25 YEARS OF P53 RESEARCH

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FOREWORD TO THE PAPERBACK EDITION

This book was assembled in 2004-2005 to mark the 25th anniversary of the discovery of p53 and to summarize the current knowledge on the multiple roles of this protein as a central tumour suppressor with potentially great clinical impact. Through 20 chapters authored by the scientists who are leading the field, this book provides an overview of how p53 rose to the status of « superstar » among cancer-related genes, catching the essential lessons of 25 years of research to identify major paths towards applications.

This paperback edition has two ambitions. The first is to make the p53 research field accessible to the largest community of scientists, clinicians, and biology and medical students. The second is to stimulate the development of clinical applications by outlining a « roadmap » for translational research.

It is probably fair to say that p53 is the most studied protein in the whole history of cancer biology so far. The knowledge accumulated on its structure, biochemistry, function and mutation in cancer is unprecedented in its detail and complexity. This information represents a gold mine in the search for novel ways to approach, detect and manage cancer. However, the p53 field is difficult to grasp for the non-p53 specialist and its complexity may act as a deterrent for clinical applications. The fact that mutations in p53 are diverse in their biological effects and that they may occur at many different stages during tumor evolution makes it impossible to derive simple messages uniformly applicable to all clinical contexts. Thus, there are several roadblocks on the path towards the clinic. Here we highlight certain key problems and questions that must be addressed in order to facilitate the clinical exploitation of p53:

1. Communication, awareness and access to information: Given the complexity of the field and the fact that data pertaining to each particular aspects of p53 biology or deregulation are scattered in many different publications, it is extremely difficult to access the full scale of relevant information of any specific p53-related topic. Review arcticles, despite their fundamental role in disseminating knowledge, usually focus only on general mechanisms and do not discuss in detail the many variations that can occur with respect to cell type, particular mutation type, as well as biological activation context. Books such as this one may help in this task by putting into perspective both general considerations on the p53 pathway and more specific information on various aspects of p53. In the longer term, however, open access to p53 complexity will require the development of knowledge bases accessible through the web and using simple navigation tools to guide users towards the specific information they need. Several efforts are currently being developed in that direction. They need to be strenghtened and better integrated within the rapidly growing galaxy of web-based information sources on molecular and individual variations in cancer.
2. Reference functional assays and structural analysis: Given the huge diversity of cellular and animal models for wild-type or mutant p53 functions, it will be important to set up standard, universally accepted assays to measure critical p53 protein functions. Yeast-based transcriptional assays, for example, have proven extremely useful to measure residual transcriptional activities of all known p53 mutants in a controlled system. However, because yeast cells do not share the sophisticated and intricated proliferation and apoptosis control systems of mammalian cells, yeast assays lack the sensitivity and specificity for measuring biologically relevant functional effects of human p53. The availability of affordable, universal mammalian cell-based assays to measure key p53 properties such as growth suppression, induction of apoptosis, dominant-negative effects of mutant p53 over wild-type and gain-of-function properties of mutant p53, would greatly boost research on designer drugs capable of rehabilitating the p53 pathway or enhancing p53-dependent growth suppression in cancer cells. Also, more detailed information on the structural consequences of specific p53 mutations from structural analysis by NMR and/or X-ray crystallography should complement the information obtained in functional assay to provide a full characterization of the most common mutant p53 isoforms.

3. Alternative mechanisms for inactivation of p53: Based on current evidence on the central tumour suppressor role of p53, one may wonder how cancer can develop from cells that retain apparently wild-type and functional p53 genes. Such cells must develop elaborate systems to trap their p53 in an off-side position, or to bypass its effect by compensating mutations in other effectors. There is solid evidence that several alternative mechanisms exist in cancer cells that inactivate the function of p53 in the absence of a mutation. These mechanisms include interactions with cellular or viral proteins, competition with paralogues such as isoforms of p63 or p73, and perhaps also competition between full-length p53 and several of its own recently discovered isoforms that lack the N-terminus required for transactivation functions. Elucidating these mechanisms is critical for the correct exploitation of p53 in the clinic. Indeed, it is possible that many cancers with wild-type p53 carry alterations in the p53 pathway that are as detrimental as inactivating mutations in the p53 gene. Further research on these aspects is required to make sure that, in future clinical trials, patients are stratified in an appropriate way with respect to the degree of deficiency in the p53 pathway.
4. Understanding and using the potential of p53 as a target in combination therapy: As stressed by George Klein in chapter 20 of this book, the idea behind combination therapy is seductively simple: if the frequency of cells resistant to drug A is 10^{-6} and resistance to drug B occurs with the same probability, the frequency of doubly resistant cells is 10^{-12}. Achieveing such low frequency of resistance would greatly increase the chances of eradicating a tumor. The availability of small molecules that either activate wild-type p53 or restore wild-type function to mutant p53 opens unprecedented opportunities for new concepts in combination chemotherapy. The combined use of drugs restoring or enhancing the activity of p53 as a critical inducer of apoptosis may increase the therapeutic efficacy of many current chemotherapy protocols with only limited side effects. Developing and validating treatment modalities based on such an approach should be considered a priority. Indeed, in contrast to many « new drug » developements, this approach is not an alternative to classical chemotherapy. It is a complementary approach, which will allow reduced cytotoxicity and increase the efficiency of current protocols and should therefore be relatively easy to incorporate within existing therapeutic regimens.
5. Coordination of p53 clinical trials: Despite overwhelming experimental evidence that p53 is a major effector in DNA

damage-induced apoptosis, we still have a very fragmentary knowledge of the significance of p53 status for predicting treatment responses and for selecting therapeutic options. This is due, at least in part, to the fact that most clinical studies so far have lacked the necessary structured design and statistical power. This can be achieved only in the context of large, structured clinical trials in which patients are recruited on the basis of specific inclusion criteria, randomized for treatment according to determined regimens, and followed up for long-term therapeutic and clinical end-points. Moreover, it is essential that such trials are not run as separate studies: the detailed understanding of the exact significance of p53 status in the clinic will derive from pooled analyses and meta-analyses assessing the strenght of evidence across large datasets and different study contexts. Databases such as the current p53 mutation databases have a critical role in collecting, structuring and disseminating such data. Trial design and coordination and efficient use of available databases will of course also be critical in clinical studies of novel compounds that target wild-type or mutant p53.

After 25 years of research, p53 has had a tremendous impact on our understanding of the molecular biology of cancer. As this knowledge develops to reveal more and more intricate pathways, the p53 protein will continue to be the « Ariadne thread » pointing out new routes in the maze of cancer biology. But the greatest hope for the 25 years to come is that concerted efforts to remove roadblocks for clinical applications will result in the effcicient transfer of p53 know-how from he lab to the bedside. We hope that this book will, it its own way, contribute to this objective by opening up the « p53 box » to the scientific and medical community.

