The p53 protein – not only the guardian of the genome

ABSTRACT

The p53 tumor suppressor protein is best known as an activator of cell cycle arrest and apoptosis. Only a fraction of p53-activated genes encode proteins affecting cellular replication and various forms of cell death (apoptosis, ferroptosis, autophagy). The p53-regulated genes can be divided into so-called the core transcriptional program, which comprises genes activated in most cell types by most activators, and into the group of genes activated in in cell- or stress-specific manner. Activation of p53 occurs *via* the extensive set of post-translational modifications, which adjust its stability, interaction with other transcription regulators, and its ability to form a tetramer. Surprisingly, in mouse models, the activation of the best-studied p53 target genes encoding the inhibitor of the cell cycle (*CDKN1A*) or the inducers of apoptosis (e.g. *NOXA*, *PUMA*) is dispensable for protection against cancers. Thus, the non-classical functions of p53 must be studied to better understand its tumor suppressive mechanisms.

INTRODUCTION

The p53 protein is a well-recognized molecule due to its prominent role in cancer biology as the major tumor suppressor. It is best known as a regulator of cell cycle and apoptosis. These are the "textbook functions" of p53. However, p53 positively regulates the expression of hundreds of genes, and only a fraction of them encode proteins involved in the cellular processes. Judging by the activities of all known genes regulated by p53, its functional role is more comprehensive than initially expected. In this review, I will highlight these other, less-known functions of p53.

THE FIRST PLOT TWIST IN p53 RESEARCH

The p53 protein and its gene *TP53* have been intensely studied since the beginning of the 90ties of the last century. The number of papers dealing with these molecules grew steadily each year. This number leveled off at the beginning of the 21st century but remains high at approximately 5,000 papers yearly. During the 80ties, nothing indicated that these molecules would become the major focus of cancer research. The first papers on a mysterious cellular molecule interacting with a protein (large T antigen) of an oncogenic virus SV40 detected as a contaminant of early polio vaccines were published in 1979 (reviewed by Levine, [1]). Initially, the data indicated that p53 promotes cancer formation and is coded by an oncogene. The expression of p53 was remarkably high in cancer cells, its gene could cooperate with other oncogenes in transforming normal cells into cancer cells. During the first decade of p53 research, less than 100 papers on p53 were published yearly. It was completely altered at the beginning of 90ties. Several events precipitated this change. First, it was discovered that not all cDNA clones of p53 promote oncogenic transformations. Some of these clones inhibited cell growth [2,3]. Second, the germline mutations of *TP53* were responsible for cancer-prone Li-Fraumeni syndrome [4]. This was a feature of the tumor suppressor gene. Third, in various common cancers, the point mutations of p53 were frequently found accompanied by the deletion of the *TP53* locus on the homologous chromosome – this is another feature of the tumor suppressor gene [5]. Thus, after a decade of research, the status of the p53 gene changed from an oncogene to a tumor suppressor gene. These discoveries initiated an extensive search for p53 mutations in various tumors. It soon became apparent that *TP53* is the most frequently mutated gene in human cancers. After more than three decades of research and the widespread use of next-generation sequencing for the analysis of gene mutations, the status of p53 remains unquestioned [6,7].

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DEDICATION

This article is dedicated to the memory of Professor Mieczysław Chorąży, my Mentor, who guided me through the maze of science.

THE MAJOR BIOCHEMICAL FUNCTION OF p53 – SEQUENCE-SPECIFIC BINDING TO DNA

With the realization that p53 is frequently mutated in cancers, it became apparent that a deep understanding of cancer cell biology cannot be reached without a comprehensive knowledge of the functioning of this protein. Many oncogenes detected during the 80ties were found to code for proteins participating in transducing the growth-promoting extracellular signals from growth factors and their receptors to proteins located further downstream the signaling pathways. So, researchers speculated what might be the function of p53. It was soon discovered that this protein can bind to DNA in a sequence-specific manner – the major feature of gene regulatory proteins [8]. These proteins contain at least two domains. One is responsible for sequence-specific binding to DNA, and the other transcription-activating domain participates in interaction with other proteins involved in the activation of transcription. In case of p53 the DNA binding domain is localized in the central part of the molecule, which is affected by most mutations. Its three-dimensional structure was determined, and it was discovered that this fragment is well-structured, with the zinc atom playing an important part in stabilizing the conformation [9]. The amino acids making direct contact with DNA were identified and coded by codons in the mutation hot spots (R248W, R273H) of *TP53* (reviewed by Chen *et al.* [7]). While the central part of the p53 protein binds specifically to DNA, the amino-terminal fragment of the protein contains two transcription-activating domains – TAD1 and TAD2. They promote the expression of specific p53-regulated genes. The domains undergo extensive posttranslational modifications (mostly phosphorylation), which regulate the interaction of p53 with other proteins, which in turn modifies p53 stability, activity, and selection of particular genes to be activated (reviewed by Wen and Wang [10]). Thus, p53 is not a simple switch in gene regulation toggling between *on* and *off* position. By introducing various posttranslational modifications to p53, a cell can select subsets of genes which are activated, which in turn allows for a precise response to the stress conditions, which elicited the activation of p53.

