

## What do we need to know and understand about p53 to improve its clinical value?

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## Summary

Few proteins are more studied than the p53 tumor suppressor, but what have we learned from these studies and what do we really know about p53 that can benefit clinical practice? The p53 sequence is frequently mutated in cancers but the functional outcomes of single mutations, in respect to loss or gain of different activities, especially in relation to immune evasion, is not clear. This illustrates p53's complexity and why, after 40 years, it remains elusive and keeps providing surprises but also, why it has not yet lived up to its potential to benefit cancer treatment. We have reassessed a few key experiments that have shaped the p53 field and we take a closer look at the interpretations of these experiments, what they have taught us, the resulting dogmas and their potential clinical importance. One outcome is a more dynamic view on p53 in terms of its activity, its regulation and downstream effectors that will benefit the clinical application of p53 for diagnosis, prognosis and therapy. Mutations and regulatory factors can have different effects on p53 activity depending on context, which are important but neglected aspects when interpreting p53 and its pathways in cancers. Even though p53 is undoubtedly unique as a multifunctional hub in different cellular pathways, the concept of a factor taking up different functions within a regulatory pathway during different conditions is not. In this sense p53 continues to lead the way for a better understanding of the cellular and molecular mechanisms underlying cancer development *in vivo*.

## *Introduction*

P53 is somewhat of an enigma despite over 40 years of intense studies and well over 100,000 publications. Part of this reflects the fact that we do not have sufficient techniques or models to address physiological questions that mimic human cancer progression and the fact that p53 plays different roles depending on cellular conditions, cell types and microenvironmental factors. This is illustrated by the frequency of which p53 mutations appear in cancers, varying greatly from few in acute myeloid leukemia and renal cancers to more frequently in breast ovarian, bone and soft tissue sarcomas, brain tumors and adrenocortical carcinomas (ADC) [1]. Animal models, mostly mice, have been, and still are, the main model system to address the role of various aspects of p53 in cancer development. These models rely to a large extent on germline mutations, thus being considered as driver mutations [2,3]. However, more recent works show that the order of genetic events during tumor development is important in cancer development and, thus, other factors and the overall pattern of genetic alterations, as well as microenvironment, need to be taken into account when validating the role of p53 in oncogenesis [4,5].

Studies on JAK2 and TET2 in hematopoietic malignancies suggest that tumors have a different phenotype depending on which mutant comes first [6]. Thus, a malignancy is not the sum of all mutations, but rather a result of an order of events and this has consequences for therapeutic strategies and molecular diagnostics as a mutation event that is required for a certain step in tumorigenesis might not be active at a later stage and therefore not be an optimal therapeutic target. Li-Fraumeni patients born with a heterozygote missense p53 mutation have an increased risk of developing sarcoma, breast cancer, leukemia and adrenal gland tumors, the so-called SBLA syndrome [7–9]. On the other hand, cancers that exhibit a high frequency of p53 mutations, such as colon and ovarian, are not overrepresented in Li-Fraumeni patients [10]. Being born with a p53 deficiency does not result in the same cancer type incidence as when the somatic mutations appear during different steps of cancer progression. Hence, p53 deficiency can be seen as a driver for some types of cancer, but not for others. In colon cancer, the driver mutation instead seems to be on the APC and the Wnt signaling pathway [11]. Mice lacking a p53 allele have an increased risk of developing cancers, such as T cell lymphomas, which are not frequently seen in Li-Fraumeni patients, pointing towards also species-specific differences [12]. Taken together, these issues make it difficult to interpret the role of specific p53 mutations, or deficiency, in cancer development and to validate pre-clinical drug development based on animal models in which p53 mutations have been introduced in the germ line. In addition, tumors evolve by natural selection through progressively evading immune eradication but the role of p53 pathways in combat with the immune system is almost completely unknown and inbred mice living in sterile conditions might not be the optimal model to study this. Nevertheless, mouse

models have to a large extent formed our current view for how we believe p53 is regulated, how it functions and its role in carcinogenesis.