August 30, 2007
Pierre Hainaut and Klas G. Wiman

Chapter 1

THE FIRST TWENTY-FIVE YEARS OF P53 RESEARCH

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SETTING THE STAGE

During the 1960s, the field of cancer research lacked clear direction. Several facts appeared to be well-established and correct, but the relationships among these observations were not apparent. Fifty years of research had demonstrated that viruses with both DNA and RNA genomes could cause cancer in animals. Over the next 45 years six new viruses were to be discovered that were able to initiate cancers in humans (Epstein-Barr Virus, Human T-Cell Leukemia Virus, Hepatitis B and C Viruses, Kaposi Sarcoma Virus and the Papilloma Viruses) (McKinnel et al., 1998). It was equally clear from the perspective of the 1960s that certain chemicals, when applied to animals, were able to initiate cancers (Yamagawa et al., 1918). Chemical carcinogenesis was a field both separate and distinct (both in the experiments one did and the experimentalists who did them) from viral carcinogenesis and very few scientists thought to find a common ground between concepts generated in each field. Thirdly, the study of mouse genetics demonstrated that some cancers were clearly inherited and these observations confirmed many prior publications that suggested a role for cancer causing genes in humans and other animals (DeOme, 1965). Finally epidemiologists, studying a variety of important variables that predispose humans to developing cancers, had made the very striking observation that the rates of cancer incidence increase exponentially with age and begin to rise dramatically by the fifth and sixth decade of life (Miller, 1991). While these four observations were all accepted facts the relationship between

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these concepts was not clear and researchers who studied viruses hardly ever discussed chemicals and those who thought about genes and viruses didn't know what to make of aging as an important variable. Literally researchers from each of these fields, virology, chemical carcinogenesis, genetics and epidemiology never got together to discuss these issues.

Things began to change when it became clear that some RNA tumor viruses packaged an extra gene in their genomes and this gene could cause the cancer (Kawai and Hanafusa, 1971; Bader, 1972). The cancer causing gene, or oncogene, was shown to be derived from the hosts chromosome and when sequenced contained mutations that activated the oncogene so that it behaved as a dominant mutation giving rise to the cancer in cells infected by the retrovirus (Stehelin et al., 1976). These studies united the concepts for the role of viruses and genes and chemicals that could cause mutations in selected genes, be they from the host or a virus carrying a host gene. The power of this explanation and the unity it gave to three of the four observations discussed above, kept everyone from focusing on two additional observations that contradicted the oncogene dogma. First, somatic cell genetics were employed to fuse a cancer cell with a normal cell in culture. The resultant hybrid, unlike the cancer cell, no longer made tumors in isogenic animals, suggesting that the gene(s) that made the cell tumorigenic was (were) recessive to the normal allele from the normal cell (Jonasson et al., 1977). When these hybrid cells occasionally did produce tumors in animals, those tumors contained cells that had lost some chromosomes. This was interpreted as the loss of genes that prevented cancer formation. The idea that humans and animals have genes that prevent cancer formation or reverse the oncogene phenotype was novel. Independently, in 1971 A. Knudson hypothesized that two independent mutations in the same gene, later called the retinoblastoma gene, could give rise to a childhood cancer of the eye (Knudson, 1971). Knudson noted that the same tumor, a retinoblastoma, had two very different presentations in young children. Some children developed this tumor within the first year after birth, and these children had bilateral tumors in both eyes and as many as three or more tumors per eye. Other children developed these tumors over several years after birth, and these children had unilateral tumors and only one tumor in one eye. He went on to explain these observations using a common hypothesis that unified both classes of tumors. The tumors that presented at a very young age were due to an inherited mutant allele followed by a spontaneous mutation in the other allele (resulting in multiple tumors in both eyes at a very early age). The other class of tumors were due to a rare event of two independent mutations one in each of the two alleles in a cell (resulting in a single tumor in one eye at a later age). This idea suggested a different type of gene than an oncogene (by definition a

dominant gene) was involved in the origins of cancer and it was variously called an anti-oncogene, recessive oncogene or a tumor suppressor gene. The idea that several oncogenes and tumor suppressor genes must sustain mutations in the same cell to give rise to a cancer, and that any one mutation is necessary but not sufficient to produce a cancer came to be appreciated, understood and demonstrated at some time in the future (Knudson and Strong, 1972; Land et al., 1983). This concept would explain why cancer was usually a disease of the elderly (it took a long time to accumulate many mutations in the same cell) and that the rate of cancer formation would rise exponentially with the age of the population. This unification of four very diverse observations into a single hypothesis for the origins of cancers in humans gave the field some confidence that these ideas might be correct.

HOW DO THE DNA TUMOR VIRUSES CAUSE CANCERS?

The small DNA tumor viruses, discovered in the mid 1950's, were quickly tested to determine if they too carried oncogenes that were derived from cellular DNA sequences. From this it became clear that the small DNA tumor viruses (SV40, polyoma, the adenoviruses and later the papillomaviruses) encoded their own genes (not the cellular genes) that caused the cancer and therefore were termed viral oncogenes. When these viruses were inoculated into a host animal, the animal would develop a tumor at the site of injection after a long latent period. It was most common that the infectious virus disappeared and that a single cell (a clone) developed into a tumor with the viral DNA integrated into a cellular chromosome. This DNA was differentially expressed and viral m-RNA made one or a few viral proteins in the tumor cells. These proteins were recognized as foreign by the host's immune system that responded by making antibodies against the viral encoded proteins. Thus, these viral oncogene products were termed tumor antigens. These antibodies were then employed to demonstrate that the viral proteins were common to all tumors made by that virus, were different when different tumor viruses were employed to initiate the tumors and that the tumor antigens were most commonly encoded by the viral genomes. An extensive genetic analysis with these tumor viruses provided strong evidence that one or two viral encoded genes were required to cause these tumors and the products of these genes were most often the tumor antigens. In every case the viral tumor antigens were also required for an efficient replication of the virus. For SV40 the proteins were called the large T-antigen and the small t-antigen, the adenoviruses encoded the E1A proteins and the E1B proteins (E1B-58K and

E1B-19K) and for the papilloma viruses the E6 and E7 proteins. Mutations in these viral oncogenes resulted in the inability to form a tumor in animals.