The sequence of p53 response element (RE) – the DNA fragment to which p53 binds specifically, is long when compared to the sequences of other transcription regulators. The canonical sequence contains two direct repeats of a decameric motif RRRCWWGYYY (R = A or G, W = A or T, Y = C or T). In some of the REs, these decameric repeats may be separated by a spacer with various lengths (1-14 nucleotides), however, the spacer containing elements usually works less efficiently. This decameric sequence consists of two pentameric elements, which are inverted RRRCW and WGYYY [11]. This arrangement reflects the fact that p53 binds to its RE as a tetramer, and each p53 molecule binds to one pentameric sequence. The domain responsible for tetramerization of p53 molecules is located on its carboxyl terminus. Some posttranslational modifications of this fragment promote p53 tetramerization, which, in principle, may lead to the alteration of the strength of p53 binding to its RE [12]. Interesting observations concerning the connection between p53 tetramerization and the selection of particular REs were published by Schlereth *et al.* [13, 14]. The authors created mutant versions of p53 with increased cooperativity of binding of monomers in tetrameric

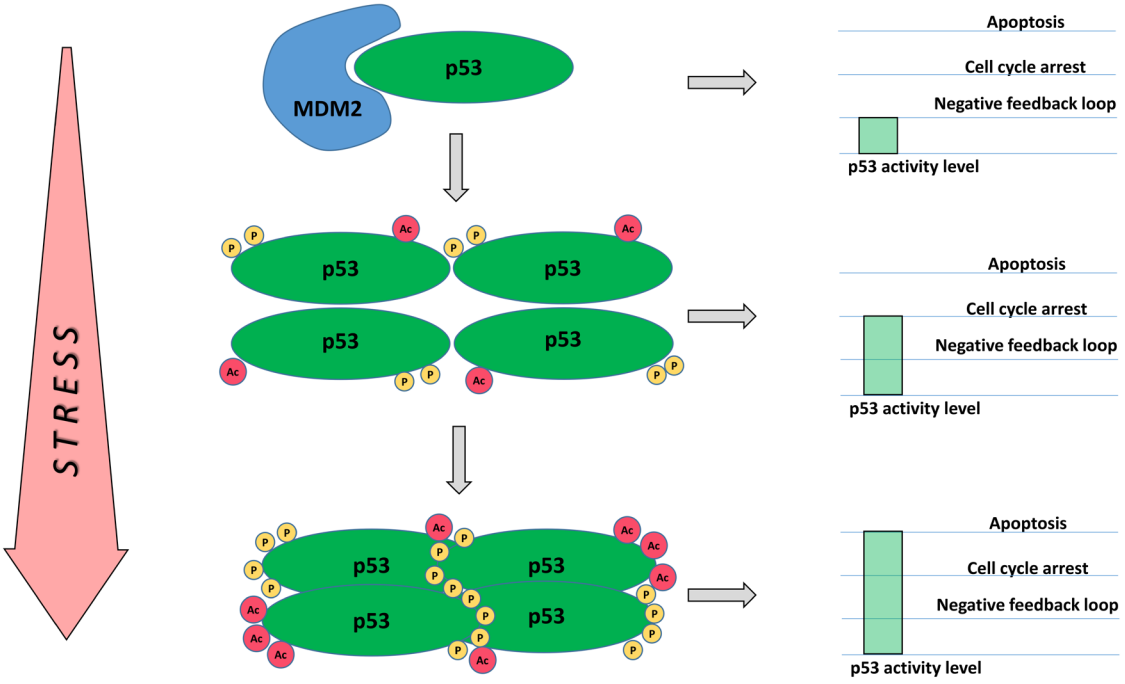

Figure 1. The activity of p53 is modulated by the nature and intensity of stress. During normal growth conditions the activity of p53 is limited by its major negative regulator – the MDM2 protein. In these conditions p53 is able to activate only a tiny set of its targets. During moderate stress, p53 is posttranslationally modified, what reduces its sensitivity to MDM2 and enables p53 to activate more genes, e.g., the ones which inhibit the cell cycle. During strong stress, the posttranslational modifications of p53 are more extensive, what can result in higher stability of p53 tetramer and easier interactions with other transcription regulators. This results in efficient activation of more genes including the pro-apoptotic ones. This is simplified model, e.g., some cells easily undergo p53-activated apoptosis, while others are more resistant. (P – phosphate, Ac – acetyl group)

p53 complex. Subsequently, using the ChIP-Seq technique (chromatin immunoprecipitation followed by sequencing), they compared the binding to DNA of wild-type p53 and the cooperativity-promoting mutant molecules. The analysis revealed that cooperativity extends the number of sites to which p53 tetramer can bind. This happens because the cooperativity allows binding to non-canonical response elements characterized by deletions, spacer insertions and base mismatches. But even in this apparent confusion, there are rules. For instance, the frequent deviation from the consensus involves mismatches only in one pentamer of the RE. This generates so called three-quarter sites, and p53 can also bind to them in some conditions [11]. Thus, genes regulated by p53 can be divided into low and high cooperativity groups. This has functional consequences because the low cooperativity group is enriched in genes coding for cell cycle regulators. While the high cooperativity group is enriched in genes coding for activators of apoptosis. In other words, to activate pro-apoptotic genes, p53 monomers must strongly attach what allows for binding response elements, which deviate to some degree from the consensus (Fig. 1). In these experiments, strong cooperativity is created by the engineered mutation, but the question is, what promotes cooperativity *in vivo*? Most likely, it is created by the proper set of posttranslational modifications. One of them (phosphorylation of Ser392) was identified years ago, but other modifications also play a role [12]. The findings of Schlereth *et al.* [14] agree with the model suggested by Kruse and Gu [15]. They suggested that there are various levels of p53 activation (Fig. 1). The lowest level are found in physiological conditions. The p53 activates genes, which participate in maintaining its low activity, e.g., *MDM2* (see below). To activate this gene, no special p53 modifications are required. At the higher level, additional p53 modifications start to appear (e.g., phosphorylation and acetylation of selected amino acids). This allows for the activation of different genes involved in cell cycle arrest or DNA repair (e.g., *CDKN1A* coding for p21 protein). At the highest level of activation, induced by strong stress factors or their combination, the posttranslational modifications of p53 are extensive. Moreover, p53 interacts with additional proteins, which, in combination with posttranslational modifications, allows for the activation of even more genes, involved, for instance, in the induction of apoptosis. These transitions are not sharp, and with the increasing stress intensity and/or duration, more genes are turned on by p53, leading to appropriate cellular response starting from cell cycle arrest and DNA repair and ending with apoptosis if cellular damage is irreparable. There are several genes activated by p53, which code for positive feedback loop elements in this signaling pathway (Fig. 2). One of them is *ISG15*, which codes for ubiquitin-like protein conjugated to other proteins. *ISG15* gene is activated by interferons and plays a role in antiviral defense. The attachment of ISG15 to p53 significantly increases its binding to the promoter regions of target genes, which forms a positive feedback loop [16]. Unexpectedly, another element of the positive feedback loop in the p53 signaling pathway is the c-Ha-Ras protooncogenic protein. Its gene (*HRAS*) is positively regulated by p53, which is very odd for a tumor suppressor. Paradoxically, inhibiting c-Ha-Ras function can block p53-mediated gene transactivation and p53-dependent apoptosis [17]. The activation of *HRAS* by p53 is not a peculiarity of the model used by these authors because this gene was identified as a p53 target in several high-throughput transcriptomic studies [18]. Other p53-activated genes that promote its activity are *SFN* [19] and *DDR1* [20]. The positive feedback loops enable p53 to activate more genes if the stress conditions are long-lasting. Thus, the fact that a gene is activated late during the stress does not necessarily mean that it is controlled by p53 indirectly. It is plausible that to activate some late genes, p53 needs assistance from the protein products of early genes.