The complexity of cancer heterogeneity goes against the comfort of our minds to use reductionist approaches as a tool to build complexity but it is important to consider that generalized models and concepts are sometimes oversimplified and even misleading. For example, mutations do not only inactivate p53, they can also change its activity and increase or suppress tumor development in a condition-dependent fashion [13]. This not only illustrates a side of p53 that harbors oncogenic activity but also that it is difficult to pin one genetic event to a certain cell biological effect, which calls for a reconsideration of the potentially misleading concept of placing genes in pigeonholes labelled as “oncogenes” or “tumor suppressors”. P53’s main regulator MDM2 is usually pigeonholed as “oncogene” as it can target p53 for degradation under normal conditions [14,15] but it also supports p53 synthesis during DNA damage and functions as a positive co-factor [16]. Thus, depending on conditions MDM2 can be both positive and negative effector of p53 (see further below). But this is not unique for MDM2 and instead reflects a broader concept of condition-dependent multi functionality of gene products in regulatory pathways. In addition, MDM2 has several isoforms that are elevated in human cancers but are largely understudied in terms of clinical impacts. For example, a “RING domain only” isoform can be oncogenic in a mouse model [2,17] and such isoforms do not have the classic N-terminal p53 binding domain and would be non-refractory to drugs of the Nutlin class (discussed further below). In this overview we will look back and see how some of the dogmas surrounding p53 have weathered over time and to highlight a more dynamic view that p53 and MDM2, as well as p53 downstream target genes, take on different functions depending on the cellular conditions.

### *P53*

With the development of gene targeting in embryonic stem cells in 1992, p53 was the first tumor suppressor gene knock-out in a murine model mimicking spontaneously arising human tumors in Li-Fraumeni syndrome patients that carry germ line p53 mutations or the somatic loss of p53 in sporadic tumors. 75 % homozygote *Trp53*<sup>-/-</sup> mice developed spontaneous tumors by an average of 6 months, while *Trp53*<sup>+/-</sup> animals develop tumors by an average of 18 months [2]. Even though this animal model shaped p53 research and supported its role as a tumor suppressor, it had its limitations [17–20]. The tumor spectra in *Trp53*<sup>+/-</sup> mice differ from that of Li-Fraumeni patients, most notably with a high frequency of T cell lymphomas (25 %) and sarcoma (57 %); whereas Li-Fraumeni patients present a predominance of breast carcinomas, observed in about 70% of affected female mutation carriers, and a high frequency of sarcoma, observed in 27% of affected carriers [12,21]. It should be noted that the

frequency of spontaneous tumors, most notably sarcomas, in inbred mice is close to 90 % by the 30<sup>th</sup> month.

This warrants the question whether tumors that develop in humans and mice due to the loss of p53 are caused by the same molecular mechanisms? Can it be assumed, even though there is a clear link to cancer development, that the human and mice proteins are functioning and doing the same job and/or via the same mechanisms and pathways in different species? We know from missense mutations that changing a single amino acid in p53 can have important impacts on its activity and the human and murine p53 are not identical proteins. In fact, only a few of all antibodies generated against p53 cross react equally well between species [22,23]. Even polyclonal sera generated in rabbits by injected either murine or human full length p53 protein do not cross react. This is, actually, quite remarkable and indicates that despite the sequence similarities, the conserved regions between the two proteins are not exposed in a similar fashion to serve as antibody epitopes. The key p53 regulatory factor MDM2 is often referred to as human MDM2 or murine MDM2 (HDM2 & MDM2) but this is rarely encountered when describing p53 and “these animal studies indicate that murine p53 is doing this or that” is not the norm -it is simply p53. The differences between p53-associated cancers in mice and human could also depend on differences in cellular factors between the species, even if the proteins are having similar functions. For example, the p14Arf (human) tumor suppressor and its murine homologue p19Arf are tumor suppressors with 50 % identity that control MDM2 activity but the human and murine proteins carry out different functions [24].

Nevertheless, murine and human p53 seem to carry out similar functions to a large extent and later works have confirmed that many p53 upstream and downstream pathways are indeed active in both species. However, it is worthwhile keeping in mind differences in tumors between species that can preclude impacts of mouse models in the human clinic. A recent review has highlighted this problem in terms of immunity [25]. For example, mice fail to develop clinical signs seen in humans after infection with *H. pylori*, *C. difficile*, or the influenza virus. In addition, T cells from mice exposed to conventional microbiomes resemble T-cells from neonatal humans. By contrast, mature T cell types seen in humans are observed only in mice colonized with complex microbiota. Fibrosis and IL-17 also exhibit striking differences between mouse and human. Considering the larger proportion of transgenic mice are “pathogen-free”, we are perhaps not gaining insights into how the microbiome can shape cancer development and perhaps we are missing a clue for some differences between mouse and human in terms of tissue-specific cancer development. Together, these data show that the overall physiological differences of the murine and human systems are likely more problematic than we like to think and may preclude a more complete understanding relevant to the human cancer clinic.

