Drugs Against Cancer: Stories of Discovery and the Quest for a Cure

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CHAPTER 32

The p53 story – guardian of the genome and the Li-Fraumeni Syndrome.

The most famous and perhaps most important discovery of a familial cancer syndrome was made in 1969 by Frederick Li and Joseph Fraumeni at NIH in Bethesda, Maryland (Li and Fraumeni, 1969) (Figures 32.1 and 32.2). The cause of the Li-Fraumeni syndrome was eventually traced to mutations in what came to be the most famous and most important of all cancer-causing genes, not only in its rare inherited form, but in at least half of all cancers. Mutation of the gene, *TP53* (*T* for transcription factor), was found to be a very frequent early step as normal cells in various tissues begin on their path to malignancy.

In their famous 1969 paper in the Annals of Internal Medicine, Li and Fraumeni reported on four families in which cancers were unusually common (Figure 32.1). What drew their attention to these families was that the cancers occurred at unusually young age and that each family had a pair of children who had rare soft-tissue sarcomas. By 1988, they had assembled data from 24 families, which confirmed their conclusions (Li et al., 1988). A mutated *TP53* gene evidently was highly potent, since several cancers of different kinds, including sarcomas, often occurred during an affected young person's life.

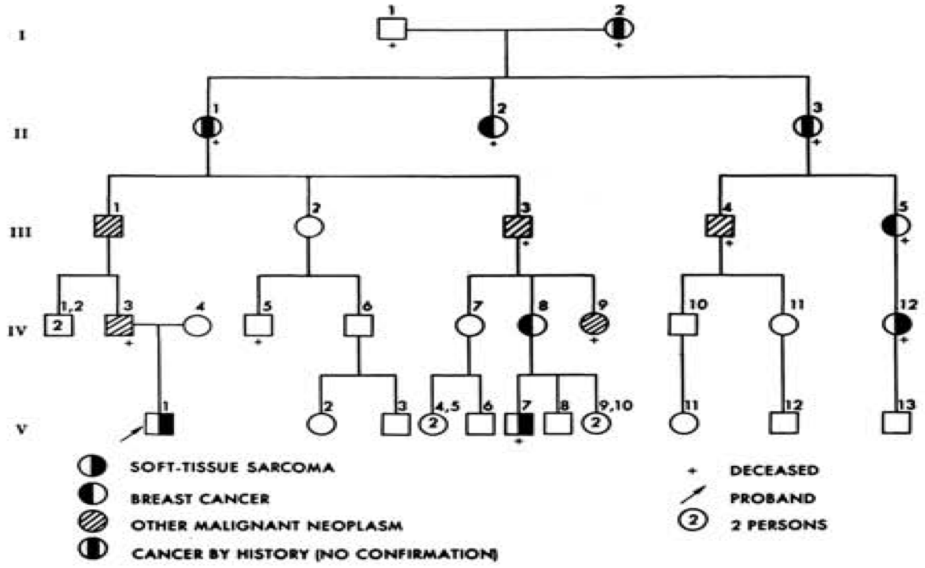


Figure 32.1. One of the cancer-prone families reported by Frederick Li and Joseph Fraumeni in 1969 (Li and Fraumeni, 1969). Both the frequency and the variety of cancers, including sarcomas, in members of this and their other cancer-prone families were remarkable.

Frederick Li and Joseph Fraumeni, 1991,

Figure 32.2. Frederick Li and Joseph Fraumeni, who in 1969 described the famelial cancer-prone syndrome that bears they name, and who later helped discover the mutations of the responsible gene, *TP53*.

The molecular unraveling of the Li-Fraumeni familial cancer disposition began in 1979, when Albert DeLeo, Wolfgang Dippold and Lloyd Old at Memorial Sloan-Kettering Cancer Center in New York City, together with Gilbert Jay, Ettore Appella, George Khoury, Garrett Dubois, and Lloyd Law at the National Cancer Institute in Bethesda, Maryland, found a protein, which they estimated to be 53,000-daltons in size, in various cancer tissues of the

mouse (DeLeo et al., 1979; Dippold et al., 1981; Jay et al., 1981) (Figure 32.3). They accordingly dubbed the protein p53 (although it later was found to be closer to 44,000 in size) – and more than 38,000 papers with "p53" in the title have since then been published. They also found a serologically similar protein in human cells, both normal and cancerous (Dippold et al., 1981).

In 1990, researchers at the Uniform Services University of Health Sciences and the National Cancer Institute in Bethesda, Maryland, analyzed the *TP53* gene of members of a Li-Fraumeni family (Srivastava et al., 1990). They found a single-base mutation, a G replaced by an A, in the gene in several family members but not in others. The mutation was in codon 245, which normally codes for glycine, but in the mutant coded for aspartate. This region of the *TP53* sequence, which codes for DNA binding, turned out to be a hot spot for mutations. The affected individuals had (in their non-cancer cells) the mutation in one chromosome but not in the other chromosome of the pair (Figure 32.4). Cancer would arise when the normal copy of *TP53* became deleted, leaving only the mutated copy. Thus, the cell would be left without a functional p53 and would lack the protein's tumor-suppressor function.

Codon 245 is in the DNA-binding region of the p53 protein (Figure 32.5), where most p53-inactivating mutations occur. Inactivating mutations in this region prevent p53 from binding to DNA, thereby abolishing p53's major functions.

Having a mutated *TP53* (as well as a normal copy of the gene) made the person a carrier of the Li-Fraumeni cancer-prone condition. Carriers developed cancer when one or more of their cells lost the normal copy of the gene. Characteristic of the Li-Fraumeni syndrome was that a great variety of cancers could develop and that they often developed at an early age (Malkin, 1993). Among the malignancies noted were childhood sarcomas (ordinarily a very rare disease), breast cancers, brain tumors, leukemias, and adrenal carcinomas. Individuals with an inherited loss-of-function-mutations in p53, as in Li-Fraumeni syndrome, had a 50% chance of developing cancer by age 30, when only 1% of the general population did so, and a 90% chance of developing cancer by 70 (Mullard, 2020).

TP53 was at first thought to be an oncogene, whose presence promoted cancer; only later was it realized that *TP53* was actually a tumor suppressor gene: it *inhibited* cancer formation (Levine, 1992). Another characteristic, however, was that the inactive mutated p53 protein was more stable than the normal p53; consequently, p53 concentration was usually higher in cancer than in normal tissues – but the high concentration did not signify cancer causation, because the p53 was mutated and inactive; its cancer suppression function was inactivated.

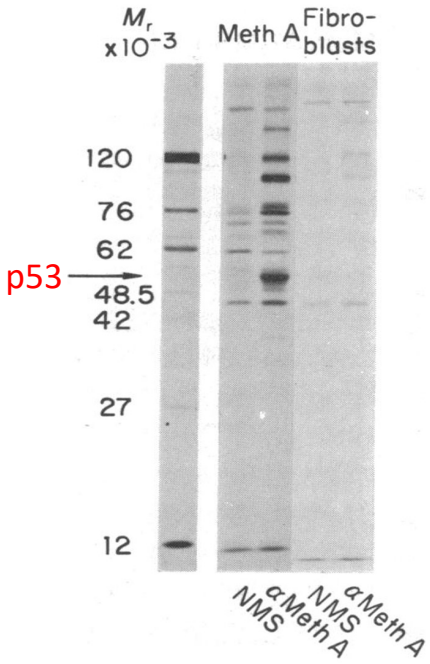


Figure 32.3. This may be the first published display of the p53 protein (DeLeo et al., 1979). It was a gel-electrophoresis experiment that separated proteins according to molecular weight. The p53 band showed up only in the lane labeled Meth A on top and αMeth A at bottom. This lane was loaded with radioactively (^{35}S-methionine)-labeled proteins extracted from a mouse sarcoma (Meth A); the extract was then incubated with Meth A antibodies, after which the resulting immune complexes were isolated and loaded onto the gel. The other 3 lanes in the group were controls showing that p53 did not show up in extracts from normal fibroblasts or when the antibodies came from normal mouse serum. (The lane at the left had molecular weight markers.) (From (DeLeo et al., 1979) with text in red added.)