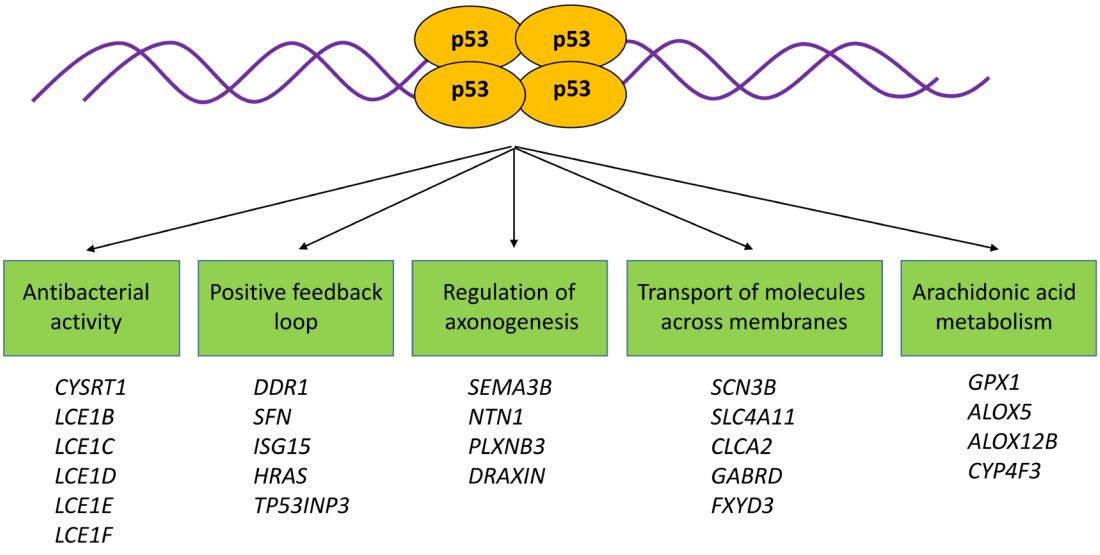

Figure 2. The less known groups of p53-activated genes. P53 activates genes for proteins with antibacterial functions, what suggest that during stress p53 may protect against infections. Moreover, p53 activates genes for proteins, which enhance its activity forming a positive feedback loop. In this way, during long-lasting stress p53 can activate more genes than during transient stress. Why p53 activates genes participating in axonogenesis is a mystery. Probably these genes have additional cancer-related functions, e.g. regulation of Wnt/β-catenin pathway. P53 also regulates transport of various molecules across cell membranes and affects metabolism of the major regulator of inflammation – the arachidonic acid.

REGULATION OF p53 STABILITY AND ACTIVITY

The p53 protein is very unstable in physiological conditions because it is rapidly degraded in proteasomes due to the attachment of ubiquitin molecules catalyzed by MDM2 protein - the major negative regulator of p53. This high turnover is a feature of many regulatory proteins. The attachment of ubiquitin molecules can be prevented by the attachment of phosphate to the selected amino acids on the amino terminus of p53. The modified p53 can no longer interact with and can no longer be destabilized by MDM2; hence, its stability and amount significantly increase, and it starts to activate various genes, as mentioned in the previous section. The MDM2 negatively regulates p53 not only by destabilizing it but also by binding it to its transcription activating domains and concealing them so they no longer function properly (reviewed by Zafar *et al.* [21]). There are also other negative regulators of p53, e.g., MDMX protein, but it negatively regulates p53 by a slightly different mechanism. For instance, MDMX inhibits p53 by binding it to its transactivation domains and thereby inhibits their activity [22]. The role of MDM2 is well-illustrated by the gene knockout experiments. The mice without murine version of MDM2 are embryonic lethal. Apparently, p53 hyperactivity due to lack of MDM2 causes too much disorder to allow for proper embryonic development. However, when MDM2 knockout is combined with p53 knockout, the mice are viable, which indicates that the major cause for embryonic lethality of MDM2-deficient mice is the hyperactive p53 [23]. The most frequent mechanism of p53 inactivation is the gene mutation [7]. However, in various cancer types, the activity of p53 is reduced by overexpression of the MDM2 protein due to the amplification of its gene [24]. This creates a therapeutic opportunity because a putative drug, which prevents p53-MDM2 interaction, would be able to bring back functional p53. Such a molecule was designed and synthesized based on the knowledge of the shape of the p53 binding pocket in MDM2. Its name – Nutlin-3a, was derived from Nutley in New Jersey, USA, where the company that created the compound had its campus [25]. Nutlin-3a could activate p53 in vitro but cannot be used in humans. Hence, negative regulators of p53-MDM2 interactions more suitable for *in vivo* use were designed by various companies and are evaluated in clinical trials [26].