Animal models using conditional gene expression (the gene can be turned on or off) to see what happens when p53 activity is restored in cancer cells developed in a p53 null background, show that lymphomas indeed respond with induction of apoptosis whereas sarcomas instead respond with senescence and activation of the immune system [26]. There is nothing to compare the activation of p53 in mouse models with a similar activation in humans, but treating patients suffering from leukemia with compounds that prevent MDM2 from binding to p53 (*i.e* Nutlin-like) to activate p53, not only has so far showed little obvious positive effects on cancer but causes bone marrow toxicity [27,28]. Apart from the target might not live up to expectations, there are other factors to take into account such as a dominant MDM2 oncogenic isoform in human sarcoma might not express the N-terminal domain of MDM2 or that Nutlin can induce PDL1 that can mediate immune suppression [29]. Mice in which p53 is activated by genetic recombination show no important toxicity outside the tumors, suggesting that in normal tissue the balance between p53 and MDM2 prevents toxicity. But why does not the induction of p53 also induce MDM2 in the animal tumors and prevent p53 from being activated? As the loss of p53 in the animal tumors is a germline driver mutation it is likely that other genetic events have taken place, making p53 activation redundant in some of the tumor types. These observations add to the notion that animal models can provide insights on pathways of interest in understanding p53's role in cancer development and for establishing which p53 regulatory and the mechanisms employed and how they function under different conditions. But some cautions are warranted to which extent mice models compares to human cancers. Ideally, in order to exploit p53 for targeted cancer therapies an intermediate comparative oncology model that complement the current mice models and that take into accounts some of the issues of tumor heterogeneity and an intact immune system would be needed.

#### *P53 oncogenic activity*

P53 is most commonly altered by missense mutations (75 %) and not by complete deletion or nonsense mutations [1]. Six 'hotspot' residues are mutated within the DNA-binding domain (R175, G245, R248, R249, R273 and R282) that either directly affect its interaction with DNA, or indirectly by disrupting the p53 protein structure [30]. It is rather unusual for a tumor suppressor to have such a high frequency of missense mutations instead of the expected nonsense mutations or deletions, like for the retinoblastoma gene [31]. Considering that a change in the p53 coding sequence can generate neoantigens, it can be argued that this positive selection of missense mutations should reflect a growth advantage. It was recently demonstrated that peptides representing mutant p53 are presented on major histocompatibility (MHC) class I molecules [32]. Indeed, early mouse models show that animals expressing mutant p53 develop tumors more rapidly than p53 null animals, and Li-Fraumeni patients gain p53 mutations in tumor cells under conditions while one allele is lost in the germline [3,33]. In

such a scenario, one could expect that the enrichment of mutant p53-carrying tumor cells is a consequence of gain of oncogenic activity, rather than a loss of tumor suppressor activity. One complication with the gain of function (GOF) concept is that most p53 mutations are all grouped together in the same category when it is unlikely that they will all change p53 functions and its interactome in a similar way. It is interesting to note that all but 7 residues in p53 have been shown mutated in human cancers, suggesting that other mechanisms, apart from DNA binding, also play roles (**Figure 1**) [34,35]. Indeed, many effects related to mutant p53 are related to protein-protein interactions [36–40] and there is little evidence suggesting that all p53 mutants would affect the p53 interactome equally [41]. It is also conceivable that the interactome induced by one mutation in one cell type will differ from another cell type and, thus, play different roles in carcinogenesis depending on where and when it arises. Another possibility is that GOF is a selective loss of function and hotspots mutations that have lost DNA binding activity retain a common DNA-independent function. We come back to what this could be under *perspectives*.

A difficulty with animal models in which a mutation is inserted in the germline as drivers for cancer development is whether this reflects the situation that occurs in spontaneously developed cancers. It is generally believed that a mutation in a certain stem cell results in survival and proliferative advantage of a clone that increases the chances for further mutations to occur that eventually results in a cancer. However, different clones will acquire different mutations, which help to explain the heterogeneity of spontaneous cancers. It also indicates why certain events follow one after the other and why the order of events differs from one tissue to the next, or even in the same tissue. This can explain why some cancers seem to have p53 as a driver while others not, and adds a different perspective to the interpretation of mutations in terms of cancer development. This can be likened with p53 being a main actor on the cancer development stage, but with each cell type representing a different stage set with different co-actors. A recent study in mice using intestinal cancer models illustrates this concept by demonstrating how the p53 mutation R172H (R175H in human) has opposite effects in different segments of the gut [13]. In the distal part it behaves as an oncogene, while in the proximal segments it acts as a tumor suppressor. It would be interesting to see what happens in this intestinal cancer model using different p53 mutations. Interestingly, the tumor suppressor activity was related to the environment and could be prevented by the gut microbiota. When gallic acid was supplemented to animals with a sterile gut, the tumor suppressor activity of mutant p53 was lost. It should be pointed out that several mechanisms of the anti-cancer and anti-inflammatory action of gallic acid have been put forward [13]. Nevertheless, this study illustrates the dynamics of how a mutation can have different effects depending on where and when it occurs which would affect the selection of p53 mutations. Considering that one of the holy grails of the therapeutic field is to convert

mutant p53 to a wild-type conformation, this should stimulate further studies that exploit the microbiome as a preventative therapy to reduce cancer incidence.