The next question that came under study was how did the viral tumor antigens act to initiate tumors in animals or transform cells in culture? It was in the pursuit of this question that several groups uncovered the p53 protein. In 1979 David Lane and Lionel Crawford (Lane and Crawford, 1979) demonstrated that the immunoprecipitation of the SV40 T-antigen also detected a second protein of 53,000 molecular weight, called p53. They could show that the dilution of their tumor antisera always produced the same ratio of T-antigen and p53 which demonstrated that there was a T-antigen –p53 complex in the cell extract (it is unlikely that the antibodies to two different proteins were in equal concentrations). The SV40 T-antigen bound to the p53 protein in the cell. At the same time Daniel Linzer and Arnold Levine (Linzer and Levine, 1979) employed antisera from animals bearing SV40 induced tumors to detect both p53 and the viral T-antigen in SV40 transformed cells. Antibodies in this sera also immunoprecipitated the p53 protein from teratocarcinoma cells in the absence of the SV40 T-antigen. The peptide maps of the p53 proteins from the SV40 transformed cells and the teratocarcinoma cells were identical. These results demonstrated that the p53 protein was a cellular protein, animals bearing SV40 induced tumors also made antibodies against the p53 protein, and monoclonal antibodies to the SV40 T-antigen co-immunoprecipitated the p53 protein demonstrating the T-antigen p53 complex (Linzer and Levine, 1979). The concentration of the p53 protein in SV40 transformed cells was much greater then in normal cells in culture. In teratocarcinoma cells in culture p53 was higher in its concentration than in normal cells but lower than in SV40 transformed cells [13]. The presence of an SV40 T-antigen – p53 complex and the higher levels of p53 in transformed cells suggested that p53 might act as a transforming gene product or oncogene. At the very least the presence of antibodies directed against the p53 protein demonstrated that it was a tumor antigen. In SV40 transformed cells that contained a temperature-sensitive mutation in the SV40 T-antigen gene, shifting to the non-permissive temperature inactivated T-antigen function, made the cell revert to a non-transformed phenotype, and drastically lowered the levels of p53 in the cell (Linzer et al., 1979). This demonstrated that T-antigen really did control p53 levels in a cell. At this time, Llyod Old and his colleagues (DeLeo et al., 1979) demonstrated that animals immunized with spontaneous transformed and tumorigenic cells also made antibodies to the p53 protein and so it was clear that the p53 protein could well be called a tumor antigen in its own right. At a later time L. Crawford and his colleagues showed that some humans with cancers made antibodies directed against the p53 protein (Crawford et al., 1984).

The generality of these observations received a big boost when it was shown that an adenovirus tumor antigen, the E1B-58k protein, which was quite distinct from the SV40 T-antigen, bound to the p53 protein in adenovirus transformed cells (Kao et al., 1990; Sarnow et al., 1982). Similarly a human papilloma virus oncogene product, the E6 protein, bound to the p53 protein in cells derived from human tumors caused by this virus (Werness et al., 1990). Thus three distinct tumor virus groups encoding diverse proteins evolved a mechanism to complex with the same cellular protein, the p53 protein. About this same time the retinoblastoma (Rb) gene was identified and cloned (Friend et al., 1986). The Rb protein, the product of a tumor suppressor gene, was shown to bind to the adenovirus E1A gene product (Whyte et al., 1989) the SV40 T-antigen (DeCapricio et al., 1988) and the papilloma E7 protein (Munger et al., 1989). Thus three different tumor viruses encoded oncogene products that bound to the cellular proteins p53 and Rb. The real meaning of these observations was only poorly understood until the functions of the p53 protein and the Rb protein were elucidated, but they made everyone feel confident that they were on the right track.

CLONING THE P53 GENE: IS IT AN ONCOGENE OR A TUMOR SUPPRESSOR GENE?

The cloning of the p53 c-DNA and gene were carried out by several groups from a wide variety of cellular sources including both transformed and normal cells (Crawford et al., 1984; Beinz et al., 1984; Oren et al., 1983; Pennica et al., 1984). Once these c-DNA and genomic clones were in hand the biological activities of these clones were tested. The fact that the SV40 T-antigen regulated and increased the levels of the p53 protein made most think that p53 was an oncogene whose over-expression (mutant or not?) resulted in transforming the cell. At the time there were two assays for testing an oncogene, one group of oncogenes was able to immortalize cells in culture but not change other properties of these cells (E1A, Myc) while other oncogenes could fully transform immortalized cells (E1B, Ras) but could not transform non-immortalized cells in culture unless myc or E1A were added as well (Land et al., 1983). Very quickly three groups demonstrated that the p53 c-DNA clones were like myc or E1A and could immortalizes cells or could fully transform cells when added to the Ras oncogene clone (Eliyahu et al., 1984; Jenkins et al., 1984; Parada et al., 1984). p53 was declared an oncogene. Moshe Oren's laboratory had a genomic clone of p53 and A Levine's group had a c-DNA clone of p53 and they exchanged these clones for further experiments. There were two

complicating aspects to these results; first the c-DNA from the Levine laboratory did not immortalize cells in culture nor did it transform cells along with the ras oncogene. The Levine laboratory could repeat the results of M. Oren's showing that p53 was an oncogene when they used his clones but could not reproduce these results when the Levine c-DNAs were employed. Second, the amino acid sequence of the Oren and Levine clone differed at codon 135 (a valine and alanine difference). In a series of experiments several things became clear; 1. the wild type p53 c-DNA does not transform cells (Hinds et al., 1989), 2. mutant p53 c-DNAs or mutant genomic clones are commonly found in cells that are grown in culture, in fact p53 mutations are commonly selected for as cells adapt to long term culture conditions (Harvey and Levine, 1991), and 3. a mutant p53 c-DNA or genomic clone can act in a dominant negative fashion (the p53 protein is a tetramer and faulty subunits will inactivate the wild type p53 function) and transform cells (Eliyahu et al., 1988; Kraiss et al., 1988; Finlay et al., 1989). The Levine group went on to show that the wild type p53 c-DNA and its protein can actively inhibit oncogenes from transforming cells in culture (Finlay et al., 1989). In fact a very similar observation had been observed in murine erythroleukemia cells transformed with a retrovirus containing an oncogene (Munroe et al., 1988; Ben David et al., 1988) where the integration of the viral DNA disrupted the p53 gene function in these cancer cells. Thus p53 was behaving as a tumor suppressor gene in all of these assays. These conclusions were independently demonstrated by Vogelstein and his colleagues when they sequenced three human colon carcinomas and showed that p53 mutations were found in their p53 genes and the other allele was lost or reduced to homozygosity (Baker et al., 1989; Nigro et al., 1989). This is the hallmark of a tumor suppressor gene.

Thus three different approaches all led to the conclusion that the p53 gene product acted as a tumor suppressor protein and that the viral oncogene products bound to the p53 protein must therefore inactivate it. Mutations in both p53 alleles were selected for in non-viral induced cancers. Adding back the wild type p53 gene to a cancerous cell in culture killed the cell or blocked the action of oncogenes. At this time there were two examples of tumor suppressor genes (retinoblastoma and p53) and the field turned its attention to elucidating the functions of these genes and their products.