The crucial step in the activation of p53 as a transcription regulator is its posttranslational modifications (mostly phosphorylation of serine or threonine residues and acetylation of lysine), which prevent interaction with MDM2 and promote interaction with elements of the transcription machinery. Thus, the activity of p53 is regulated by kinases and acetyltransferases, which modify p53, and by phosphates and deacetylases, which reverse the modifications. Because there are dozens of modification sites on p53 and there are other modifications as well, e.g., methylations and the attachment of small proteins like ubiquitin, SUMO or NEDD8, it appears that regulation of p53 activity is dauntingly complicated, yet there is the underlying order, which allows p53 to precisely respond to stress factors or other activating signals [10].

THE FIRST BIOLOGICAL FUNCTIONS – INHIBITION OF CELL CYCLE, INDUCTION OF APOPTOSIS, STIMULATION OF DNA REPAIR

When it was discovered that p53 acts as an activator of gene expression, the race was on to identify genes regulated by p53. One of the first genes found (*CDKN1A*) codes for the p21 protein, which is a negative regulator of various cyclin-dependent kinases (CDKs) – these enzymes promote the progression through the cell cycle. By inhibiting CDKs, the p21 protein causes cell cycle arrest [27]. This observation perfectly conformed to the picture of the tumor suppressor, which inhibits cell divisions. However, p53 not only promotes the expression of the negative regulators of the cell cycle, but it also negatively regulates genes indispensable for cell divisions [28]. This exemplifies a mechanism used by p53 to regulate other cellular processes – p53 impacts cellular function by regulating several genes. In the case of cell cycle arrest, it became gradually apparent that p53 negatively regulates a plethora of genes, which code for proteins indispensable for cell growth, DNA replication and recombination, replication-associated DNA repair, etc. According to the initial model, p53 is directly or indirectly bound to gene regulatory elements within negatively regulated genes, preventing their transcription. However, later it became evident that p53 inhibits these genes indirectly. The indirect inhibition is started by the production of p21 protein, which, by inhibiting CDKs, prevents phosphorylation of RB tumor suppressor protein and two other so-called pocket proteins (RB-related p107 and p130). The mechanism of indirect inhibition is the following. Many of the cell cycle genes are regulated by DNA sequences called CDE (cell cycle-dependent element) or CHR (cell cycle genes homology region). The CHR sequence can be occupied by the MuvB protein complex. If this complex is additionally bound by the B-Myb protein (coded by a protooncogene!), the transcription of adjacent cell cycle genes ensues. Alternatively, the MuvB protein complex can be bound by the p130 pocket protein and its two partners, E2F4 and DP1. The entity composed of MuvB, p130, E2F4, and DP1 is known as DREAM, and it represses the transcription of nearby cell cycle genes. The p21 protein, by inhibiting CDKs, prevents the phosphorylation of p130 RB-related protein, which in a hypophosphorylated state promotes the formation of DREAM repressive complex. Thus, p53, by activating p21, promotes the formation of repressive DREAM complex on numerous cell cycle genes [29]. A similar mechanism is used by p53 to repress cell cycle genes controlled by RB protein, which, with its binding partners E2F and DP, forms another repressive complex in the cell cycle genes (reviewed by Engeland [30]). The high throughput analyses of gene expression and other experimental evidence indicate that p53 does not directly repress genes. It is only the gene activator, and the repression is executed indirectly through p53-p21-DREAM/RB pathways [31]. However, it must be mentioned that there is some controversy over the mechanism of p53-mediated repression [32].

The p53 also positively regulates the expression of several DNA repair genes. One of them – *DDB2,* codes for a protein, which is crucial for global genome repair of DNA damaged by UV radiation. The homozygous germline mutations

of this gene cause human cancer-prone syndrome known as *xeroderma pigmentosum*, associated primarily with cancers of the skin exposed to UV. Interestingly, p53 does not activate this gene in mice, which is an example of differences between humans and mice regarding the functioning of the p53 pathway [33]. The p53 activates another gene associated with *xeroderma pigmentosum* – *POLH*, which encodes DNA polymerase with a special task to replicate damaged DNA fragments [34]. Another gene associated with *xeroderma pigmentosum* and activated by p53 is *XPC*, which codes for a protein recognizing DNA fragments damaged by UV [35]. Thus, out of the eight genes whose mutations cause *xeroderma pigmentosum*, three are positively regulated by p53. The *RRM2B* gene activated by p53 codes for ribonucleotide reductase protein, which catalyzes the conversion of ribonucleoside diphosphates to deoxyribonucleoside diphosphates, which is necessary for DNA synthesis [36]. Thus, p53, by activating the gene for this enzyme, supplies nucleotides needed for DNA synthesis during repair.

The next process regulated by p53 is programmed cell death – apoptosis (Fig. 3). There are two branches of pro-apoptotic signaling pathways – extrinsic and intrinsic. The intrinsic pathway is triggered by cellular stress, e.g., DNA damage, and the extrinsic pathway is triggered by death receptors activated by cognate ligands. Both branches converge on the activation of so-called executioner caspases, which start to cleave other proteins, which leads to all morphological and biochemical features of apoptosis. The crucial step of the intrinsic pathway is the release of cytochrome *c* from mitochondria to cytosol. Cytochrome c, together with APAF-1 protein, forms a multi-subunit structure called apoptosome, which activates caspase-9, which in turn activates executioner caspase-3. Permeabilization of the outer mitochondrial membrane is tightly controlled by a set of proteins belonging to the BCL-2 family. These proteins are of two types – pro-survival and pro-apoptotic. Their balance decides whether apoptosis occurs or not. Furthermore, the caspases are kept inactive by interaction with inhibitors of apoptosis (IAPs) like survivin (coded by *BIRC5* gene). Those proteins, in turn, are inactivated by other molecules like Smac, which are released from mitochondria together with cytochrome c. Thus, the activation of apoptosis is a multilevel process with built-in failsafe mechanisms, which prevent accidental induction of apoptosis, but when it is triggered, it occurs quickly, within less than 2 hours. The activation of extrinsic apoptosis starts with the binding of the cognate ligand to the death receptor (e.g., FAS, DR5). This activates caspase 8, which either, in specific cells, directly activates executioner caspase 3 or leads to the permeabilization of mitochondria with all its proapoptotic consequences [37, 38].