Sequencing different segments of the same tumor has shown areas with different p53 mutations, or no p53 mutations [42,43]. A study focused on p53 in glioblastoma showed some tumors with wild type p53 while some had three different mutations in different regions [42]. Another recent study that focused on intra-tumoral heterogeneity (ITH) in human colorectal cancer, phenotyped, genotyped and topography analysis of 12 microdissections of human colorectal tumors to represent the clonal evolution. In one of the tumors, three different mutant p53 sub-clones with their own private mutation and distinct morphologic boundaries were found. Each cell with mutant p53 had a different phenotype (superficial or invasive) that was unrelated to arising early or late during tumor development, confirming that p53 mutations are not equal and suggesting that the genetic background of the ancestors of the different clones modulates the function of the different mutant p53 [44]. Other studies come to a similar conclusion that tumor heterogeneity reflects the specific genetic background of the clone they arise from, when they arise or the microenvironment they exist in. These arguments should apply for any gene involved in cancer development.

#### *P53 activation and therapeutics*

A biochemical study was first to open the door to the possibility that p53 activity could be regulated via therapeutic interventions. A peptide from the C-terminus of p53 was shown to activate the DNA binding capacity of p53 and even though this did not *per se* suggest a therapy, it stimulated others to try to find ways to activate p53 [45]. A compound that could activate mutant p53 was later identified by screening in cell lines using a p53 reporter gene construct that later developed into a drug that is currently being tested in clinical trials against a variety of different cancers [46–48]. This concept has the broader interesting implication of “repairing” dysfunctional proteins. Another early study showed that a short peptide derived from p53 conserved BOX-I domain could prevent MDM2 from binding p53 and later crystal structures showed how the peptide interacts with a hydrophobic pocket at the N-terminus of MDM2 [49]. This paved the way for the identification of drugs, most notably the Nutlin series, which bind this hydrophobic pocket and thereby prevent MDM2 from suppressing p53 activity with the aim to activate p53 in cancers, predominantly cancers overexpressing MDM2 [50]. However, these compounds do not disrupt the MDM2 – p53 protein-protein interaction but acts as agonists [51]. A closer look at the mechanistic aspects of these compounds reveals that they impose allosteric changes in MDM2, including a second interaction site for p53 and stimulates p53 monoubiquitination and chromatin binding. Hence, what initially seemed like a straight forward concept of targeting a protein interaction to control cancer growth, turns out to be more complicated in terms of drug target physiology, mechanism of action as well as alternative oncogenic MDM2 isoforms that do not bind the



compounds. Several pharmaceutical companies have developed their own MDM2 inhibitory compounds but as of today, this approach has overall not been successful in the clinic. Both the therapeutic tactics of MDM2 ligands and the activation of mutant p53 had their origin in academic labs in the late 1980s but the clinical trials are only currently being carried out, 30+ years later. This gap in the application of translational aspects of well-established findings by the basic academic research, is not a reasonable practice and it should not take over 30 years from the discovery of a potential new cancer therapeutic approach to reach clinical trials. As new therapeutics have their origin almost exclusively in academic research, this highlights the difficulty of gapping academic (discovery) with industrial (execution) research.

The above examples illustrate how p53 folding and expression have been targets for therapeutic intervention strategies. Early studies showed that p53 is also subjected to post translational modifications. In particular, phosphorylation of residues in the N-terminal transactivation domain of p53 following DNA damage have been implicated in p53 activation [52]. Animal models in which these phosphorylation sites have been mutated support that idea these residues are indeed important but no therapeutics targeting the post translational regulation of p53 activity have been developed, as yet.

## *MDM2*

Based on a few key experiments, MDM2 has emerged as the main regulator of p53. It was shown to i) bind the N-terminus of p53 ii) to act as an E3 ubiquitin ligase that targets p53 for 26S proteasomal degradation and iii) that the embryonic lethality at E5.5-6.5 caused by *Mdm2* deletion is rescued by simultaneous deletion of *Trp53* [15,53–56]. The interpretation of these experiments had far reaching consequences. One was the idea that the sole function of MDM2 is to suppress p53 activity and, thus, everything MDM2 does and every factor it binds, somehow relates to p53. Consequently, preventing the interaction between p53 and MDM2, aiming to activate p53 in cancer cells, became a hot topic for therapeutic development. Unfortunately, a negative consequence was that any data implying that MDM2 could have functions not directly related to p53 became hard to sell. The dogma was set and a simple, but yet important, aspect to keep in mind is that just because the manipulation of a factor in animals or tissue culture cells has a certain effect, it does not mean that this is the only effect. Nevertheless, a vast amount of new data has been published since these early experiments, including the observation of oncogenic MDM2 isoforms [57] and there is an urgent need to update the role of MDM2 in terms of p53-dependent and non-dependent functions. More generally, teaching new tricks to old dogmas is vital for addressing such complex mechanisms, especially in view of the constantly enriched findings being acquired by sequencing and imaging data and by modelling predictions.