THE FUNCTIONS OF THE P53 GENE AND THE DOWNSTREAM PROGRAM

One of the first clues about the function of the p53 protein came from the observation that it bound to DNA and that tight binding to DNA occurred in

a sequence specific fashion (Funk et al., 1992; Zauberman et al., 1993; el-Deiry et al., 1992). Steinmeyer and Deppert (1988) selected for DNA sequences that would bind to the p53 protein even at low concentrations and sequence analysis of these DNA's gave a consensus for the optimal DNA binding sequence: RRRCWWGYYY where R is a purine, W is A or T and Y is a pyrimidine. A core fragment of the p53 protein containing its DNA binding domain was co-crystallized with this consensus oligonucleotide and the protein was found to make strong contacts with the C and G residues and weaker contacts with the other sequences (Cho et al., 1993). At this time a series of experiments demonstrated that as a result of this DNA binding p53 behaved as a transcription factor (Fields et al., 1990; Raycroft et al., 1990; Kern et al., 1991). This set off a search for the genes regulated by the p53 protein and these target genes are discussed further later in the chapter.

At about this same time the p53 protein in cells was shown to bind to another protein and temperature sensitive mutants of the p53 protein regulated the levels of this interacting protein (Momand et al., 1992). The purification and sequencing of this p53 binding protein identified it as the MDM-2 protein (Momand et al., 1992) which had recently been shown to be an oncogene in mouse cells (Fakharzadeh et al., 1991). The MDM-2 protein was found to bind to the p53 protein and block its ability to act as a transcription factor (Momand et al., 1992) and the MDM-2 gene in humans, called HDM-2, was shown to be amplified and over-expressed in some human sarcomas (Oliner et al., 1992). Furthermore the MDM-2 gene was shown to be transcriptionally regulated by the p53 protein, containing a number of DNA sequences in the first intron of the gene related to the p53 DNA consensus sequence (Zauberman et al., 1995). This meant that p53 and MDM-2 formed an autoregulatory loop where increased p53 activity increased MDM-2 levels which in turn decreased p53 activity resulting in declining MDM-2 levels (Piksley and Lane, 1993; Wu et al., 1993). This forms a failsafe mechanism to prevent p53 activity from getting too high in a cell. Subsequently it was shown that MDM-2 is an E3 ubiquitin ligase that transfers ubiquitin to p53 resulting in its degradation (Honda et al., 1997). This type of circutry between p53 and MDM-2 means that the levels and activities of these proteins in a cell oscillate out of phase with each other over time (Bar-Or et al., 2000) and this has been shown to be the case in single cell experiments (Lahav et al., 2004). This relationship between p53 and its negative regulator MDM-2 can be disrupted in several different ways; 1. The p53 gene can be mutated so that the cell doesn't make MDM-2 proteins (present in 50-55% of cancers), 2. the MDM-2 gene can be amplified so it blocks p53 functions (found in 30% of sarcomas), 3 .p53 protein modifications (phosporylations) can occur in or near the p53-MDM-2 binding sites (Unger et al., 1999; Lin et al., 1994) and disrupt this protein-

protein interaction as is the case after p53 activation in response to the appropriate signals, 4. MDM-2 can be inactivated by the ARF protein or by interaction with some ribosomal proteins (Zhang et al., 2003; Lohurm et al., 2003).

Another gene regulated by the p53 transcription factor is the p21/ Waf-1/ Cip-1 gene (el-Deiry et al., 1993). This gene contains perfect p53 DNA binding consensus sites that regulate it by the activation of the p53 protein. One of the functions of the p21 protein is to bind to the cyclin E cdk-2 protein kinase that must act in late G1 of the cell cycle and block its activity (Harper et al., 1995; Xiong et al., 1993). This is in part the reason why p53 activation can lead to cell cycle arrest in G1. Similarly the 13-3-3 sigma gene is regulated by p53 and this protein binds to the CDC-25 protein, keeping it in the cytoplasm where it is unable to function as a nuclear phosphatase thus permitting cells to go from G2 to M phase (Draetta and Eckstein, 1997; Taylor and Stark, 2001). This contributes to a G2-M block that is sometimes observed after p53 activation. Thus some of the downstream genes regulated by p53 contribute to a cell cycle arrest. Another set of p53 responsive genes promotes apoptosis in a cell by helping to activate the release of cytochrome c from the mitochondria (bax, noxa, perp, etc.) and contributing via the production of APAF-1 (Rozenfeld-Granot et al., 2002) to the activation of caspase 9 and 3 followed by apoptosis. p53 also activates a second apoptotic pathway increasing the levels of the Fas ligand and the KILLER DR receptor in the caspase 8 and 3 pathway (Sheikh et al., 1998). Thus a second major p53 response is programmed cell death. p53 also regulates some genes that participate in DNA repair reactions in the cell (p53R2 an alternative ribonucleotide reductase subunit) and a set of gene products that produce secreted proteins after a p53 response (thrombospondin, maspin, inhibitors of plasminogen activators). These gene products may alter the extracellular matix and could impact upon the regulation of cell division, metastasis, angiogenesis, or other functions. Figure 1 depicts these pathways and see also the recent review by Nakamura (2004).

Among the more interesting aspects of the p53 inducible and regulated pathway is the elaborate negative feedback loops that are formed by three p53 regulated genes and their products. First is the p53 -MDM-2 feedback loop that has been discussed above. p53 also regulates the Cyclin G gene that makes a protein that combines with the PP2A phosphatase and removes a phosphate residue from the MDM-2 protein (Okamoto et al., 2002) thus increasing the MDM-2 activity and lowering p53 levels in a cell. A mouse with the Cyclin G gene knocked out is viable but has higher constitutive p53 levels in its cells (Jensen et al., 2003). The phosphate group removed from

MDM-2 by Cyclin G -PP2A is added to MDM-2 by one of several cyclin-cdk kinases suggesting a link to cell cycle events.

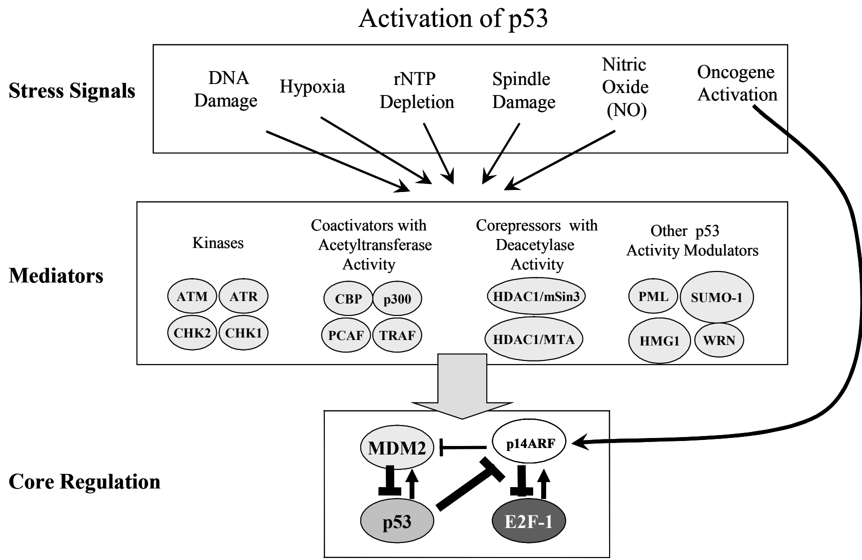

Figure 1a. Downstream of p53: known transcriptional response of the activated p53 protein.