Predominantly, p53 promotes apoptosis by upregulation of genes coding for pro-apoptotic proteins and repression of genes for pro-survival proteins of both signaling pathways (Fig. 3). In 1993, two research groups demonstrated that p53 is indispensable for apoptosis triggered by ionizing radiation in immature mouse thymocytes [39, 40]. However, the mechanism of this pro-apoptotic effect was not known.

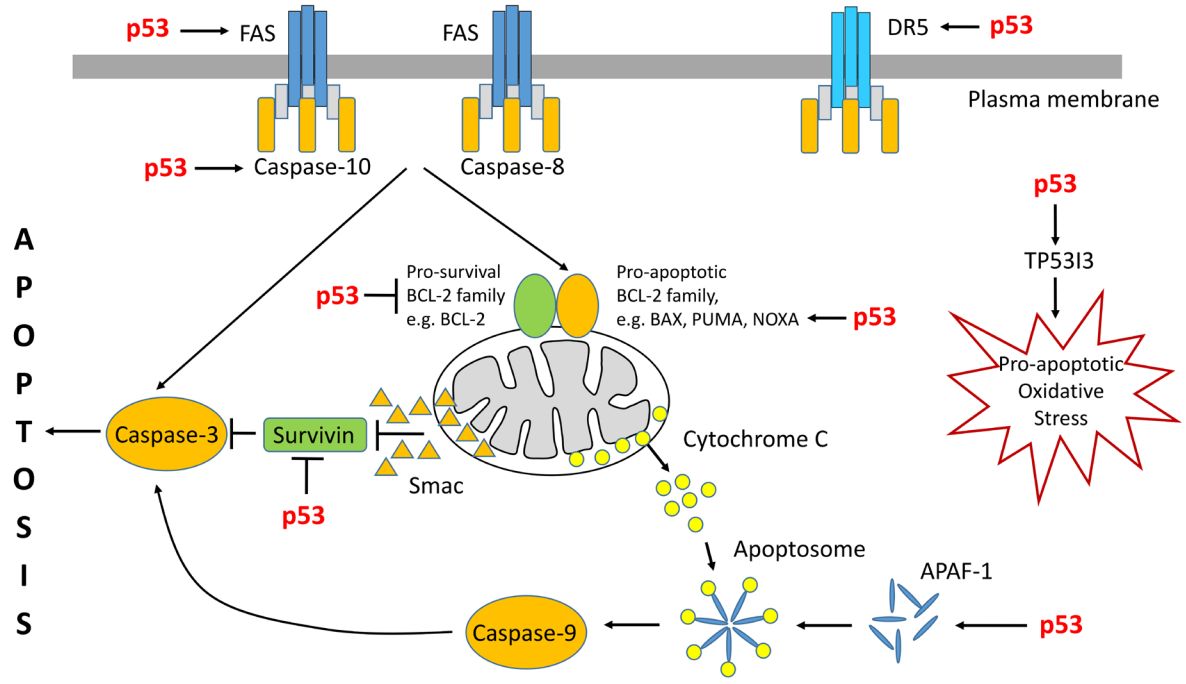

Figure 3. The simplified diagram of apoptosis regulation by p53. Apoptosis is triggered either by extracellular ligands of death receptors (e.g. FAS, DR5) located in plasma membrane or by intracellular stress or regulatory factors, which impact on outer mitochondrial membrane. The most prominent trigger of apoptosis is the permeabilization of outer mitochondrial membrane. This is governed by the interaction between various members of BCL-2 protein family, which decide when to open the pores in the membrane. The opening releases two important proteins from mitochondria – cytochrome *c* and Smac. Cytochrome *c* together with APAF-1 protein activates caspase-9, which in turn activates caspase-3, which is an executioner of apoptosis. Caspases are kept inactive in part by binding to a group of proteins known as IAPs (inhibitors of apoptosis). One of them is called survivin, which inhibits caspase-3. IAP proteins are inactivated by Smac released from mitochondria. The death receptors bound by cognate ligands activate caspase-8 and, when expressed, also caspase-10. Activated caspase-8 can either directly activate caspase-3 or it can lead to opening of mitochondria by modifying one of the proteins of Bcl-2 family. Moreover, apoptosis can be triggered by strong oxidative stress. The p53 can promote apoptosis by repressing pro-survival genes (e.g. *BCL-2*, *Survivin*) and by stimulating pro-apoptotic ones (e.g. *BAX, APAF1, FAS, CASP10, TP53I3*). Not all apoptosis-related, p53-regulated genes are shown.