The *Trp53* and *Mdm2* double null animals showed that the combined loss of p53 and MDM2 results in animals that develop past E5.5-E6.5 and are viable adults [58,59]. However, this does not show that MDM2 has no other functions or, for that matter, that embryo survival is the sole consequence of MDM2 negatively regulating p53. Data from human cancers were not entirely supportive of the model that MDM2 solely negatively regulates p53 as MDM2 is overexpressed in cancers with mutant p53 [60]. Moreover, overexpression of MDM2 in p53 null animals increase tumor incidence [58]. Nevertheless, animal and *in vitro* models on MDM2 were, thereafter, primarily focused on p53. Later conditional animal models showed that depletion of MDM2 gives tissue-specific phenotypes, suggesting that p53 might not have the same function in all tissues, or MDM2 controls p53 equally in all tissues, or that MDM2 has other tissue specific effects [61]. Studies using an MDM2 that is E3 ligase dead towards p53 (MDM2(C462A)), resulted in embryonic lethality at E7.5, supporting a role of MDM2 in targeting p53 for degradation during this stage of embryonic development [62]. However, it is important to bear in mind that changing a cysteine residue in MDM2 is likely to have severe effects on protein folding and other functions, apart from E3 ligase activity towards p53. The MDM2(C462A) protein still interacts with p53, implying that binding to the N-terminal transactivation domain of p53 is not sufficient to suppress p53 activity. An example of how minor changes in MDM2 can have drastic effects on activity was shown in mice in which the ATM kinase phosphorylation site at serine 394 (S395 in human MDM2) was substituted with alanine. This resulted in a decreased sensitivity to irradiation, demonstrating the importance of this site in controlling p53 DNA damage response [62,63]. The explanation to this observation is to be found in the fact that *TP53* mRNA levels do not change during DNA damage [64] but its rate of synthesis does [65]. The highly conserved Box-I RNA sequence of p53 that encodes the peptide that binds the N-terminus of MDM2 and promotes p53 degradation, binds to the C-terminus of MDM2 and stimulates p53 synthesis. This switch from binding the p53 protein or the *p53* RNA is what the phosphorylation of MDM2 by ATM controls [66]. This illustrated not only the importance of testing animal models under stress conditions but also that MDM2 can be both a positive or negative regulator of p53 depending on conditions. It also added a twist to the fact that one of the first identified p53 early target genes is *Mdm2*. It had been suggested, and accepted, that this provides the major negative feedback loop to ensure low levels of p53 under normal conditions. But as MDM2 stimulates p53 synthesis during p53 activation, this feedback loop becomes positive. The physiological importance of this feedback loop was tested by generating mice that lack the p53 binding site on the *Mdm2* promoter [66,67]. These animals develop normally but have a suppressed DNA damage response, showing that it is this positive feedback loop that is physiologically important. Hence, the dogma from the early animal that MDM2 is only a negative regulator of p53 is not accurate.

An interesting, and unravelling, aspect of the p53 – MDM2 interaction came from studies on the early metazoan *Trichoplax* which showed that the conserved regions of the interaction might have evolved during early evolution and, thus, had little to do with cancer protection but instead suggested a functional origin that later evolved to control the activity of one of the more important interactions in cancer biology [68]. Further studies comparing pre-vertebrates with vertebrates showed that pre-vertebrate and vertebrate *p53* RNA sequences bind MDM2 but only the vertebrate p53 protein interacts with MDM2. The p53 amino acid sequence that binds MDM2 is highly conserved but the C'-flanking sequence is not, and when this region is removed, MDM2 binds the pre-vertebrate p53 protein [69]. Hence, the *p53* mRNA – MDM2 protein interaction evolved in pre-vertebrates and the protein-protein interaction evolved later in vertebrates.

