seems to be, at least partly, p53-independent. Of note, EGFR over-expression may also induce the activation of the PI-3K/AKT signalling pathway. This activation could explain why we observed that BT-20 and SK-BR-3 cells were resistant to radiation.

Tumours

Chemotherapy

There is evidence from *in vitro* (Lowe *et al.* 1993) and animal studies (Lowe *et al.* 1994) that defective p53 is associated with resistance to chemotherapy. Furthermore, loss of p53 function correlates with multidrug resistance in many tumour types (Wallace-Brodeur & Lowe 1999).

It has been suggested in the past that p53 abnormalities could not be used as a predictor of a response to therapy (see, for instance, Elledge & Allred 1998). Indeed, the great majority of studies performed at this time were based on IHC detection of over-expressed p53. They either argued against a predictive role for p53 status or were not conclusive. For instance, several neoadjuvant studies have failed to detect a predictive value to p53 staining with regards to chemo responsiveness in breast cancers (MacGrogan *et al.* 1996, Niskanen *et al.* 1997, Bonetti *et al.* 1998, Rozan *et al.* 1998). However, the p53 over-expression detected by IHC does not necessarily correlate directly with p53 mutations.

In fact, this lack of sensitivity and specificity account, in part, for the incongruity of these findings (Cleator *et al.* 2002, Feki & Irminger-Finger 2004).

Bergh *et al.* (1995) examined a series of 316 consecutively presented breast cancers. These authors found 69 internal mutations. Mutations in the conserved regions II (codons 117–142) and V (codons 270–286) were associated with worse prognosis. Adjuvant systemic therapy, especially with tamoxifen, together with radiotherapy, appeared of less value to tumours with a p53 mutation.

In a study of 243 patients with advanced breast cancer and receiving either tamoxifen or upfront chemotherapy, Berns *et al.* (2000) found that mutations in codons that directly affected DNA binding or within the zinc binding domain L3 showed the lowest response to tamoxifen (202 patients). p53 mutations were also associated with a poor, although not significant response to chemotherapy (cyclophosphamide/Methotrexate/5-fluorouracil (CMF) in 22 patients; cyclophosphamide/Adriamycin/5-Fluorouracil (CAF) in 16 patients; doxorubicin in 1 patient; platinum-containing chemotherapy in 2 patients).

p53 detected by IHC has recently been shown to be associated with worse clinical outcome, irrespective of ER status, in a study of 97 postmenopausal patients with axillary lymph node metastasis treated with an antioestrogen for a period of 3 years after primary surgery and radiotherapy. Thus, adjuvant therapy with antioestrogens appears insufficient in this patient population with p53-positive tumours (Rahko *et al.* 2006).

In a study of 63 patients with locally advanced breast cancers receiving doxorubicin in a neoadjuvant study, there was strong evidence that specific mutations disrupting the zinc binding domains correlate with primary resistance to the drug, and the presence of such mutations was predictive of an early relapse (Aas *et al.* 1996). These findings were further supported in an updated study from the same group including 90 patients (Geisler *et al.* 2001). Of note, a number of these mutations were not associated with enhanced staining for p53, which would explain why immunohistochemical studies have been inconclusive.

Geisler *et al.* (2003) also investigated 35 patients with locally advanced breast cancer for Tp53 mutations before receiving combination chemotherapy with 5-fluorouracil and mitomycin. Mutations in the *Tp53* gene, in particular those affecting loop domains L2 or L3 of the p53 protein, were associated with lack of response to chemotherapy. On the other

hand, no statistically significant correlation between *Tp53* loss of heterozygosity (LOH) and response to therapy was seen. Together with the previous finding that such mutations predict resistance to doxorubicin, these data suggest that mutations affecting this particular domain of the p53 protein may cause resistance to several different cytotoxic compounds applied in breast cancer treatment.

In another study, p53 staining and mutations were studied in relation to the response of 67 breast tumours to neoadjuvant 5-fluorouracil epirubicin cyclosphate (FEC) or paclitaxel chemotherapy. In the FEC group, treatment failure was related to both the presence of Tp53 gene mutations and a positive IHC. Apoptosis was almost exclusively found in tumours having normal p53 in both parameters. In the paclitaxel group, treatment response was neither related to apoptosis nor to normal p53. Combination of sequencing and IHC results revealed a significant association between abnormal p53 and response to paclitaxel. The efficiency of paclitaxel during mitosis might be supported by lack of G_1 arrest due to p53 deficiency. This suggests that patients with p53-deficient tumours may benefit from paclitaxel (Kandioler-Eckersberger *et al.* 2000).