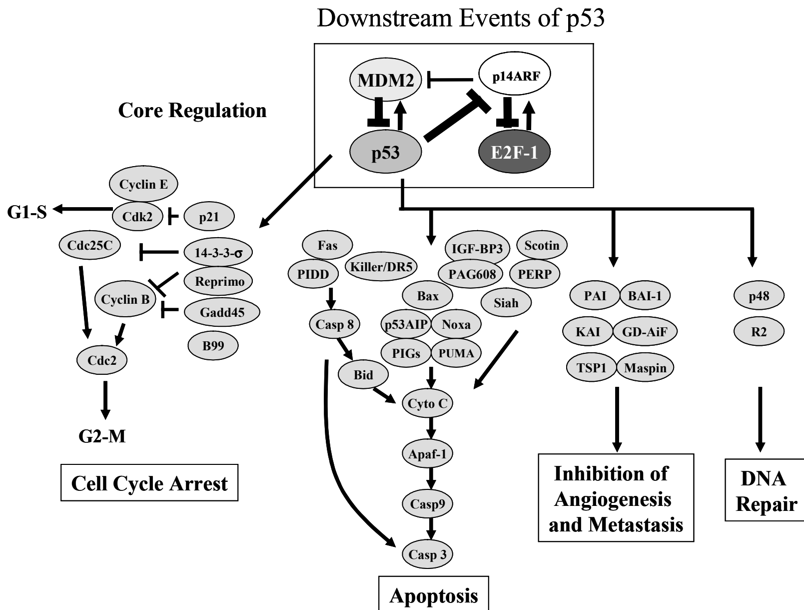

Figure 1b. Downstream of p53: known transcriptional response of the activated p53 protein.

Another negative regulator of p53 that is encoded by a p53 responsive gene is Wip-1 (Fiscella et al., 1997). Wip-1 is a phosphatase that acts upon MAP kinase that in turn can phosphorylate the p53 protein at two sites resulting in its increased activity as a transcription factor. The dephosphorylation of MAP kinase by Wip-1 lowers MAP kinase activity and reduces p53 activity (Takekawa et al., 2000). Thus MDM-2, Wip-1 and Cyclin G are all p53 regulated genes that in turn negatively regulate p53 activity or levels and both MDM-2 (Taubert et al., 2003) and Wip-1 (Bulavin et al., 2002; Sinclair et al., 2003) genes are found to be amplified in selected cancers. Figure 2 summarizes this negative feedback loop and network.

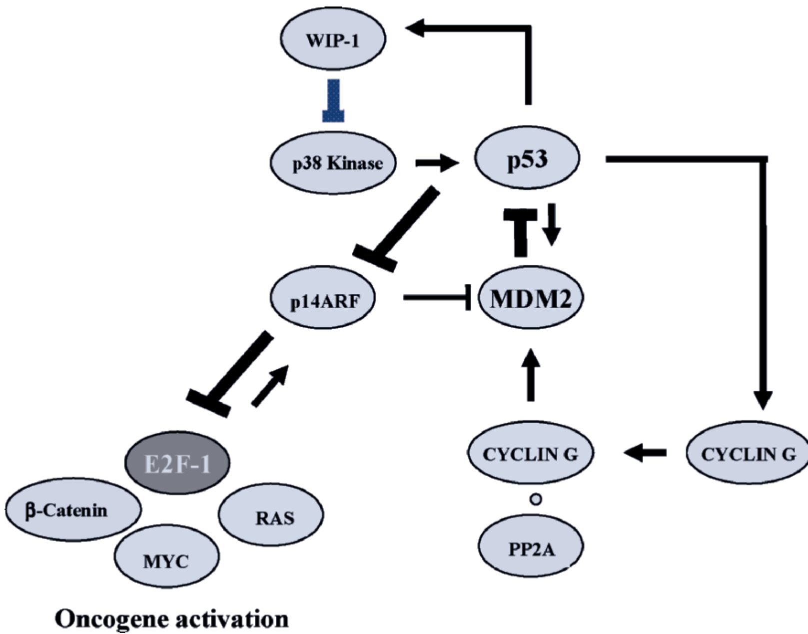

Figure 2. Negative feedback loops that control p53 activity in the cells.

To date some 30-35 genes have been shown to contain the p53 responsive elements in their DNA and, by one criteria or another, have been shown to be regulated by p53 either in a positive or negative fashion. Hoh and her colleagues (Hoh et al., 2002) have formulated an algorithm that scans the human or mouse genome for p53 responsive DNA elements or sequences adjacent to genes that may be regulated by the p53 protein (see http://linkage.rockefeller.edu/p53). They identified in the mouse and human genome 16 genes that had excellent p53 responsive elements and tested these genes for their transcriptional regulation after p53 activation in cells in

culture (Hoh et al., 2002). To date 12 of those genes have been shown to increase or decrease their abundance after p53 activation. There is some cell or tissue type specificity in some of these responses and this has been observed in mice as well. A survey of the p53 responsive DNA sequence elements in many p53 regulated genes demonstrates that there are always two RRRCWWGYYY palindromes separated by a 0-21 base pair spacer of any sequence and a good deal of sequence degeneracy is permitted in these sites. An oligonucleotide chip analysis of genes up- or down-regulated after p53 levels rise in a cell identified a number of genes that have p53 responsive elements (as in Figure1) and many genes whose m-RNA levels change but don't have recognizable p53 responsive elements (Zhao et al., 2000). This suggests that their may well be a program of gene activation or repression begun by p53 regulated genes that is no longer dependent upon p53 for its activity and that among the p53 regulated genes might well be transcription factors that carry out this program. Figure 3 shows the kinetics of mRNA levels (increased or decreased) for a series of genes in lymphoblastoid cell lines, as detected by Affymetrix chips after exposure to gamma irradiation, a known activator of the p53 pathway. Using the data provided by Jen and Cheung, (2003) but utilizing different clustering algorithms, we addressed the problem of identifying among 126 IR-responsive genes in common between 3 Gy and 10 Gy exposure, clusters of genes which are highly correlated in their temporal expression patterns at the two doses (Figure 3 and http://www.csb.ias.edu/Research/clusters.htm). As in Jen and Cheung our analysis reveals a complex program of gene expression in these cells after p53 activation. A similar program was observed in carcinoma cells undergoing a p53 response (Robison et al., 2003).