MDM2 is a complex hub with a large interactome and, for example, interacts with three different tumor suppressors (p53, the retinoblastoma protein (pRb) and p14Arf) but also with factors controlling DNA repair and metabolism. It is interesting to note that while p53 is frequently mutated in cancers, MDM2 is not. One might have expected that there would be some gain or loss of particular functions of MDM2 that would benefit tumor development. Perhaps this reflects a need for the cancer cell to have MDM2 as both a positive and negative regulator of cell growth and proliferation? MDM2 is, nevertheless, overexpressed in cancers and the emerging consensus is that non-p53 functions of MDM2 have been overshadowed by its regulation of p53 and it is timely to start looking at some of MDM2's non-p53 functions that are also potential therapeutic targets. Predicting the effects of targeting MDM2 can, however, be hazardous. For example, apart from binding the *TP53* mRNA, MDM2 has also been shown to interact with other mRNAs including *XIAP*, *SLUG*, *N-myc*, *RB1* and *E2F1* and with some key cell growth regulatory gene products [70–74]. The interaction with *p53* and *RB1* mRNAs is induced during the DNA damage response [16] and promotes tumor suppressor activity, while the *E2F1* mRNA interaction stimulate growth-promoting pathways [70]. In addition, in the case of *TP53*, *RB1* and *E2F1* mRNAs MDM2 also binds the encoded proteins [75,76]. Together, this implies that depending on conditions, MDM2 can support tumor suppression or tumor growth.

Some of the more recent examples of MDM2 activity and regulation that are not p53 dependent include the DNA damage repair MRN (Mre11/Rad50/Nbs1) complex that was found to interact with MDM2 via Nbs1 independently of p53 [77]. Increased MDM2 levels reduced MRN activity, ATM kinase signaling and DNA break repair, thus indicating a suppressive function that can enhance cell transformation in p53 null cells [78]. The reduced genome stability in *Mdm2* transgenic mice is linked to an increase incidence of tumors and MDM2 stabilization using Nutlin-like compounds in p53 null cells increases the number of DNA breaks [79]. MDM2 has also been linked to chromatin via the ATF3/4 transcription factors in response to metabolic changes, resulting in the activation of an ATF3/4-

dependent gene expression program controlling amino acid and glutathione metabolism. The phosphorylation sites of MDM2 involved in this process were identified [80]. MDM2 levels can be enhanced either via gene amplifications such as sarcomas and oesophageal carcinoma carry *Mdm2* gene amplification [81] or via transcriptional activation by p53. There are also cases where the levels of RNA do not correspond to protein levels such as Burkitt's lymphoma and melanoma, that express high levels without genetic changes [82]. It was shown more recently that MDM2 stability is controlled by the PI3K $\delta$  pathway in response to mRNA translation stress, independently of its own E3 ligase activity, offering alternative therapeutic opportunities to target MDM2 in cancers [70].

#### *Condition-dependent effects of p53 target genes.*

Considering that the regulation of p53 by MDM2 is context-dependent and that the activity of mutant p53 differ from one location to another, could the activities of its downstream target genes also depend on conditions and cell type? This is quite likely and even though some p53 target genes seem to be cell-type independent it is conceivable that gene expression patterns are adapted to tissue and microenvironment and, hence, that induction of a factor in one cell-type can have a different effect than in another. One mechanism of differentiating p53 activity under different conditions via target genes is to express isoforms. One such p53 isoform, p53/47, is induced following stress of the endoplasmic reticulum by alternative initiation of translation at a second downstream methionine and lacks the first 40 amino acids, including the MDM2 binding site and the transcription activation domain. But it retains an RNA binding domain and the capacity to suppress mRNA translation [83,84]. How these two isoforms control cell cycle progression via controlling p21<sup>waf1</sup> expression nicely illustrates alternative condition-dependent cell biological effects of p53. The full length p53 binds the *CDKN1A* (p21<sup>waf1</sup>) gene promoter during DNA damage, resulting in higher p21 levels and G1 arrest, while during ER stress the p53/47 binds the *p21* mRNA and suppresses its translation causing G2 arrest [85,86]. Interestingly, transgenic mice who overexpress the p53/p47 isoform show a dramatic phenotype with premature ageing and early death [87]. This phenotype is dependent on the presence of the wild type p53, suggesting that the two isoforms act together in controlling tissue generation and ageing.

#### *Conclusions and Perspectives*

The p53 field can pride itself for being open minded to new ideas and concepts, which is a reason for its success. From its discovery it has been in the forefront of cancer biology but some dogmas have, unfortunately, slowed down progress in terms of translating knowledge to the human clinic. The early p53 transgenic mouse models did not consider tumor heterogeneity and physiological difference between mouse and humans in several disease indications, or the microbiome effects on the immune

response or even p53 conformation itself. It is therefore important to revisit some of the dogmas from these early animal models in view of later models of cancer origin. Neither p53 status, nor MDM2 levels, do *per se* give an insight into their respective contribution to tumor development and however uncomfortable as it might be, genetic events and gene expression patterns need to be interpreted with caution when addressing their role in tumor development, and as potential therapeutic targets.