Intriguingly, p53 is not needed for apoptosis triggered in thymocytes by other factors. Thus, p53 was not a universal inducer of apoptosis. In another experimental model, the Bcl-2 protein was found to antagonize p53-induced apoptosis [41]. One of the first papers showing the mechanism of proapoptotic activity of p53 demonstrated that mice deficient in p53 have increased levels of bcl-2 protein and decreased levels of proapoptotic bax protein (which also belongs to Bcl-2 family of proteins regulating mitochondrial permeability) [42]. This suggested that p53 may regulate the expression of these two genes oppositely. However, the paper published in the same year indicated that activation of p53 can trigger apoptosis without the need for gene transcription. This suggested that the induction of apoptosis by p53 does not involve the activation of proapoptotic genes [43]. On the other hand, it was demonstrated that p53 directly activates the expression of the proapoptotic *BAX* gene [44, 45]. Later it was suggested that p53 can induce apoptosis by two mechanisms, one that requires gene activation and the other, which is transcription-independent [46]. More than three decades of research that followed revealed molecular details of both mechanisms. The p53 was found to directly bind to the outer membrane of mitochondria which promoted its permeabilization and induction of apoptosis without the need for activation of pro-apoptotic genes (reviewed by Ho *et al.* [47]). On the other hand, dozens of p53-regulated, pro-apoptotic genes were identified. Their proteins promote apoptosis by various mechanisms. For instance, p53 activates two genes for death receptor FAS [48] and DR5 [49] (Fig. 3). This type of death is frequently used by lymphocytes with membrane-bound FAS ligand (FASLG) to induce apoptosis of cancer or infected cells (bearing FAS and DR5 receptors). The p53 activates genes for proteins of Bcl-2 family members, which shifts the balance of pro- and anti-apoptotic proteins regulating mitochondrial membrane permeabilization towards apoptosis. For instance, p53 activates the expression of at least three genes coding for proteins belonging to pro-apoptotic members of the BCL-2 family: PUMA [50], NOXA [51] and the BAX [45]. Moreover, p53 activates the gene for APAF-1 protein, which, together with cytochrome *c*, forms the apoptosome activating caspase 9 [52]. On the other hand, p53 represses the expression of the *BIRC5* gene for the pro-survival protein known as survivin [53]. The p53-activated gene *TP53INP1* codes for protein, which helps in posttranslational modifications of p53, making it more active in the upregulating expression of pro-apoptotic genes [54]. This is another example of the positive feedback loop in p53 signaling (Fig. 2). The p53 also promotes the expression of genes coding for proteins of the extrinsic pathway. In addition to the genes for death receptors FAS and DR5, p53 activates genes for caspase-10 [55] and CDIP1 [56]. The molecular details of the action of other proapoptotic p53 targets are less known. For instance, *TP53AIP1*, a p53-regulated gene, codes for a protein that promotes the release of cytochrome c from mitochondria [57]; however, the molecular mechanism of its proapoptotic activity is unknown. *TP53I3* is another p53-activated gene [58]. It also codes for proapoptotic protein; however, its mechanism of action has long been unknown. More than 10 years after its discovery, it was demonstrated that TP53I3 (alias PIG3) protein is a NADPH-dependent quinone oxidoreductase, which generates oxidative stress leading to apoptosis (Fig. 3) [59]. Interestingly, this gene is regulated by p53 from a response element, which does not conform to the known consensus but is a polymorphic microsatellite sequence $(TGYCC)_n$. The comparison of this element between various species of primates revealed that the sequence with multiple repeats appeared only in apes and humans, whereas the number of TGYCC repeats is lower in monkeys. As a result, p53 can activate the *TP53I3* promoter in humans and chimpanzees but not in marmoset monkeys. Thus, the responsiveness of *TP53I3* to p53 appeared recently in primate evolution and may regulate cancer susceptibility [60].

The decision of whether p53 promotes life (cell cycle arrest) or death is influenced in part by the expression of proteins known as ASPP (apoptosis-stimulating proteins of p53). There are three family members ASPP1, ASPP2, and iASPP. They bind to p53 and other proteins regulating apoptosis (e.g., BCL-2, RELA). Biochemical and genetic evidence shows that ASPP1 and ASPP2 activate, whereas iASPP inhibits the apoptotic function of p53 (reviewed by Sullivan and Lu [61]). Thus, the factors regulating the expression of ASPPs will decide whether the activation of p53 will lead to cell cycle arrest or apoptosis.

P53 can also regulate apoptosis in another noteworthy manner. During the apoptosis, the polarity of the plasma membrane switches, and the phospholipids (prominently phosphatidylserine), which face cytoplasm, now contact the extracellular space. This constitutes the eat-me signal for macrophages, which engulf the apoptotic bodies. One of the proteins that bind phosphatidylserine is MFGE8. It is also bound by integrin receptors on the plasma membrane of macrophages, which helps in the phagocytosis of apoptotic bodies [62]. Interestingly, as of this writing, no individual paper reported the regulation of *MFGE8* by p53. However, the meta-analysis by Fisher *et al.* [18] demonstrates that this gene is upregulated by p53 in 30 out of 57 high-throughput studies, and its promoter and enhancer are bound by p53. Thus, p53 not only induces apoptosis but also prepares the apoptotic cells to be engulfed by macrophages.

THE SECOND PLOT TWIST IN p53 RESEARCH

Having learned that p53 is a transcription regulator, which promotes cell cycle arrest (transient or permanent known as senescence), DNA repair or apoptosis, it seemed perfectly obvious that this protein exerts its anticancer activity by preventing the formation of mutant cell clones either through repair of DNA or through the elimination of cells with damaged DNA by apoptosis or senescence. However, papers published in 2011 and 2012 contradicted this belief. Jiang *et al.* [63] generated a knock-in mouse strain expressing a p53 mutant compromised for transcriptional activation. These mice carry double mutation p53(25,26) in the transcription-activating domain. This mutant cannot activate classical p53 target genes, like p21 (cell cycle arrest), Noxa, and Puma (apoptosis), but it keeps the ability to activate a small subset of p53 target genes, including Bax. Surprisingly, this mutant can nonetheless suppress tumor growth. In contrast, a quadruple mutant p53(25,26,53,54) that is completely defective for transactivation, fails to suppress tumor