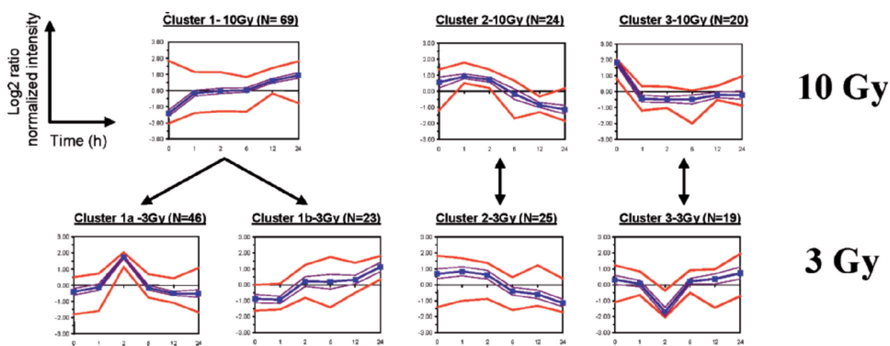

Figure 3. This analysis was performed to better identify clusters of genes whose expression following IR insult display similar temporal patterns. The data that consisted of Affymetrix chip analysis of gene expression in lymphoblastoid cells, was provided by Jen and Cheung [78] (see also http://www.csb.ias.edu/Research/clusters.htm for details). The consensus

profiles show a highly coordinated IR response of genes after 3 Gy and 10 Gy IR exposure: (i) more than 90% of the genes in two of the four 3Gy profiles (Cluster 3Gy-1a and Cluster 3Gy-1b) display similar temporal patterns after 10 Gy exposure (in Cluster 10Gy-1), and (ii) more than 85% of the genes in each of the remaining two 3Gy profiles (Cluster 3Gy-2 and Cluster 3Gy-3) display similar temporal expression patterns after 10 Gy radiation (in Cluster 10Gy-2, and in Cluster 10Gy-3, respectively). Each profile identified in this analysis is stable to data perturbation with white noise, has high homogeneity, and has a very low likelihood to occur by chance. Additionally, Jen and Cheung identified 16 profiles of smaller size in response to 3Gy, and 10 Gy radiation exposure.

THE ACTIVATION OF P53 AND THE UPSTREAM PROGRAM

The p53 protein is synthesized in most cells in the body and has a very short half-life of 6-30 minutes depending upon the cell or tissue type. Under these circumstances there is very little p53 regulation of p53 responsive genes. A variety of stress signals will activate the p53 protein so that there is an enhanced transcription of the p53 responsive genes. Activation is associated with and is caused by protein modifications of p53 (phosphorylation and acetylation). This in turn results in an increased half-life and increased concentrations of the p53 protein (Maltzman and Czyzyk, 1984; Price and Calderwood, 1993; Maki and Howley, 1997). While activation of the p53 protein was first carried out using temperature-sensitive mutants of the p53 gene, the first demonstration by a real physiological stress that activated p53 was by Maltzman and Czyzyk (1984) who showed that UV light damage increased the level of the p53 protein in cells. This was roundly ignored by the field for quite a while until Kastan (Kastan et al., 1991) and others demonstrated that a wide variety of DNA damaging agents producing very different DNA lesions, each can activate the p53 response (Huang et al., 1996). UV damage involves the formation of thymine dimers, gamma radiation results in single or double stranded breaks in DNA, alkylating agents often react with guanine residues producing alkylated G-residues and each of these lesions has a distinct set of repair activities in the cell. Associated with this repair process are a series of enzymatic activities; protein kinases, histone acetylases, and possibly histone methylases, sumo ligases, or other such activities, that recognize the type of DNA damage and modify the p53 protein, signaling to it the existence of that type of damage. Although it has been difficult to assign a specific kinase to a specific signal, the available evidence suggests that the ATM kinase (Canman et al., 1998; Banin et al., 1998) and a CHK kinase (Zhao et al., 2002; Gatei et al., 2003) may well play a role in the single and double strand break stress signals and

the ATR kinase could be involved in UV damage recognition (Unsal-Kacmaz et al., 2002). The patterns of phosphorylation after such stress signals have been intensively studied and it appears that different kinases may yield different combinations or patterns of phosphorylation on the p53 protein. UV damage or gamma radiation produces distinct p53 transcriptional responses as examined by oligonucleotide arrays (Zhao et al., 2000). These data suggest that the p53 protein integrates the input signals from different stresses and responds accordingly with a distinct transcriptional output. Thus, removal of each of these lesions might be by a different mechanism in cells. There are also sets of p53 responsive genes that are always transcribed in a p53 response independently of the type of input stress or the cell type under study. These genes include p21, MDM-2, Gadd-45, 14-3-3 sigma, and Cyclin G. Today we recognize a variety of stress signals that activate the p53 pathway such as DNA damage, hypoxia, spindle poisons, the size of the ribonucleotide triphosphate pools in a cell, NO signaling, cold shock, denatured or altered proteins and even some oncogene mutations result in enhanced p53 activity in a cell (reviewed recently by Nakamura (2004). Thus the p53 protein is modified by a wide variety of stress signals or alarms, it then processes this information using a protein modification code yet to be elucidated and responds by activating a transcriptional program (Figure 3) resulting in either cell cycle arrest, apoptosis, DNA repair, the modification of the cellular matrix and communication with neighboring cells.

Clearly these upstream signals that communicate with p53 can mobilize a large number of enzymatic functions such as kinases and phosphatases, histone acetylase complexes and histone deacetylases, PML bodies, helicases, ubiquitin ligases, etc. which all may play a positive or a negative role in modulating p53 activity and p53 responses (see Figure 1). The modified p53 protein must then enter into the transcriptional machinery of the cell, which may respond to the protein modification code, and promote p53 interactions with other proteins to enhance the rate of transcription of selected genes. Together these upstream inputs and their downstream responses create a highly regulated network that responds to the stress signals (Figures 1, 3).