The development of compounds designed to prevent MDM2 from binding p53 do have not the desired clinical effects, partly due to the unappreciated complexity of p53 regulation but also due to the shortcoming of cancer models in mice. For example, the first generation of MDM2 binding ligands in fact stabilize the MDM2-p53 interaction resulting in p53 activation and it is not clear if these drugs will enhance the gain-of-function of mutant p53 in a tumor with a mixed p53 status. The clinical response to drugs activating mutant p53 is still under evaluation.

One also has to consider condition-dependent roles of p53 target genes. For example, p53-dependent induction of p21 during DNA damage leads to G1 arrest, while active suppression of p21 by the p53 isoform causes G2 arrest. This illustrates how not only p53 or its regulatory factor (MDM2) but also its downstream target genes, can have different functions/effects under different conditions. Hence, the two classic p53 response target genes *p21* and *Mdm2* play different roles depending on conditions and levels of expression.

One is forgiven for wondering if we have so far missed something in terms of p53, a piece of the jigsaw that would help bring it all together? Or, do we have to embrace the idea that it will get more complicated before becoming, if ever, more comprehensible. So, what could this something be in terms of p53? Since early in its research history, it has been known that p53 not only binds DNA but also RNA, but so far this activity has not been studied with any great effort. An early study indicated that p53 can bind its own mRNA and later works show that p53's mRNA binding capacity can suppress translation of messages such as p21 or the ER chaperone BiP [85,88,89]. The *TPp53* mRNA is a target for regulation via many cellular factors and the *TPp53* has an overrepresentation of synonymous, or whisper, mutations that change the RNA sequence but not the encoded peptide [90,91]. Considering that evolution has put so much information into the *TP53* gene, it is conceivable that also the messenger RNA plays a role in determining p53 activity [92].

The first thought when learning that elephants have 19 p53 copies or isoforms is to wonder why does it need so many [93]? A further contemplation leads to the question -why do we only have one? Would it not make more sense that *TP53* duplicate during evolution in humans as well and that each copy specialized in specific stress or signaling pathway? We can also ask how the elephant p53s

regulated and if, indeed, they each have a specific function? This is an interesting emerging field where comparative cell biology/oncology might be able to tell us more on how p53 works.

Considering that humans only have one p53 and that this protein is capable of carrying out a vast amount of activities in response to the type of cell damage the question is *how, when and where* p53 activity is determined? Is it possible that the native p53 protein “hangs around” until either MDM2/MDM4 targets it for degradation, or until an enzyme modifies it such that it binds to co-factors leading to a certain activity? What speaks against a model of a free p53 waiting to be activated is the assumption that non-modified p53 binds DNA and there is nothing to prevent it from also interacting with other proteins. We have discussed the importance of the order of genetic events in oncogenesis and the same concept also applies for p53 activation. The order of events in which p53 is modified and interacts with cellular factors determine its final activity. Hence, a free p53 waiting to be activated is presumable an illusion. Some data instead suggest that p53 activity is decided during its synthesis. For example, the nascent p53 polypeptide is phosphorylated by the DNA damage-response kinase ATM during synthesis [94]. In an extended scenario this could indicate that different signaling pathways merge on the p53 polysome so that the fate of the newly synthesized p53 is determined before it leaves the ribosome. Obviously, these types of models have interesting implications also for mutant p53 and its activity and have far-reaching implications for response pathways outside of p53.

Finally, will the animal models developed with various genetic alterations in the p53 pathway help to develop new therapies? As discussed above, mice grown in pathogen free environments are poor physiological models for developing new cancer treatments for humans and less than 1 % of potential therapeutics that pass the pre-clinical stage are of any use in the clinic. Yet still, this is the “gold standard” for drug development. Daniel Kahneman in his book “Thinking fast, thinking slow” used the term “dart-throwing monkeys” to describe the accuracy of experts to predict certain outcomes and this phrase comes to mind when describing cancer drug development using mouse models. One issue is the false positive data and treatments that later turn out not to work in the clinic, but what about the false negative data –drugs or treatments that actually could work but show no effect on cancers in mice? With this in mind, the increased interest in using old drugs for new purposes is understandable as well as to develop better and more reliable comparative oncology animal models [95]. An interesting alternative model is provided by our dogs (*Canis lupus familiaris*) a species displaying a huge diversity in traits as well as body sizes following hundreds of years of highly selective breeding for specific traits. This strict selection had the inevitable result of significant in-breeding effects including significant likelihoods of specific tumors linked to certain breeds [96]. The molecular signature of canine cancers look similar to the human cancers and dogs might thus, provide a model

to examine not only p53's effects on age and size and the effect mutations, but also the effects of generic compromises of an immune system as the result of key mutations [97,98].