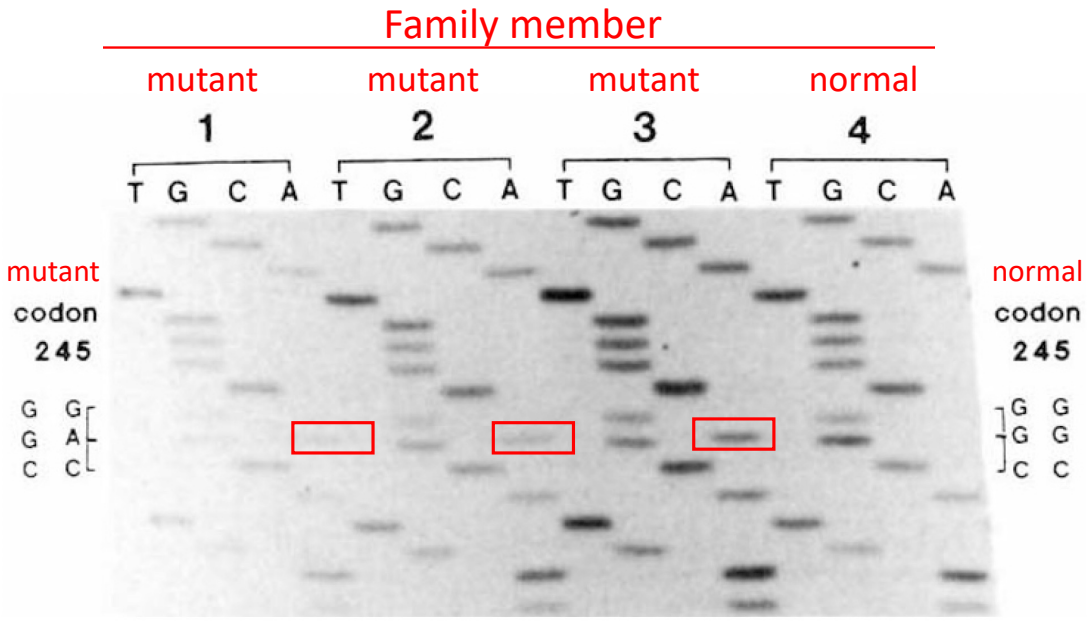


Figure 32.4. Mutations in the *TP53* gene in three members of a Li-Fraumeni cancer-prone family. The mutation was in one copy of the gene; the other copy – on the other chromosome of the pair – was normal. The affected individuals had both a normal and a mutant copy of *TP53* codon 245 (showing both a G and an A), one in each of the two chromosomes of the pair. The mutation changed a G to an A – which changed the codon from glycine G**G**C to aspartate G**A**C (red boxes). The fourth family member was normal and had a GGC sequence in both copies of codon 245. These DNA sequences were from non-cancer cells of the patients; their cancer cells would usually have one chromosome with the inherited mutation and the other chromosome deleted by a random cancer-causing event; thus, the cell had no functional *TP53* to suppress cancer formation. (From (Srivastava et al., 1990) with markings in red added.)

Another major finding came in 1988 from David Meek of the Salk Institute in San Diego, California; he found that cells often phosphorylate their p53 and identified several amino acids in the protein that were phosphorylated (Meek and Eckhart, 1988). This was the beginning of countless and seemingly endless studies in many laboratories disclosing the enormous complexity of the interactions of the p53 gene. Reviewing the intricacies of all these interactions in 2009, Meek, then at University of Dundee, UK, and Carl Anderson at Brookhaven National Laboratory in Upton, New York, displayed all known at the time in a diagram so complicated that I reproduce it here only to indicate the complexity of p53's functions (Meek and Anderson, 2009) (Figure 32.5.). We had represented some of these interactions in another way in a comparably complicated molecular interaction map (Kohn and Pommier, 2005) (Figure 32.6.). Another view of the central role of p53 in DNA repair according to Lindahl in 1995 is reproduces in Figure 32.7 (Lindahl et al., 1995) .