formation [63]. More experiments from the same laboratory yielded similar conclusions. The loss of primary mediators of p53-induced cell cycle arrest (*CDKN1A* – p21) or apoptosis (*PUMA* alias *BBC3)* does not cause increased tumor susceptibility observed in p53-deficient mice [64]. Other researchers reached similar conclusions. They generated mutant mice with single (p53 K117R) or triple mutations (p53 K117R+K161R+K162R), which prevent p53 acetylation on the respective amino acids. The single mutant cells are competent for p53-mediated cell-cycle arrest and senescence, but they are unable to undergo apoptosis. All three of these processes are ablated in the cells with triple mutation. And now comes the surprise, in contrast to p53 null mice, which quickly succumb to spontaneous thymic lymphomas, early-onset tumor formation does not occur in either single or triple mutant mice. These results indicate that unconventional activities of p53 are critical for the suppression of early-onset spontaneous tumorigenesis [65]. Researchers corroborated these conclusions. They generated mice deficient for three genes: p21, Puma, and Noxa. Cells from these animals were deficient in the ability to undergo p53-induced apoptosis, cell cycle arrest, and senescence, yet these mice remained tumor-free for at least 500 days in contrast to p53-null mice, which succumbed to sarcoma or lymphoma by 250 days [66]. Thus, at least in the mouse models, the activation of the cell cycle arrest (transient or permanent) or apoptosis is not critical for tumor suppressive function of p53. If not cell cycle arrest or apoptosis, then what is?

THE p53 AS A COMPREHENSIVE REGULATOR OF CELL PHYSIOLOGY

In the group of genes constituting the core p53 transcriptional program, the largest group is composed of the genes with unknown functions [67]. This indicates that our understanding of the functioning of this tumor suppressor is far from complete. What we know for sure is that p53 protects against cancer, as demonstrated by observations in various p53-deficient mouse models [68]. The cancer-prone Li-Fraumeni syndrome caused by germline mutations of *TP53* indicates that p53 functions as the tumor suppressor in humans [4]. This cancer syndrome is inherited in an autosomal dominant fashion because the mutation in one allele is already enough to elevate the risk of cancer. For a long time, it was not known if humans with homozygous mutations of *TP53* are viable. Such an individual with bi-allelic missense germline mutation was reported in 1999 [69]. In early childhood, this individual presented multiple primary cancers, but the child had completed normal embryonic development. Another individual with biallelic mutation was identified, this time causing protein truncation that did not affect embryonic development but led to early onset cancer [70]. Thus, it appears that in humans, as in mice, p53 is dispensable for normal embryonic development, but it acts as a tumor suppressor.

As already demonstrated in the previous section, the mechanism of tumor suppression by p53 in mice is not well understood. We may guess that the mechanism of this suppression in humans is even more complicated because we are much longer-lived than mice so our anticancer protection must be much more efficient. This is why mice and humans significantly diverge in the set of genes activated by this tumor suppressor. The majority of p53 target genes are mouse (509) or human (329) specific, and only 86 genes have been identified as conserved between both species [71]. Many of the *in vivo* functions of p53 in humans remain elusive. We may make educated guesses about them by learning the functions of the known p53-regulated genes. Initially, we learned that p53 regulated cell cycle, DNA repair, and apoptosis because these cellular processes can be easily studied in cells cultured *in vitro*. We do not have widely used experimental models that would study other functions of p53, which require, for example, extensive communications between various cell types, which occur either in the cancer microenvironment or in healthy tissues.

p53 activates the transcription of specific genes coding for enzymes involved in various metabolic pathways. These enzymes may have cancer-suppressive properties. Cancer cells display significantly altered metabolism. Its most prominent feature is enhanced glycolysis and enhanced uptake of glucose, which results in large production of lactate, which is the end product of glycolysis (technically speaking, it is lactate fermentation). However, in cancer cells, in contrast to muscle tissue, lactate fermentation takes place even during a sufficient supply of oxygen (the Warburg effect). To uptake glucose, cells need various glucose transporters (GLUT) in the plasma membrane. There are more than ten genes coding for various GLUT transporters in different tissues. P53 represses the transcription of the genes for GLUT1 and GLUT4, which significantly cuts the supply of this sugar inside the cells (Fig. 4A) [72]. Moreover, p53 inhibits the translocation of GLUT1 to the cellular plasma membrane to suppress glucose uptake. This process is indirectly mediated by the RRAD protein, coded by the p53-activated gene [73]. Moreover, p53 suppresses glycolysis by activating the gene for Parkin protein (Fig. 4B) [74]. It is E3 ubiquitin ligase, which promotes the degradation of Hif-1α transcription factor [75], which promotes the expression of some key glycolytic enzymes. P53 also positively regulates the expression of another gene, regulating the metabolism of glucose [76]. The gene codes protein named TIGAR (TP53-induced glycolysis and apoptosis regulator), which is fructose-2,6-bisphosphatase catalyzing transformation of fructose-2,6-bisphosphate into fructose-6-phosphate. Because fructose-2,6-bisphosphate is a strong activator of a glycolytic enzyme PFK1, its removal by TIGAR inhibits glycolysis (reviewed by Tang *et al.* [77]). Fructose-2,6-bisphosphate is produced by an enzyme named PFKFB3. The gene coding for this enzyme is repressed by p53 [78]. Thus by upregulation of TIGAR and repression of PFKFB3 p53 is predicted to redirect glucose from the glycolysis to secondary reactions such as the pentose phosphate pathway (PPP). This pathway stimulates the production of the reduced form of NADP dinucleotide (NADPH) and ribulose-5-phosphate. NADPH is a key substrate of the antioxidant enzyme, which produces reduced glutathione (the antioxidant metabolite). Thus, p53, by increasing the production of TIGAR and reducing the expression of PFKFB3, inhibits glycolysis and promotes the production of reduced glutathione for antioxidant defense and nucleotides for DNA repair (Fig. 4B) [77,78]. The increased apoptosis in TIGAR-deficient cells is thought to occur through elevated oxidative damage [76]. More detailed information