Rahko *et al.* (2003) examined the predictive relevance of a mutated p53 in a series of 254 samples from primary breast cancer patients. The response rate to anthracycline-based chemotherapy in metastatic disease was low in the p53-positive cases.

It has recently been suggested that the status of codon 72 polymorphism (resulting in a Pro or an Arg) could affect the response of cancer cells to chemotherapy, notably through a different interaction between p53 and P73 (Bergamaschi *et al.* 2003). For instance, breast cancer patients with the Pro/Pro variant may be less sensitive to anthracycline-based treatment than those with the Pro/Arg or Arg/Arg variant (Xu *et al.* 2005). More generally, the response of cancer cells to chemotherapy could be influenced not only by p53, but also by the status of a network that contains p53, p73 and perhaps the closely related p63. However, interactions between these three proteins are expected to be cell type-dependent (i.e. p63 is expressed mainly in basal/myoepithelial breast cells (Matos *et al.* 2005) and its role remains to be clearly established in breast cancer).

In summary, p53 mutations, particularly those affecting the DNA binding core regions, are generally associated with tumour cell resistance to

chemotherapeutic drugs, with the notable exception of mitotic spindle poisons. However, drug sensitivity of tumour cells might be related to additional parameters, as suggested by the higher qualitative and quantitative gene expression response of luminal cells to doxorubicin and 5-fluorouracil. The exact mechanisms underlying these different behaviours remain to be elucidated.

Radiotherapy

It is presently unclear whether the p53 status may provide an advantage in resistance to radiotherapy. Clinical studies examining the relationship between clinical radiosensitivity and tumour p53 status have largely failed to demonstrate a significant effect. Thus, many factors other than p53 status are expected to determine the sensitivity of tumour cells to irradiation (Ross 1999).

Additional comments on p53 and therapy

p53 isoforms

The difficulties in linking p53 status to the biological properties and drug sensitivity of cancer cells could be partly explained by the recently discovered differential expression of the p53 isoforms in cancer. Indeed, as previously observed for *Tp63* and *Tp73*, *Tp53* contains an alternative internal promoter in intron 4 and can transcribe 9 splice variants. p53 isoforms can bind differentially to promoters and can mediate p53 target gene expression and apoptosis. However, the pattern of isoform expression may vary from tumour to tumour (Bourdon *et al.* 2005), generating a complex landscape of possibilities.

p53 as a survival factor during therapy

According to a common view, p53 should sensitize tumour cells to therapy, as p53 is expected to trigger apoptotic events. However, it is likely that in some tumours the apoptotic function of p53, either mutated or not, could be lost, but not the ability of the protein to direct prolonged cell growth arrest and DNA repair. One may speculate that such a mechanism could explain why tumours expressing a mutant p53 are generally more sensitive to paclitaxel and other mitotic spindle poisons. In these cases, p53 could favour the recovery of cells damaged by therapy, thus acting as a survival factor preventing mitotic catastrophe, and p53 inhibitory therapies could be envisaged. Prediction

of tumour response to p53 inhibitors would require determination of the status of p53 in the tumour and, specifically, whether it can function to induce apoptosis (Gudkov & Komarova 2003, 2005).

p53-independent apoptosis

p53-independent apoptosis in response to IR and chemotherapy exists. It may be the consequence of mitotic catastrophe, which occurs after extended DNA damage. The mechanisms of mitotic catastrophe are unknown, but it likely results from a combination of deficient cell-cycle checkpoints (in particular the DNA structure checkpoints and the spindle assembly checkpoint) and cellular damage (Castedo *et al.* 2004). For instance, it has been shown that the primary mechanism of death in BCC lines exposed to the mitotic spindle poison docetaxel was mitotic catastrophe, as determined by scoring of micro nucleated cells and cells undergoing aberrant mitosis (Morse *et al.* 2005). More generally, there are indications that cells of epithelial tumours may often die by mitotic catastrophe during radiation therapy and chemotherapy (Hendry & West 1997).