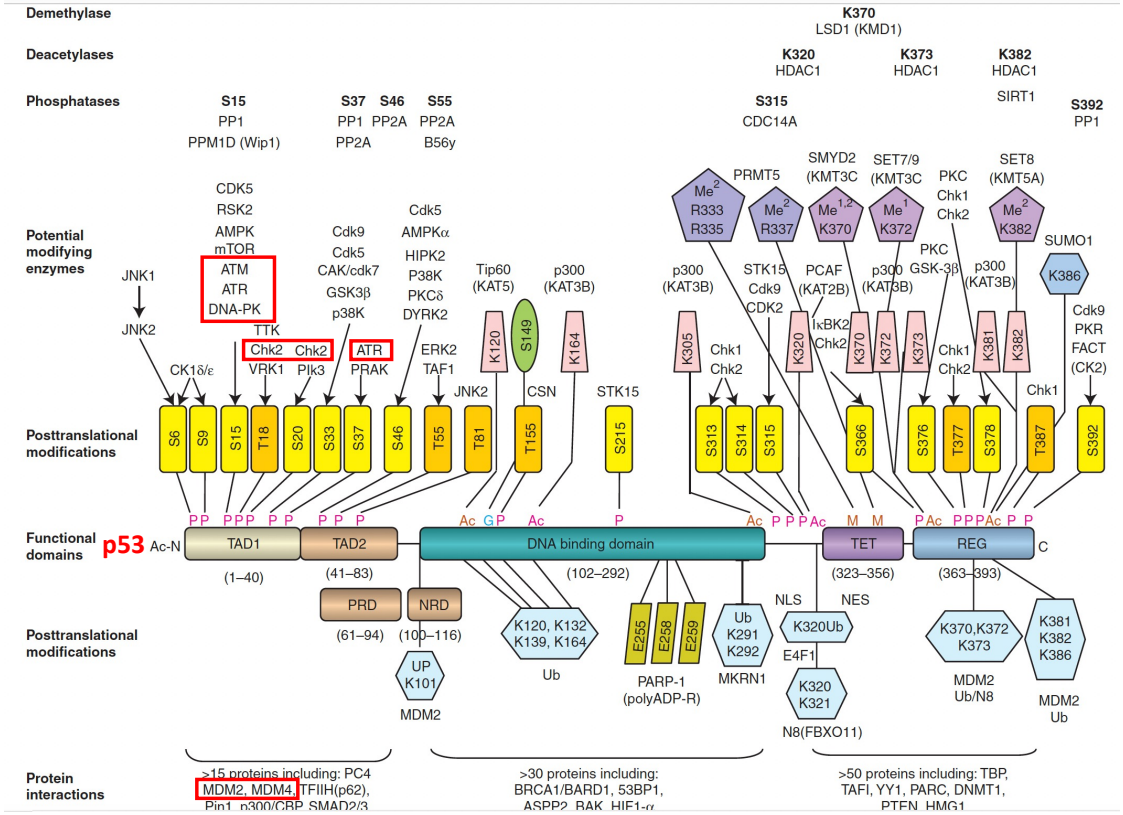
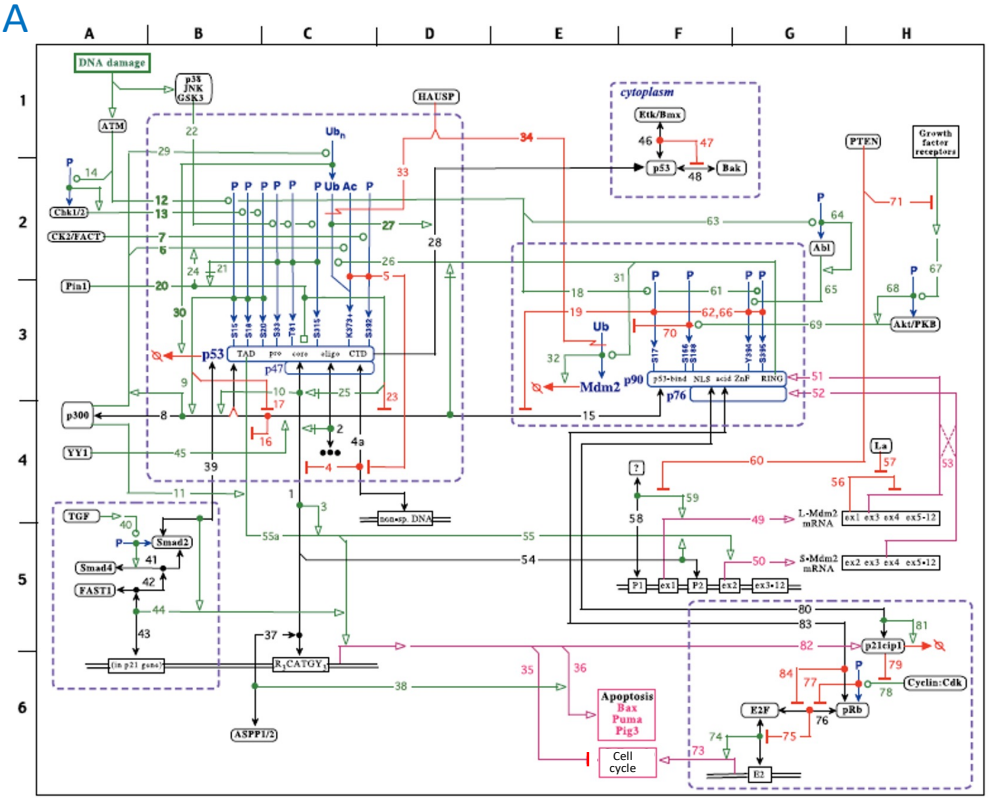


Figure 32.5. A comprehensive diagram by David Meek and Carl Anderson in 2009 of the then-known interactions of p53 (Figure from (Meek and Anderson, 2009) with markings in red added.)

A



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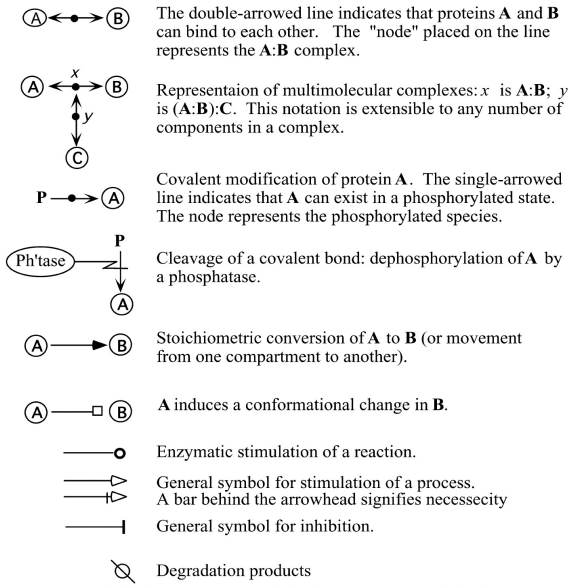

Figure 32.6. (A) Molecular interaction map focusing on p53 and Mdm2 (Kohn and Pommier, 2005). (Figure from (Kohn and Pommier, 2005) with correction made at bottom: reaction *73* stimulates the cell cycle, reaction *35* inhibits it.)

(B) The symbols used in the map (Kohn, 1999, 2001).

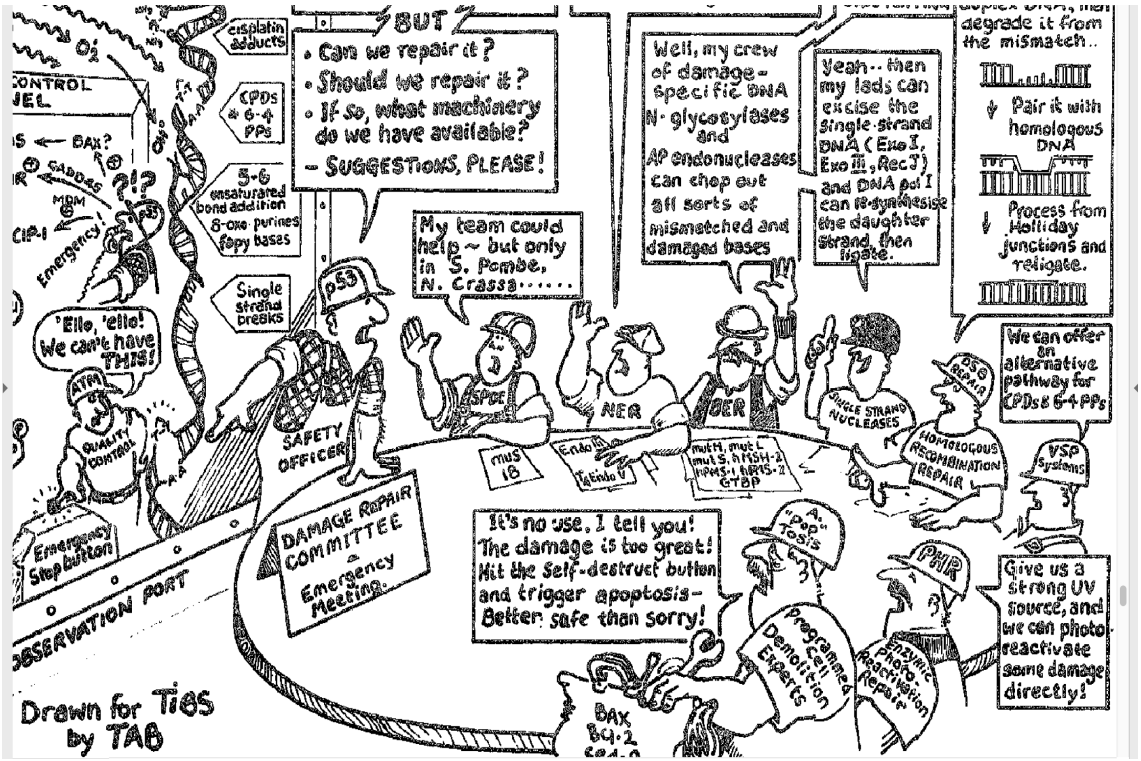

Figure 32.7. Part of a DNA damage repair cartoon showing the central role of p53 (Lindahl et al., 1995). (Drawn for *Trends in Biochemical Sciences,* October 1995, based on information from Tom Lindahl.)