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### Legends

Figure 1. The multifunctional aspects of p53 are illustrated by its interactions with DNA, RNA and proteins. One of p53's main functions is to act as a transcription factor and downstream target genes span a broad range of different reversible or irreversible cellular functions such as cell cycle arrest and repair or senescence and apoptosis. It also interacts with cellular proteins including apoptotic regulators Bax/Bac and Bcl2. P53 activity is tightly regulated and differentiated via post translational modifications and protein interactions. Hot spots mutations that prevent DNA binding show gain of function and is attributed to protein – protein interactions that can indirectly target p53 to gene promoter regions or interfere with the activity of p53 family members (p53, p63 and p73) with effects on gene expression. Mutant p53 also show growth suppressing activity in a condition-dependent fashion. Some less studied and, thus, more speculative effects of p53 are also indicated such as the isoform (p53/47) that lacks the N-terminal gene transactivation domain but retains RNA binding capacity and interacts with full length p53 and suppresses gene expression via mRNA translation control. Green represents growth promoting activities and red suppressive.

Figure 2. MDM2. A few selected interactions between MDM2 and cellular factors are shown and the effects of these in terms of promoting (green arrows), or suppressing (red), cell growth are suggested. MDM2 is unique in the sense that it can stimulate protein synthesis via mRNA interactions or promote degradation via protein interactions. Even though MDM2 targets the p53 protein for degradation during normal conditions, other MDM2-dependent events also take place. The cartoon also points out a few events that have been shown to be cell condition-dependent (*italic*). The consequences of MDM2 overexpression depend on cellular and environmental conditions and are therefore difficult to interpret. Green represents growth promoting activities and red suppressive. Some interactions can serve both.

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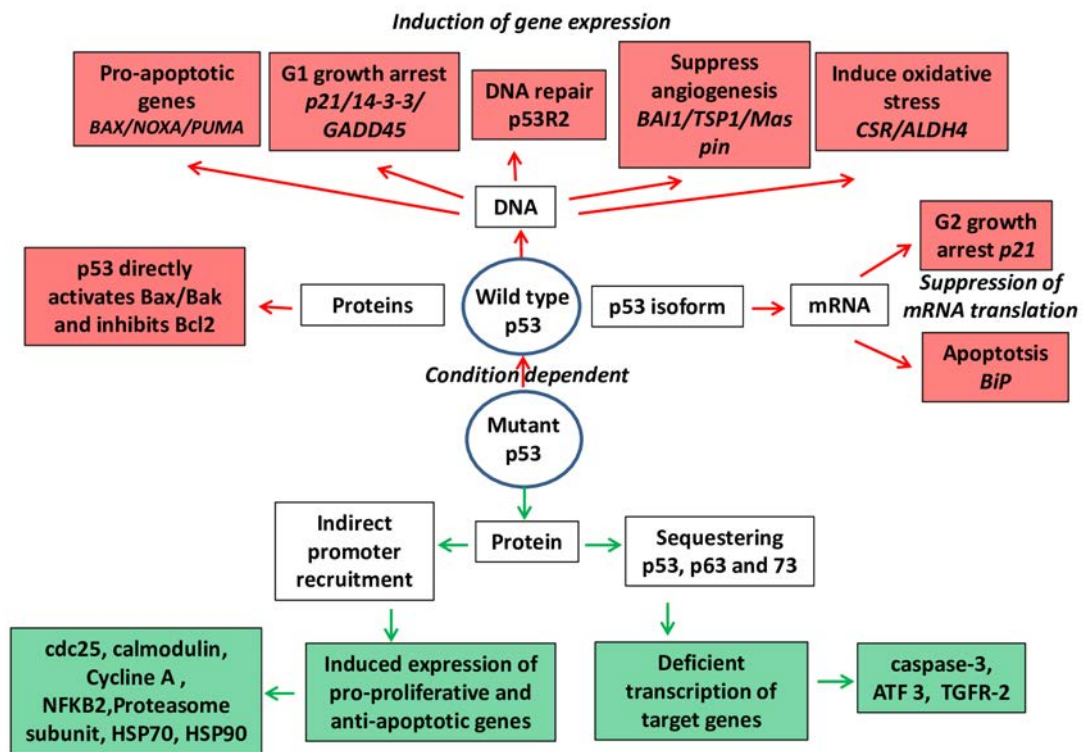
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**Figure 1.**



**Figure 2.**