INACTIVATION OF THE P53 PATHWAY IN CANCERS

The p53 pathway is composed of hundreds of genes and many of them will have single nucleotide polymorphisms that impact upon the efficiency of p53 function. Thus we can expect that genetic difference between people will contribute to the cellular and molecular responses to stresses and this

may well impact upon the age of onset of cancers, the incidence of cancers and the responses to therapy that results in DNA damage. A small number of families inherit one p53 mutant allele in the germ line. These families have the Li-Fraumeni syndrome with early age of onset of cancers, and in some cases multiple independent cancers. The penitrance of the p53 mutant allele is almost 100% in people and the tumors frequently, but not always, reduce to homozygosity for the mutant allele. The tumors are commonly sarcomas, but breast, colon and several other types of cancers are observed (Frebourg et al., 1992; Malkin et al., 1990). The mouse with no p53 alleles develops thymic lymphomas at a young age and these tumors are not observed in humans. The heterozygous mouse frequently develops sarcomas and this is similar to the human spectrum of tumors (Jacks et al., 1994). About 50 percent of all cancers have p53 somatic mutations in both p53 alleles (Hollstein et al., 1991). A few cancer types do not usually have any p53 mutations (teratocarcinomas), and others (melanomas, some leukemias) have a very low frequency of p53 mutations (about 10% of the time) (Hollstein et al., 1991; Drexler et al., 2000). In teratocarcinomas, which are germ cell tumors, the p53 protein is not functional (Lutzker and Levine, 1996) but has a wild type DNA sequence. Because it is not functional there is no selection pressure to inactivate it via mutation. The p53 protein can be activated and kill the cell by apoptosis after DNA damage. Interestingly teratocarcinomas respond very well to chemotherapy and are cured most of the time. Similarly leukemias rarely have p53 mutations and respond very well to chemotherapy. When they relapse these tumors often, but not always, now harbor p53 mutations.

The International Agency for Cancer Research (IARC) maintains a database of p53 mutations (http://www.iarc.fr/p53) that encompasses 18,585 examples of somatic and 1114 of germ line mutations. Of the somatic mutations 82% are point mutations whereas 18% are insertions, deletions or more complex rearrangements. When the entire p53 c-DNA or gene is sequenced the great majority of the mutations are located in the DNA binding or core domain of the p53 gene and protein. Because of this most researchers have sequenced only exons 5-8 encompassing codons 108 to 298 which is the DNA binding domain. Possibly because of this bias 94% of all point mutations in the p53 gene have been localized to codons 100-310. About 35% of these point mutations are localized in six hot spots in the gene at codons 175, 245, 248, 249, 273, and 282. When tested, the proteins with these p53 mutations fail to bind to the p53 DNA response element efficiently (Bullock et al., 2000; Kern et al., 1991; Epstein et al., 1998) and fail to transcribe p53-regulated genes. The hot spot mutations correspond to amino acid residues that make contact with the nucleotides in the p53 response element. This suggests that mutations resulting in a loss of function of the

DNA binding and transcription factor properties of the protein are being selected. A mutational analysis of the amino terminal region of the p53 protein, where the transactivation domain resides, indicates that at least two independent point mutations are required to inactivate the transcriptional activity of this domain (Lin et al., 1995) so it is clearly easier and more common to obtain point mutations in the DNA binding domain.

Some other tumor suppressor genes, such as the APC gene and the p16 gene, are commonly inactivated by point mutations that result in stop codons that lead to a loss of function. In the p53 database both missense and nonsense mutations are found in many cancers. The missense proteins are not transcriptionally functional and so no MDM-2 protein is made in most cells. Due to the decreased abundance of the negative regulator protein, mutant p53 protein is found in much larger amounts in cancer cells than in normal cells with the wild type p53 protein (Hinds et al., 1990). When the missense protein is produced in large amounts in a cell along with wild type p53 protein both wild type and missense proteins are synthesized and enter into a tetrameric protein complex that is inactive because of the mutant or faulty subunits. Thus p53 mutant c-DNA clones show a dominant loss of function phenotype and can transform cells in culture (Hinds et al., 1989). It is not at all clear that this has any functional significance in vivo or in tumors. However many of the missense mutations in the p53 gene have been shown to have a potential gain of function phenotype (Blandino et al., 1999; Dittmer et al., 1993). When a p53 missense c-DNA clone is added to a cell that is normal but has no p53 gene, the cell can grow more rapidly, become more tumorigenic when inoculated into animals and can gain a drug resistant phenotype. These experiments have been carried out by a number of different research laboratories, they appear to be quite reproducible and even show some allele specific phenotypes, all of which suggests that the missense p53 mutations generated in cancers could have a gain of function phenotype. If this was the case then one could understand why missense mutations would occur more frequently in the p53 gene in cancers than nonsense mutations that are true loss of function mutations.

One of the questions never answered properly is whether or not missense p53 mutations are selected for over and above the frequency of nonsense mutations or neutral mutations observed in the database. The IARC database was employed to ask this question for all possible point mutations in codons 100-310 that could lead to a missense mutation or a nonsense mutation. The way to accomplish this is by comparing the number of ways a mutation in any base in a codon can result in a missense mutation or a nonsense mutation with the number of times this has occurred in cancers with p53 mutations. The necessary assumption is that the database contains a large enough set of mutations that differences in the point mutation rates are balanced out. In

addition it is important to consider the set of mutations that were selected, above the noise, by using the silent mutations as background noise. The frequency of silent mutations (a nucleotide change that does not result in an amino acid change) in the database suggests a background level of unselected mutations such that a mutation should occur in four or more separate tumors so as to be above this background level. Then the mutation is clearly selected for some property. In Table 1 we carry out this exercise for mutations that are found in 4 or more tumors. Although it is not shown, increasing this cutoff has very little effect on the results. In column A is shown the number of different nucleotide changes that have led to a missense, nonsense, or silent mutation, respectively, and were represented in the database as least 4 times. The total number of tumors in the database that have the set of nucleotide changes described in column A is represented in column B. Finally, in column C, we calculate the average occurrence of each type of mutation (column B divided by column A). The surprising result of this analysis is that when we correct for the number of positions in which these mutation types could occur, missense mutations occur at about the same frequency or ratio (B/A) as nonsense mutations.

Table 1. Comparison of missense, nonsense and silent mutations in p53. Numbers correspond to mutations that are found in 4 or more tumors in the IARC TP53 database. The total number of tumors in the database that have the set of nucleotide changes described in column A is represented in column B. Column C: average occurrence of each type of mutation (column B divided by column A).

	A	**B**	**C**
MISSENSE	529	12296	23,2
NONSENSE	50	1145	22,9
SILENT	76	445	5,9

We have added the analysis in column A because different bases in different sequence contexts have different rates of mutation as is shown in Table 2. By far the most common mutation in the p53 gene is a C to T change in the dinucleotide CpG (see table 2) whether or not the mutation results in a nonsense, missense or silent amino acid change. For nonsense mutations C to T and G to T changes make up 75% of the mutations observed. For missense mutations C to T, G to A, and G to T changes make up 63% of the mutations in the database. For silent mutations the C to T and G to A changes make up 62% of the mutations found. With these base pair biases in the rate of mutations taken into account, it does not appear that missense mutations or nonsense mutations are preferentially selected in the cancers. Recently Yang et al. (2003) applied a variety of mutation rate models to this same p53 database and also concluded that selection for missense and nonsense mutations is about equal in the DNA binding region.