The Mdm2 story complicates and clarifies the p53 story.

In 1991, Donna George and her colleagues at the University of Pennsylvania identified a gene, *Mdm2*, that became known to be a central controller of p53 function and cancer initiation. They isolated the gene from a mouse cell line that contained amplified genes present in numerous extrachromosomal nuclear bodies, called double minutes (Figure 32.8.). Working with that cell line for some time, George had noted that the presence of the double minutes seemed to be giving the cells a growth advantage, and she imagined that maybe the amplified DNA in those double minutes might contain a previously unknown oncogene. It may have seemed a far-out possibility but turned out to be spectacularly correct (Fakharzadeh et al., 1991; George and Powers, 1982).

Focusing on the DNA in the double minutes in their cell line, they isolated three genes that they called *Mdm1*, *Mdm2*, and *Mdm3* (mouse double minute). They cloned each gene and transfected it into mouse cells that they then implanted into mice to see if it would cause tumors in the mice. Of the three genes, only *Mdm2* produced tumors (Figure 32.9.)

(Fakharzadeh et al., 1991). Moreover, they found that humans had a gene that was homologous to the mouse *Mdm2*. So, whatever *Mdm2* did in mice, the human homolog likely did the same thing in humans.

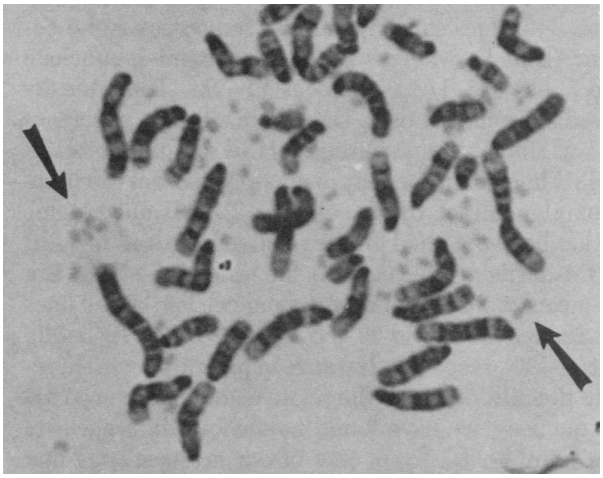

Figure 32.8. Chromosome spread from a mouse cell line containing extrachromosomal nuclear bodies (arrows) from which George and her colleagues later isolated the *Mdm2* gene (George and Powers, 1982). The double minutes lacked centromeres and therefore did not connect to the spindle during mitosis, enabling them to multiply and causing the genes they contain to become amplified. (From (George and Powers, 1982).)

Table I. Tumorigenicity testing of transfectants

Cell line	Tumorigenicity*	Cell line	Tumorigenicity*
N/mdm2	12/12**	R/mdm2	4/4
N/mdm1	1/8	R/mdm1	0/4
N/mdm3	0/8	R/mdm3	0/2
N/pCV001	0/8	R/pCV001	2/8

Figure 32.9. Donna George and her colleagues transfected each of the three genes they had isolated from double minutes into cell lines that they implanted into animals to see if tumors would be produced. Only mdm2 consistently produced tumors. The experiments were done in mice (N) and in rats (R). pCV001 was the empty vector that served as control. (From (Fakharzadeh et al., 1991).)

Well, what was it that made *Mdm2* an oncogene? It did not take long to find out. In a collaboration between Donna George at University of Pennsylvania and Arnold Levine at Princeton, they found that Mdm2 binds and inhibits p53 (Momand et al., 1992). They found that Mdm2 worked by inhibiting the tumor suppressor functions of p53, thus behaving as an oncogene.

The role of p53 in DNA damage repair -- synopsis

A key component of the DNA damage response network was found to be *TP53*, a gene that is mutated in more than half of all cancer cases, whose transcription product was the p53 protein. When DNA damage signals arrive, *TP53* was found to respond in two ways: its first action was to delay the onset of DNA replication, which gave more time for the DNA repair machinery to do its job. If the damage signal persisted, *TP53*'s second action was to activate genes that caused the cell to commit suicide in an orderly fashion (by apoptosis, which is Greek for the falling of Autumn leaves). When *TP53* was inactivated by mutation, as it was in most cancers, the cancer cells did not wait long enough for repair to be completed, and the cells entered mitosis while DNA damage was still in place; consequently, chromosomes would become scrambled. In some of the cells – perhaps only a small minority -- the resulting change in chromosome complement would give those cells a greater growth and metastasis potential. Thus, *TP53* mutation was found often to be an early step in the route to a malignant tumor.

Another, albeit less common, way that *TP53* function was impaired was by overexpression of Mdm2, for example by amplification of the *Mdm2* gene.

Initiation of apoptosis by TP53 can play a major part in the anti-tumor effect of chemotherapy; thus, cancers whose TP53 is inactivated by mutation do not respond well to cisplatin. The situation is not so simple, however, because sometimes the opposite is observed (O'Grady et al., 2014). In those cases, it may be that lack of TP53 functions in tumor cells allows them to start DNA replication before the DNA damage has been fully repaired; then most of the tumor cells undergo abnormal mitoses and die. If the cancer's *TP53* is defective, DNA damage is less likely to kill cancer cells by apoptosis, but more likely to kill the cells because they don't wait for DNA damage to be repaired before they start DNA replication. Thus, the dependence of the sensitivity of a tumor to a DNA-damaging drug, such as cisplatin, on TP53 function may depend on the balance of those two TP53-dependent actions -- cell cycle arrest or apoptosis -- in a particular tumor (Vogelstein et al., 2000).

Another p53 response to DNA damage was stimulation of DNA repair (Figure 32.7; (Lindahl et al., 1995)).

Molecular interaction map notation

The sections below describe some of the details of p53 function, as understood in 2005 (Kohn and Pommier, 2005), and refer to the molecular interaction map in Figure 32.6 that uses graph symbols defined in the lower part of the figure. The molecular species appear only once on a map and are connected by various types of lines to show their interactions, such as binding, phosphorylation, enzyme action, stimulation, and inhibition. The product of an interaction is represented by a small filled circle placed on a connecting line. For example, a node on a binding interaction line indicates the product of the binding (e.g., a dimer); a node on a phosphorylation line indicates the modified (e.g., phosphorylated) protein. When a line branches, it indicates alternative possibilities, such as competitive binding. The number on a line is used in the text (italicized in brackets) when referring to the function indicated by the line.

How p53 and Mdm2 respond in a controlled manner to DNA damage.

Cells that are not under stress, normally keep p53 function at a very low level. That is because p53 arrests the cell cycle and, if the stress is high or continuous with DNA damage, causes cells to die by apoptosis. Hence, p53 helps tissues – both normal and cancerous – survive DNA damage, but at a cost. Among the several molecular mechanisms that keep p53 function low, the two most import were binding of Mdm2 and phosphorylation of p53 (Koo et al., 2022).