Besides p53, another important determinant of breast cancer cell apoptosis is nuclear factor kappa B (NF-κB). It exerts strong anti-apoptotic functions in cancer cells. Many studies have demonstrated that inhibition of NF-κB activity by different means increased sensitivity of cancer cells to the apoptotic action of diverse effectors such as tumour necrosis factor-α (TNF-α) or chemo- or radiotherapies (Magné *et al.* 2006). Activation of NF-κB has been associated with ER negativity in tumours and cell lines. For instance, NF-κB was found to be constitutively active in the ER-negative MDA-MB-231 and MDA-MB-435 BCC, but not in the ER-positive MCF-7 and T-47D cell lines. This could partly explain the increased sensitivity of these latter two cell lines to most drugs and to IR (Nakshatri *et al.* 1997). Constitutive DNA binding of NF-κB was also observed with extracts from ER-negative, poorly differentiated primary breast tumours. As these tumours are frequently p53 mutated, some resistances attributed to p53 could, in fact, be due to NF-κB (Zhou *et al.* 2005).

The environment of tumour cells may also play a role in modulating the p53 response in these cells. For instance, activators of the PI-3K/AKT and NF-κB pathways might be produced by normal cells in the vicinity of tumour cells and contribute to the resistance of these cells to therapy.

p53 pathway-based therapies

The importance of p53 in cell death and the high frequency of mutations affecting this protein have generated a significant interest in exploiting the p53 pathway for novel cancer therapies. Various approaches have been exploited.

Small compounds have been used for the restoration of p53 function to lesions that carry full-length p53 protein with one amino acid change in the DNA-binding core domain. In theory, such compounds should only have an effect on cancer cells, because the core domain of wild-type p53 in normal cells is already structurally intact. Ellipticine (5,11-dimethyl-6*H*-pyrido[4,3-*b*]carbazole), the styrylquinazoline CP-31398, and PRIMA-1 (2,2-Bis(hydroxymethyl)-1-azabicyclo[2,2,2]octan-3-one) have been shown to restore function to a subset of p53 mutants (Foster *et al.* 1999, Bykov *et al.* 2002, Peng *et al.* 2003). PRIMA-1 (for p53 reactivation and induction of massive apoptosis-1) may synergize with chemotherapy (cisplatin) in inducing apoptosis in tumours, indicating the potential advantage of combined therapies (Bykov *et al.* 2005a). Screening of a chemical library identified another small molecule named RITA (for reactivation of p53 and induction of tumour cell apoptosis). It prevents p53-MDM2 interaction *in vitro* and *in vivo* and has anti-tumour activity (Issaeva *et al.* 2004). The maleimide-derived molecule, MIRA-1, can reactivate DNA binding and preserve the active conformation of mutant p53 protein *in vitro* and restore transcriptional transactivation to mutant p53 in living cells. The structural analogue MIRA-3 shows anti-tumour activity *in vivo* against human mutant p53-carrying tumour xenografts in SCID mice (Bykov *et al.* 2005b).

Attempts have been made to disrupt the p53-MDM2 interaction, thereby enhancing p53 activity. The first evidence that this approach could be successful came from peptide studies that culminated in the discovery of an optimized p53 octapeptide (Boettger *et al.* 1997). This, however, as well as the fungal metabolite cyclic peptide chlorofusin, was found to be poorly efficient *in vivo*. Small molecules have been designed to competitively inhibit the p53-MDM2 interaction. They include the following: synthetic chalcones (1,3-diphenyl-2-propen-1-ones), norbornane derivatives, cis-imidazoline derivatives (nutlins), a pyrazolidinedione sulphonamide, 1,4-benzodiazepine-2,5-diones, tryptophan derivatives, and the nine amino-acid peptide CDB3. The most promising drugs seem to

be the nutlins, as they have been shown to activate selectively the p53 pathway both *in vivo* and *in vitro* in human tumour cell lines that possess wild-type p53 and over-express MDM2, thus leading to growth inhibition and apoptosis. However, they appear to be significantly less cytotoxic to cancer cell lines harbouring mutant p53 (reviewed in Fischer & Lane 2004).

Chemosensitization of cancer cells has been obtained with anti sense oligonucleotides targeting the *MDM2* gene, which may inhibit the proliferation of tumour cells that possess wild-type as well as mutant p53 (reviewed in Bianco *et al.* 2005, Zhang *et al.* 2005). Radiosensitization has also been observed in cell lines from various cancers exposed to an anti-MDM2 oligonucleotide (Zhang *et al.* 2004). On the other hand, the use of an *MDM2* siRNA has also proved successful in inhibiting p53-dependent breast cancer (Liu *et al.* 2004*c*), suggesting that such molecules could be promising gene-specific drugs.