Table 2. Frequency of mutation types in p53 IARC database. Breakdown of nonsense, missense and silent mutations in the IARC TP53 database according to the nature of base change. The total number and percentage of each base change is shown.

Type	nonsense				missense				silent			
	mutation	tot number	percent	CpG	mutation	tot number	percent	CpG	mutation	tot number	percent	CpG
	A->C	0	0		A->C	243	2		A->C	7	0.8	
	A->G	0	0		A->G	1365	10		A->G	37	4	
	A->T	51	4		A->T	412	3		A->T	9	1	
	C->A	72	5		C->A	353	3		C->A	54	7	
	C->G	58	4		C->G	501	4		C->G	35	4	
	C->T	712	52.5	480	C->T	2549	19	1580	C->T	316	38.3	24
	G->A	103	8		G->A	4198	31.2	2533	G->A	201	24.3	31
	G->C	0	0		G->C	737	5		G->C	24	3	
	G->T	299	22.5		G->T	1790	13.3		G->T	25	3	
	T->A	36	3		T->A	398	3		T->A	14	2	
	T->C	0	0		T->C	492	4		T->C	86	12.1	
	T->G	7	0.5		T->G	397	3		T->G	18	2	
Sum		1338				13435				826		

These data contradict the gain of function hypothesis and suggests three possible explanations for the contradiction. 1. The gain of function phenotypes observed with missense p53 mutations are observed in cell culture and animal models but are not operative in human cancers, 2. There are times that cancers select for nonsense mutations and others where missense mutations that have a gain of function are selected for by a tumor. Here the genetic background and the nature of the oncogene and tumor suppressor gene mutations could influence whether a gain of function mutation is selected. Even the cell or tissue type of the tumor could influence this. We could use the IARC database to look at mutations from individual tumors to see if they maintain the equality of selection between missense and nonsense mutations. However, specific mutation rates are modified so strongly by particular carcinogens that we would no longer trust that the large database could smooth out the varying rates. 3. Something is fundamentally wrong with the interpretation of the gain of function experiments (the mutant p53 still acts as a transactivator that changes the cell, it still binds to DNA and alters the cell) but these properties are not important in cancers and are not selected for by tumors. This gain of function hypothesis remains one of the unresolved problems in the field.

The IARC database does not permit one to examine the frequency of p53 mutations in one or more cancers because data are not presented for the total number of tumors where the p53 gene has been sequenced so as to know what percentage of these tumors had p53 mutations. Based upon a large number of studies in the literature it would be conservative to claim that about 30% of all tumors examined contained p53 mutations (likely to be >50%). If we accept a 30% cutoff then the IARC database contains about 18585 mutations (out of an estimated 62,000 tumors sequenced) of which 826 are silent mutations. These are usually considered neutral mutations that are not selected for or against in a tumor or in the evolution of an organism. Because this may be as pure an estimate of a mutation frequency in the

absence of selection (not a rate as we know nothing of the number of cell divisions) in tumor cells in vivo, we attempted to estimate this number. There are 826 silent mutations in about 62,000 tumors (a third of which have p53 mutations) which implies that 1.3% of tumors have silent mutations. 268 of 630 nucleotides in codons 100-310 can yield silent mutations. Therefore, the estimate for the neutral mutation rate per nucleotide in the tumor is .013/268 = 5x10^(-5). An estimate of the frequency of germ line changes per base pair is commonly about 3x10^(-8) or about 1,000 times lower than the silent mutation frequency suggested from this analysis. There are many estimates and assumptions in this calculation, but it does suggest something that was intuitively thought to be correct, namely that the spontaneous mutation frequency (as measured using only neutral mutations) in a cancer is about 1,000 fold higher than in a normal cell. Thus it implies that mutator gene phenotypes are involved in raising the frequency of mutations in cancer.

The possibility that these silent mutations are actually polymorphisms and not somatic mutations can be ruled out. There are 6 known SNPs in the coding region of p53 (also 1 in the promoter region and 12 in introns). Of these 6 SNPs, 4 are silent and 2 change amino acids. Only one of the 4 silent SNPs (Arginine 213) is in the region of the gene being studied (codons 100-310). This change could account for at most 2 of the 826 silent mutations.

Finally it should be pointed out that mutations that do not alter an amino acid in the protein may not be selectively silent. Such changes could alter RNA folding, the rate of RNA processing or the rate of translation. RNA–protein interactions might fail when there is a change in the structure or sequence of the m-RNA brought about by a so called neutral mutation. While we don't usually think of these changes as critical to function, these ideas have been poorly tested.

There are a number of other mutations in the p53 pathway that alter p53 functions. As reviewed in Figure 2 there are four negative and one positive feedback loops for p53 regulation. Thus MDM-2 and WIP-1 gene amplifications in sarcomas and breast cancers respectively reduce p53 activity (Taubert et al., 2003; Bulavin et al., 2002). Cyclin G over-expression also reduces p53 function and cyclin G knockout mice have more p53 protein (Jensen et al., 2003). The AKT kinase in the IGF- PI3K-PTEN pathway has been shown to phosphorylate the MDM 2 protein resulting in the movement of MDM-2 into the nucleus where it more effectively degrades the p53 protein (Ashcroft et al., 2002; Mayo et al., 2001). Several other signal transduction pathways produce transcription factors that enhance ARF synthesis or activity, which in turn inhibits MDM-2 and positively regulates p53. Beta catenin-TCF-4 (the product of the WNT-APC pathway), E2F-1 (the product of the Cyclin D-Rb pathway), MYC, RAS and

p38 MAPK which acts via the ETSandAP-1 transcription factors, are all examples of interconnections between several signal transduction pathways and p53. Thus we are beginning to understand not only the p53 pathway but also its many connections to signal transduction pathways that play central roles in the origins of cancers. It will now be up to the new field of systems biology to construct these pathways, model them and make clear predictions which can be tested in experiments and ultimately shown to benefit cancer patients with predictive and prognostic outcomes.

After 25 years of research with the p53 gene and its protein we have built an infrastructure upon which to extend our detailed understanding of its functions. The p53 gene and its protein are not essential for life (i.e. the knock out mouse is born alive) but it is quite clear that it is essential for life to faithfully reproduce itself. The p53 gene in worms and flies protects the germ line from stress and mutations. In the mouse and human these functions still operate effectively but the role of p53 has also been adapted to faithful cellular reproduction of somatic cells, as vertebrates regenerate their tissues. Responses to stress that disrupt our homeostatic mechanisms, cause mutations that impact information transfer, and result in pathogenic outcomes, are the business of the p53 pathway. We need to understand this business better.

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