How Mdm2 binds and inhibits p53, as we understood it in 2005, is shown by interaction *[15]* in Figure 32.6 (Kohn and Pommier, 2005). The N-terminal region of Mdm2 binds to the N-terminal region of p53, where p53 has transcription-activation domains (TAD). Mdm2 blocks those domains and prevents p53 from activating genes *[16]*, such as apoptosis gene *Bax [36]*, cell cycle arrest gene *p21cip1 [82]*, and *Mdm2* itself *[55]*. Thus, there was a negative feedback loop wherein p53 stimulated the production of Mdm2, and Mdm2 inhibited that action by binding p53 (McCoy et al., 2003; Weinberg et al., 2004).

Another likely control of p53 and Mdm2 was the degradation of both proteins in a ubiquitin (Ub) dependent manner (reactions *[30]* and *[32]*), which appeared to depend in a complicated way on several components of the network (Kohn and Pommier, 2005).

Further intricacies in the controls of p53 and Mdm2 were the effects of phosphorylations of several sites on both proteins. These actions implemented the responses to DNA damage via the activation ATM (the product of the ataxia telangiectasia gene discussed Chapter 29), as shown in the molecular interaction map (Figure 32.6). ATM responded to DNA double-strand breaks and acted in part through the cell-cycle checkpoint regulator, Chk2, *[14]*. A group of mostly parallel interactions (not shown in Figure 32.6) were carried out by the ATM-related protein, ATR, which responded to DNA single-strand breaks and acted via Chk1 in parallel to Chk2.

ATM, ATR, Chk2 and Chk1, as well as a few other protein kinases, were able to phosphorylate selected sites on p53 and Mdm2 with a variety of effects as shown in Figure 32.6. ATM phosphorylated serine-15 in the N-terminal region of p53, thereby blocking the binding of Mdm2 to that site (reactions *[12]* and *[17]*). ATM also phosphorylated Chk2 *[14]*, which in turn phosphorylated two additional p53 sites *[13]* that cooperated to block the binding and inhibition by Mdm2 of p53's transcription-activation domain (TAD) *[17]*. Consequently, ATM efficiently relieved p53 of Mdm2 binding and inhibition. But that's not all. ATM also phosphorylated Mdm2 *[18]* and did so in Mdm2's N-terminal region where Mdm2 binds p53, thereby blocking that binding. The net effect of all those phosphorylations was the robust activation of p53 by ATM in response to DNA damage.

Activated by DNA damage via ATM and/or ATR, p53 then acted as a tumor-suppressor by stimulating the transcription of genes exerting two major actions: (1) arrest of the cell cycle by the cyclin-dependent-kinase inhibitor gene *p21cip1* and (2) initiating cell demise by the apoptosis-activating genes *BAX* and *PUMA* (Tyteca et al., 2006). Cell cycle arrest helped normal cells survive DNA damage unscathed by allowing more time for repair; the cells were then less likely to sustain cancer-promoting mutations. The stimulation of apoptosis served as a backup to kill badly DNA-damaged cells that had a high likelihood of becoming cancerous.

The p21cip1/WAF1 story – cell cycle arrest in response to DNA damage.

In 1993, Wafiq El-Deiry, working in Bert Vogelstein's laboratory at Johns Hopkins School of Medicine in Baltimore, Maryland, was investigating how p53 suppresses cell division. They knew that p53 was often inactivated in human cancers, perhaps by mutation, and that normal p53 suppressed tumor growth. But how p53 suppressed cell division and tumor growth in response to DNA damage was yet unknown. There were some clues however: p53 could bind to DNA at certain sequences, and it could enhance transcription, but it was not known of which genes. That was what they sought to find out. They thought that the genes induced by p53 may mediate its biological role as a tumor suppressor (Vogelstein and Kinzler, 1992).

They found a gene whose expression was stimulated by normal but not by mutated p53 (El-Deiry, 2016; el-Deiry et al., 1993). Introducing the gene into human cancer cells, in the form of a cDNA, suppressed the growth of the cells. Also, they found that p53 in fact binds to the promoter region of the gene. They concluded that the gene's expression was induced by p53 and could be an important mediator of p53-dependent tumor growth suppression (el-Deiry et al., 1993) (Figure 32.10.).

They initially called the gene WAF1 after the researcher's first name, Wafiq (el-Deiry et al., 1993) – an unusual practice. The protein product of the gene had molecular weight 21,000 and came to be called p21cip1/WAF1; "cip1" for "cdk-interacting protein" was added when the protein was found to inhibit cyclin-dependent kinases (Harper et al., 1993) (reaction

[79] in the lower right of Figure 32.6). The use of the researcher's name as an eponym for the gene was criticized but still often continued, perhaps mostly by authors unaware of its eponymic origin. It nevertheless stimulated the whimsy of my lab colleague and friend, Al Fornace. When El-Deiry's first paper about WAF1 came out in 1993, Al looked over the collection of genes he had isolated and called "gadd" for "growth arrest and DNA damage"; he had isolated those genes on the basis of their being associated with DNA damage and arrest of cell growth (Fornace et al., 1992); he had investigated some of them, like gadd45, which became famous. And indeed, there it was, still waiting to be investigated, Wafiq's new gene. Whereupon Al quipped with some regret that, had he chosen to investigate that gene earlier, he could have called it ALF1!

In 1994, Patrick M. O'Connor, then a post-doctoral fellow in our Laboratory, and Wafik El-Deiry collaborated in studies of p21WAF1 that included members of Vogelstein's lab and my lab (el-Deiry et al., 1994). We already knew that p53 was a transcription factor and tumor suppressor that responded to DNA damage by arresting the cell cycle before onset of DNA synthesis and by initiating apoptosis. We also knew that p53 induced p21WAF1, and we wanted to find out whether p21WAF1 acted to arrest the cell cycle or to initiate apoptosis. At about that time, Wade Harper and Steve Elledge at Baylor College of Medicine in Houston, Texas, found a 21,000 molecular weight protein (Harper et al., 1993)in bound in protein complexes of cyclin-dependent kinases (cdk's) (Harper et al., 1993). The cdk's, consisting of a kinase whose activity depended on it being bound to a cyclin protein, moved the cell cycle through the G1/S transition where DNA synthesis begins. The 21,000 dalton protein bound cdk's, and they called the protein CIP1 for cdk-interacting protein. It was the same as p21WAF1 and thus came to be called p21cip1. We found that DNA damage triggered p53 to induce p21cip1, which then bound to cdk's and blocked the cell cycle at G1/S. The path from p21cip1 to effect on cell cycle includes a sequence of three inhibitory steps ([*79, 77, 75*], bottom right in Figure 32.6A) the net effect of which is inhibition. (Step [*35*] is a redundant indicator of this.)

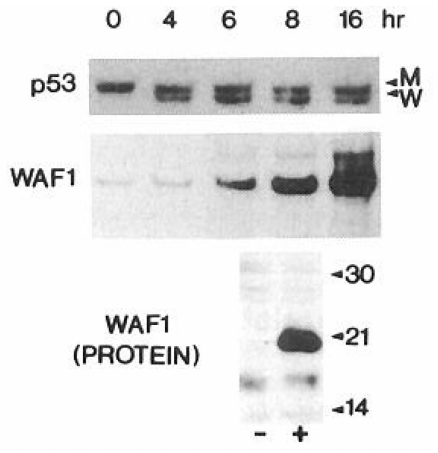

Figure 32.10. Identification of p21cip1 (also called WAF1) as a 21,000 molecular weight protein whose expression was stimulated by a normal (W) but not by mutated (M) p53

gene (el-Deiry et al., 1993). The cells were treated with dexamethasone to stimulate gene expression. The cell's p53 gene was inactive because of a mutation. However, a normal p53 was induced in the cells by way of a cDNA. The upper part of the figure shows that the inactive mutant gene (M) was already expressed at zero hours, while the normal "wild type" gene (W) began to be by or before 4 hours. The p21cip1 gene (WAF1) began to be expressed increasingly after 6 hours; hence its expression became induced by normal p53 but not by the mutant p53. The lower part of the figure shows that the p21cip1 (WAF) protein was relatively pure and had a molecular weight of 21,000. (From (el-Deiry et al., 1993).)