A 'global suppressor motif' involving codons 235, 239, and 240 has been identified in p53. With changes in these three amino acids, Baroni *et al.* (2004) were able to rescue 16 of 30 p53 cancer mutants. These rescued mutants are located within the beta-sandwich (codons 141, 157, 158, 163, 205, and 220), the L2 loop (codon 173), the L3 loop (codons 245 and 249), and the loop–sheet–helix motif (codons 272, 273, and 286), supporting the idea of a suppressor motif with a global rescue mechanism. Understanding the structural basis of this mechanism will allow the pursuit of small compounds able to achieve a similar stabilization of p53 cancer mutants.

ONYX-015 is a replication-conditional adeno-virus. It induces wild-type p53 response, which halts viral replication and allows the cell to survive. However, in cells expressing a mutant p53, ONYX-015 replicates freely, causing cell death. A similar lethal effect is observed if wild-type p53 expression is abrogated by high MDM2 expression (an indirect way of p53 inactivation in some tumours). Although ONYX-015 as a single agent did not impress in initial clinical trials (in head and neck, ovarian, prostate, and lung cancers), it is being actively pursued in combination with chemo- and radiotherapy (Haupt & Haupt 2004, Stoklsa & Golab 2005).

Most p53-based therapeutic approaches aim to restore p53 function. However, in some tumours, p53 could have lost its apoptotic function but not its ability to direct prolonged cell growth arrest and DNA repair. In such cases, p53 could favour the recovery of cells damaged by therapy and prevent them inducing a mitotic catastrophe. Thus, p53 inhibitory therapies could be of interest in such cases. One molecule able to inhibit p53 activity is pifithrin-α. (Gudkov & Komarova 2003, 2005), but it seems to have limited solubility (Gary & Jensen 2005). There is thus a need for additional specific and stable p53 inhibitors.

Learning how p53 controls apoptosis through its targets might help devise better cancer therapeutics and prognostic tests. For example, the expression of p53 apoptotic targets might predict the prognosis in p53 gene therapy or other therapies designed to reactivate p53 in tumour cells. Unlike p53, most p53 apoptotic targets are relatively rarely mutated in human cancer. Therefore, small molecules that can activate these genes independent of p53 might afford new anticancer therapies. Some of the p53 apoptotic targets, such as *PUMA*, exhibit higher potency in apoptosis induction than p53. They can potentially be used as targets for identifying such small molecules, or as targets for gene therapy (Yu & Zhang 2005). Interestingly, in a recent study, core biopsies were taken from nine patients with locally advanced breast cancer, before and at 6 h after initiation of doxorubicin-based chemotherapy. Both samples were co-hybridized on the same microarray containing 18 000 cDNA spots. The analysis revealed marked differences in gene expression profile between treated and untreated samples. The gene that was most frequently found to be differentially expressed was *PUMA*. This gene was up-regulated in eight of nine patients with an average factor of 1.80 (range, 1.36–2.73). Another p53-regulated gene, *FXDR*, was also found to be induced. *In vitro* MCF-7 breast cancer cells exposed to clinically achievable doxorubicin concentrations for 6 h also revealed marked induction of *PUMA* mRNA, together with *Tp53INP1* (Middelburg *et al.* 2005). Another potential candidate for therapy (notably based on siRNA) is *RAI3* (Nagahata *et al.* 2005).

General conclusion

The crucial role of p53 as a mediator of stress in various cell types is demonstrated; however, its contribution to breast cancer has been difficult to evaluate. Indeed, the number of functions that it controls, the diversity of its mutations, the multiplicity of the proteins constituting its 'interactome', and the genetic variability inherent to cancer cell

progression may result in a tumour suppressor effect as well as an oncogenic action of p53. As an illustration of this complexity, the link between p53 and prognosis and prediction remains largely unclear, despite numerous studies.

Further investigations are needed to determine under which conditions a therapeutic approach targeting p53 could be of real benefit to breast cancer patients. The potential importance of this approach is, however, underlined by the number of compounds that are being developed to increase p53 level and/or to correct the mutant protein.

Funding

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