Activation of apoptosis by p53 in response to DNA damage.

In addition to cell cycle arrest, the second major tumor-suppressor action of p53 was to activate the transcription of genes, such as BAX and PUMA, that stimulated apoptosis of cells that had persistent DNA damage. The molecular interaction map in Figure 32.6 showed phosphorylations that controlled gene expression by p53 generally. Since then, however, acetylation sites were discovered that controlled apoptosis-inducing genes specifically. But the control and effects of an increased number of phosphorylation and acetylation sites on p53 has made the full story too complicated for me to try to unravel here (Sabapathy and Lane, 2019). We could note, by the way, that phosphorylation and acetylation have similar effects on the electrostatic environment of the proteins they bind: phosphorylation adds a negative charge to the serine or threonine they bind: acetylation removes a positive charge from the lysine it binds.

An important aspect of the control of apoptosis by p53 was uncovered soon after the map in Figure 32.6 was made (Sykes et al., 2006; Tang et al., 2006; Tyteca et al., 2006). In its response to DNA damage, p53 was shown to become acetylated at a lysine located in p53's DNA-binding domain (K120) by an enzyme known as Tip60 (as well as by a closely related enzyme). If the lysine was mutated to arginine (K120R), the mutated p53 could still bind to DNA and activate the transcription of p21cip1 and arrest the cell cycle, but it could no longer activate the transcription of BAX or PUMA and cause apoptosis. Lysine (K) and arginine (R) both bear a positive charge and, when lysine becomes acetylated, it loses its positive charge. Moreover, the K120R (lysine to arginine) mutation still allowed the p53 to bind to the *BAX* and *PUMA* promoters but could not induce them to make the proteins. It is as if the positive charge was required for p53 to bind to the *BAX* and *PUMA* promoters (as well as to the *p21cip1* 2awpromoter) but had to be removed to activate specifically the former two promoters. Thus, K120 acetylation of p53 seemed to tip the balance of the DNA damage response toward apoptosis as opposed to cell cycle arrest (Tyteca et al., 2006).

More recently, many additional acetylation sites on p53 have been found and another class of acetylating enzymes has been characterized, exemplifies by an enzyme called p300 (Xia et al., 2022). The molecular interaction map in Figure 32.6 already showed p300 and some of its actions on p53. Reaction [*5*] indicated its acetylation of several lysine sites on p53. This acetylation was favored by p300 binding to the N-terminal region of p53 at a site

where Mdm2 also binds (reaction [*8*]); the two proteins may compete for binding in this region. p300 was found to bind directly to p53 and to acetylate several lysines in the p53 C-terminal region (Xia et al., 2022) as shown in the map. However, it did not acetylate K120, which was an unusual site that was acetylated only by the Tip60 family. The role of this increasingly complex set of acetylations on the tumor suppressor function of p53 however still remained to be determined.

Therapy by targeting the p53-Mdm2 interaction.

In 1999, Arnold J. Levine, who had led a team 20 years earlier to discover p53, proposed a novel strategy for cancer therapy based on inhibiting the binding between p53 and Mdm2. The *TP53* gene was known to be mutated in about half of cancers that arose in part due to lack of the mutated p53 protein's functions: primarily the DNA damage responses of cell cycle arrest and apoptosis, which normally prevented tissue cells from becoming cancerous. Most of the other cancers, whose *TP53* was normal, had overactive Mdm2, often due to amplification of the *Mdm2* gene, which suppressed p53 excessively. The new strategy was to inhibit p53-Mdm2 binding with the idea to relieve the excessive suppression of p53 and allow its cell cycle and apoptosis effects to act against those cancers. Based on that idea, researchers at Hoffmann-LaRoche in Nutley, New Jersey, embarked on efforts to find such inhibitors (Vassilev et al., 2004). They expected that a small molecule inhibitor could be effective because of the way p53 bound to the Mdm2 structure (Figure 32.11): the Mdm2 protein had a deep hydrophobic groove into which part of a hydrophobic amino acid chain of p53 could bind securely. They hoped to find a molecule that would bind in that groove and prevent p53's peptide chain from binding there.

They began by screening a large number of divers synthetic compounds and found a few that exhibited some degree of Mdm2 binding. Modifications of those compounds to optimize their binding strength and specificity led to potentially useful inhibitors that they called nutlins (for Nutley inhibitor) (Figure 32.12). The nutlins prevented the inhibition of p53 by Mdm2 and allowed the p53, as expected, to inhibit the cell cycle and to kill cancer cells that had normal p53, but not those that had mutated p53 (Vassilev et al., 2004).

The discovery of p53-Mdm2 inhibitors after much hard work "was spectacular stuff," recalls Michael Andreeff, an oncologist at MD Anderson Cancer Center. "I was super impressed, and I jumped up and down when I saw it in *Science*" (Mullard, 2020).

Pharmaceutical companies worked hard to develop p53-Mdm2 binding inhibitors good enough for clinical use, but, although better and more promising compounds were made, clinical trials were disappointing and discouraging. Many chemical variants were tried and the outcomes with the resulting new compounds were thoroughly reviewed in 2022, but none of potential drugs had so far passed beyond phase-3 clinical trial (Koo et al., 2022).

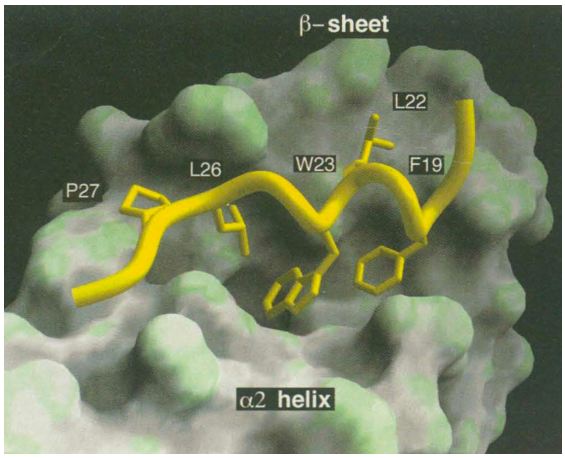

Figure 32.11. Structure of a deep hydrophobic groove in the Mdm2 protein where an amino acid chain of p53 binds (Kussie et al., 1996). Three hydrophobic amino acids of the p53 chain (yellow) bind in the groove; they are phenylalanine-19, tryptophane-23, and leucine-26. The aromatic rings of F19 and W23 lie face-to-face in the groove; the consequent interaction between the rings helps stabilize the binding structure.

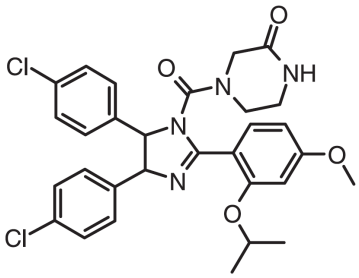

Figure 32.12. The chemical structure of nutlin-3, the strongest inhibitor of p53-Mdm2 binding among the three chemical relatives, called nutlins, made by Hoffmann-LaRoche scientists in 2004 (Vassilev et al., 2004). The compound had two enantiomer (mirror-image) forms, of which only one, called nutlin-3a, was active -- because only that one had the proper 3-dimentional structure to fit in Mdm2's p53-binding groove. (The mirror-image symmetry arises from the two asymmetric carbon atoms in the 5-membered ring.)

Additional control of p53: Mdmx/Mdm4.

As guardian of the genome and suppressor of cancer, p53 helps repair damaged DNA in cells that have sustained such damage or to kill cells with excessive DNA damage. When a cell detects DNA damage, its p53 becomes activated. However, p53 activation becomes harmful to cells undergoing normal cell division in tissues, especially during the development of the embryo. To keep p53 inactive, as we have seen, is the job of Mdm2. Evidently, this job was so critical that evolution added another actor, called Mdmx or Mdm4, that refines and further complicates the control network.

In 1996, researchers in the laboratory of A. G. Jochemsen in Leiden, the Netherlands, discovered a protein, similar to Mdm2, that also bound and inhibited p53. They called the new protein Mdmx (Shvarts et al., 1997). The p53-binding domains of the two proteins had 53.6% amino acid identity (Figure 32.13), and the same amino acids in p53 mediated p53-Mdm2 and p53-Mdmx binding.

In critical cell types, both Mdm2 and Mdmx were required to fully inhibit p53 activity. There were however significant differences between the actions of the two p53 inhibitors (Marine et al., 2007). p53 stimulated the transcription of Mdm2 but not Mdmx. Absence of Mdmx induced the consequent increase in p53 to transcribe more Mdm2, thereby partially making up for the lack of Mdmx. That was also why absence of Mdm2 produced greater increase in p53 and more severe consequences than absence of Mdmx. Also contributing to that difference was that, in contrast to Mdm2, Mdmx did not induce ubiquitylation and degradation of p53. Mdm2 and Mdmx were able to bind to each other via their RING domains (Figure 32.13). Mdm2 could ubiquitylate Mdmx, as well as itself by way of its RING domain, leading to degradation of both proteins (Marine et al., 2007).

It is remarkable that the TP53 gene is inactivated by mutation in most cancers, and, moreover, that in most of the cancers whose TP53 gene is normal, p53 (the product of the TP53 genes) is nevertheless inactivated by Mdm2 and/or Mdmx; full inactivation of p53 required both Mdm2 and Mdmx.

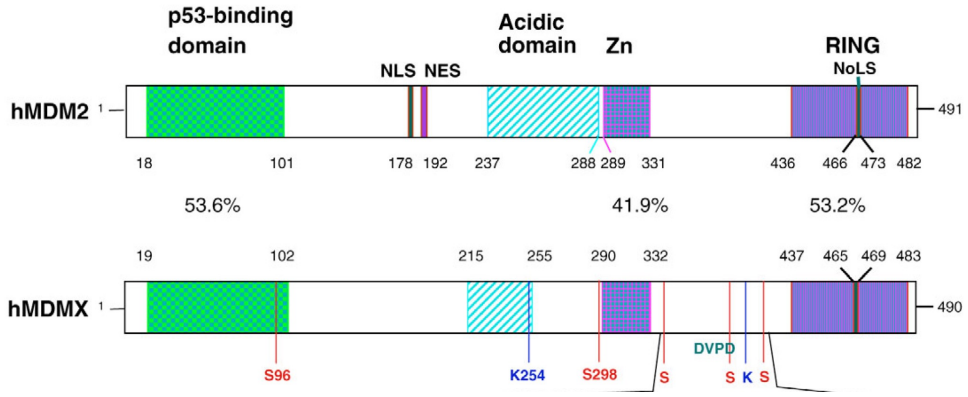

Figure 32.13. Comparison of the domain structures of the human Mdm2 and Mdmx proteins. The greatest similarities were in the p53-binding domain (53.6% amino acid identity) and the RING domain (53.2% amino acid identity). From (Marine et al., 2007)).

The p53-Mdm2-Mdmx interaction network and feedback regulation loop.

Mdmx added complexity to the p53 control network, but also added new opportunities for therapy. In response to DNA damage, ATM can phosphorylate Mdm2 (reaction *[18]* in Figure 32.6) and inhibit the p53-Mdm2 binding (reaction *[19]*). This would release the inhibition of p53, which could then activate the transcription of Mdm2 (reactions *[54]* and *[55]*) and to a lesser extent Mdmx. The abundance of Mdm2 and Mdmx however initially decreases, due to their ATM-induced ubiquitin ligase activity, which leads to degradation of both components of Mdm2-Mdmx dimers (Phillips et al., 2010). p53 can then resume production of Mdm2 and Mdmx, which then limits the duration of p53 activity.

The intricacies of the p53-Mdm2-Mdmx interaction network are explained in Figure 32.14. We simulated versions of the network and found domains of parameter space where p53 may have switch-like behavior or oscillations (Kim et al., 2010). It is perhaps not surprising that the network can have multiple complex behaviors. Particularly interesting and possibly important was our finding that p53 activity could switch on relatively suddenly in response to small changes in the reaction rate parameters, as might occur in response to DNA damage.

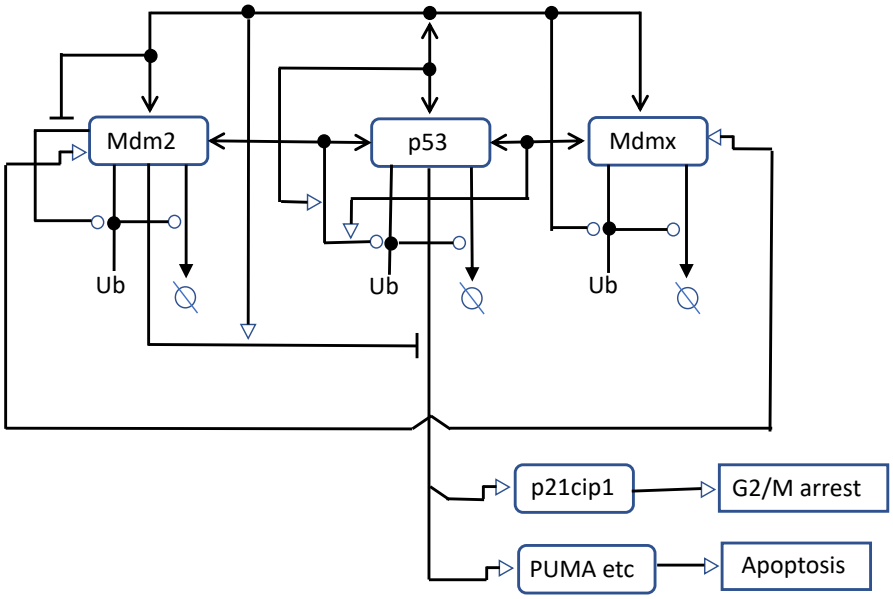

Figure 32.14. The p53-Mdm2-Mdmx interaction network displayed using the molecular interaction map notation. (Please see Figure 32.6 for symbol definitions.) The map is based on information in (Yu et al., 2020). The map shows p53 activating the transcription of p21cip1, leading to cell cycle arrest at G2/M, and activation of PUMA and other genes, leading to cell death by apoptosis. The transcription activity of p53 is inhibited by Mdm2 and further suppressed by Mdmx, which can bind Mdm2. Mdm2, p53, and Mdmx are degraded after being ubiquitinated by Mdm2. The auto-ubiquitination of Mdm2, however is inhibited by Mdmx, while the ubiquitination of p53 is enhanced by Mdmx.

Mdmx as a therapy target

As already described above, much effort to target the p53-Mdm2 interaction with Mdm2-targeted inhibitors had met with limited therapeutic success. When researchers became aware of the role of Mdmx in p53 regulation, their interest shifted to developing Mdmx-targeted inhibitors. Mdmx was frequently amplified and overexpressed in various cancers and seemed to contribute to the malignancy: it was reported to be overexpressed in a remarkably wide range of cancers, including some cases of breast (19%), colon (19%), lung (18%), stomach (36%), bone osteosarcoma (71%), brain, and thyroid cancers, as well as melanomas and some leukemias and lymphomas (Yu et al., 2020).

Many Mdmx-targeted inhibitors of different steps of the interaction network were obtained and some of some of them had promising therapeutic potential. Inhibition of Mdmx would restore the tumor-suppressor actions of p53 and suppress the tumor-promoting actions of Mdm2. The Mdmx-targeted strategies to meet those goals aimed to (1) block the p53-MDMX interaction, (2) inhibit MDMX expression, and (3) induce MDMX degradation (Yu et al., 2020). These strategies led to the development of Mdmx-targeted drugs that inhibited human xenograft tumors in mice, but clinical trials against human cancer had not yet been reported.

Addendum: Nutlin-3 in CellMiner.

CellMiner is a set of database analysis tools that can relate (among other things) the activity of a drug with expression or mutation of genes in cell lines (see Chapter 20). I applied these tools to elicit relationships between nutlin-3 activity and *TP53* expression or mutation (Figure 32.15). Although previous studies of p53-Mdm2 binding inhibitors focused on acute myeloid leukemia (AML), Figure 32.15 suggests that the nutlin-3 activity relationships may apply as well to acute lymphocytic leukemia (ALL) and non-Hodgkins lymphoma (NHL) cell lines and that there is a subgroup of cell lines with high nutlin-3 response and high expression of non-mutated p53; those may be the lines driven by high *Mdm2* expression. (See legend of Figure 32.15.) Therefore, patients potentially responsive to nutlin-3 or other p53-Mdm2-binding inhibitors might be identified as having high *Mdm2* expression but no inactivating *TP53* mutation.

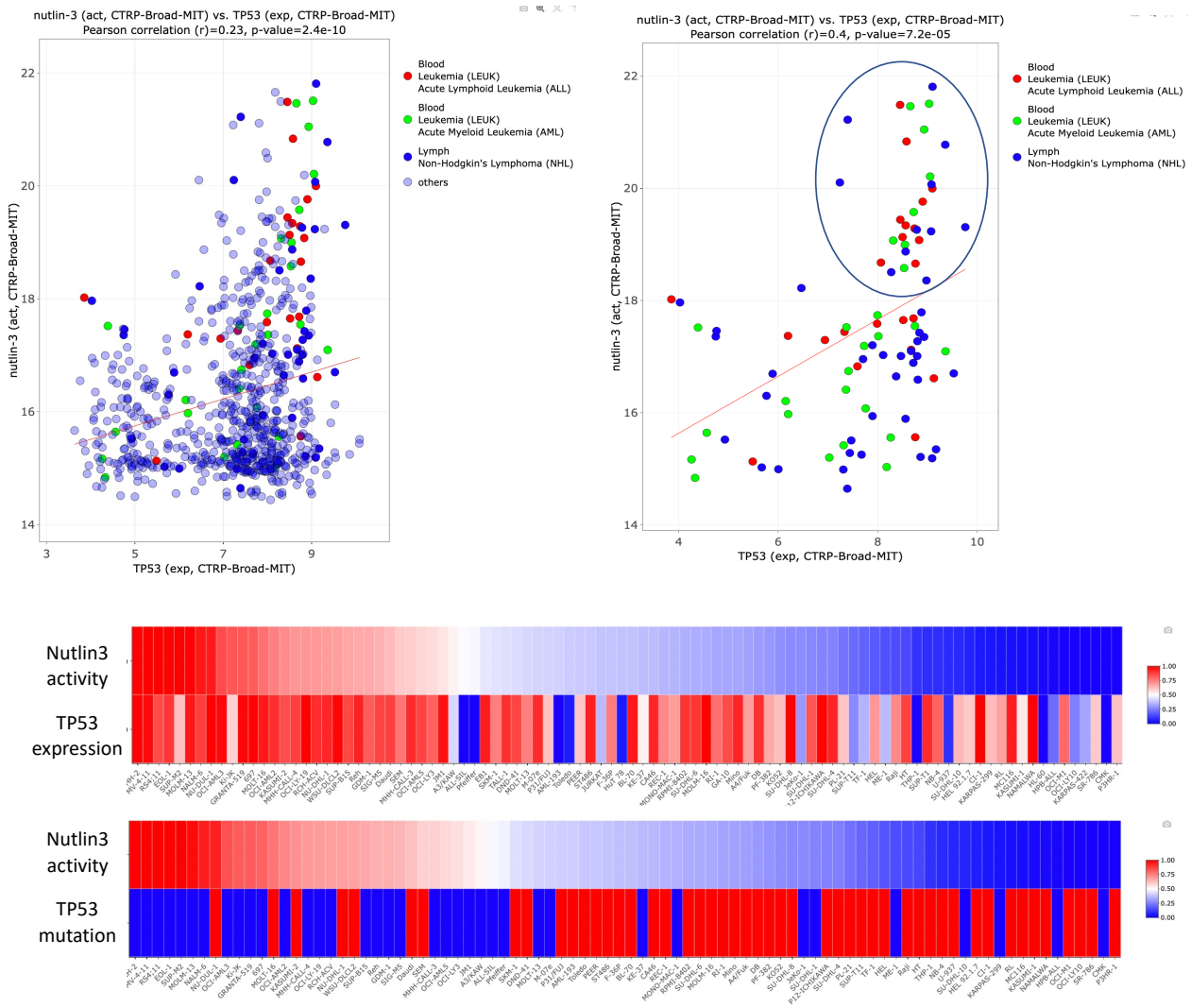

Figure 32.15. Activity of Mdm2-inhibitor nutlin3 correlated positively with *TP53* expression and negatively with *TP53* mutation in acute lymphocytic leukemia (ALL), acute myeloid leukemia (AML), and non-Hodgkins lymphoma (NHL).
From CellMinerCDB version 1.5 (April 2022 release); cell line dataset CTRP-Broad-MIT.
Upper left: nutlin3 activity versus *TP53* expression for all cell lines.
Upper right: nutlin3 activity versus *TP53* expression for ALL, AML, and NHL cell lines. A subset of the cell lines (within the oval) showed high nutlin-3 sensitivity and high *TP53* expression. The *TP53* in most of these lines was non-mutated (bottom, lower).
Bottom: (*upper*) nutlin3 activity and *TP53* expression; red-to-blue is high-to-low.
(*lower*) nutlin3 activity and *TP53* mutation (red, mutated; blue, not mutated).
High nutlin-3 sensitivity (red) correlated with absence of *TP53* mutation (blue).

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