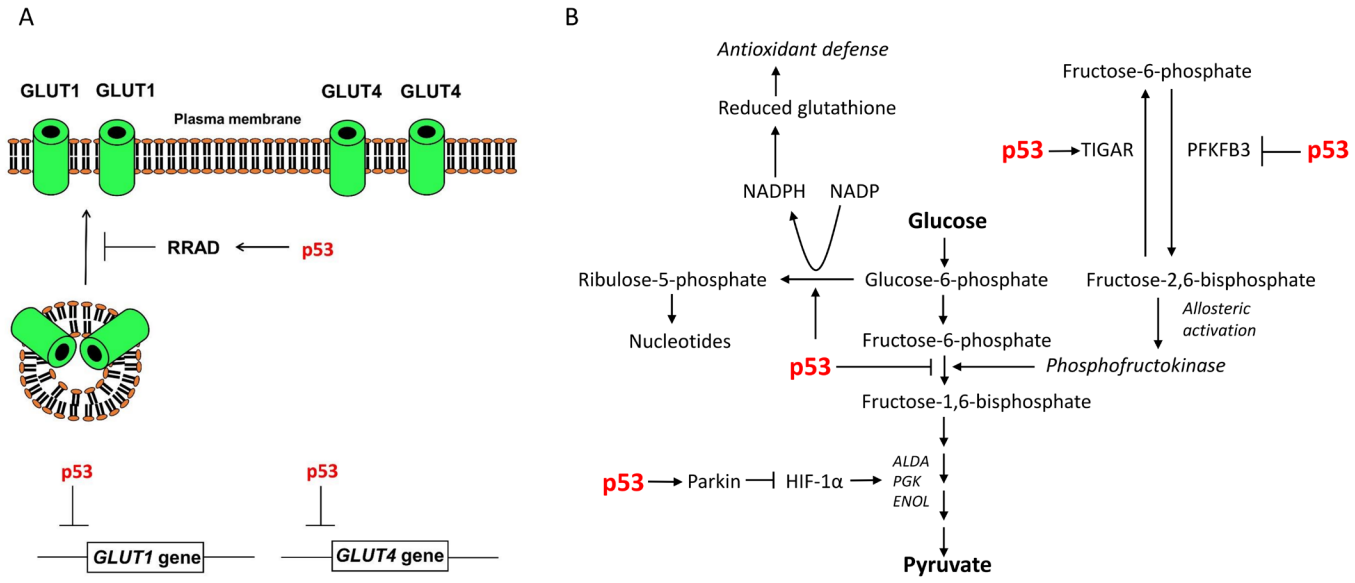

Figure 4. Regulation of glucose metabolism by p53. **A**. The p53 can reduce the intracellular supply of glucose by repressing genes coding for two of the glucose transporters (GLUT1 and GLUT4). Moreover, the protein coded by p53-activated gene *RRAD* inhibits transport of GLUT1 to the plasma membrane. **B.** The p53 can regulate the rate of glycolysis. The enzyme phosphofructokinase regulates the rate of glycolysis by catalyzing one of the irreversible reactions of the pathway. The phosphofructokinase is allosterically activated by fructose-2,6-bisphosphate, which is degraded by TIGAR (TP53-induced glycolysis and apoptosis regulator), an enzyme coded by p53-activated gene. Fructose-2,6-bisphosphate is produced by an enzyme named PFKFB3 coded by a gene represses by p53. Thus, by activating *TIGAR* and repressing *PFKFB3*, p53 inhibits the rate-limiting step of glycolysis. The inhibition of glycolytic pathway leaves more glucose to enter the pentose phosphate pathway (PPP), which generates ribulose-5-phosphate being the substrate for nucleotide synthesis. PPP also generates reduced NADP dinucleotide (NADPH), which reduces glutathione – a major antioxidant. Moreover, p53 indirectly inhibits the production of other glycolytic enzymes (ALDA, PGK, ENOL) by promoting the expression of Parkin, which promotes degradation of HIF-1α, a transcription factor, which in hypoxic conditions stimulates the expression of the glycolytic enzymes.

about the role of p53 in the regulation of metabolism is outlined in a recently published review [79].

The other ubiquitous process regulated by p53 is protein translation. The major activator of the protein translation rate is a complex of several proteins named mTORC1. This complex phosphorylates its target proteins, and this signaling cascade converges on the translation initiation complex on the 5′ end of mRNA. mTORC1 is regulated by multiple inputs like cellular energy status (AMP/ATP ratio), the presence of growth factors, amino acids, or stress. The major upstream regulators of mTORC1 are AKT kinase (positive regulator) and AMPK kinase (negative regulator). AKT senses the presence of growth factors, whereas AMPK senses the AMP/ATP ratio. The p53 codes for elements of this signaling system which leads to inhibition of TORC1. For example, p53 activates a gene (*PRKAB1*) for regulatory subunit β of AMPK [80] and a gene for LKB1 kinase, which is a positive regulator of AMPK [81]. AMPK is also positively regulated by products of two other p53-activated genes, *SESN1* and *SESN2* [82]. On the other hand, TSC2, coded by another p53 target, negatively regulates mTORC1 downstream from AMPK [80]. P53 also activates negative regulators of AKT like PTEN, but it must be pointed out that activation of this gene by p53 occurs in stress and cell specificity [80]. More details are outlined in a recent review [83]. A new report has added more data to refine this model. P53 has been shown to activate indirectly (through the RFX7 transcription factor) the metabolic regulator DDIT4. DDIT4, in turn, inhibits the activation of AKT kinase [84]. Thus, p53 negatively regulates AKT through RFX7->DDIT4 axis. The RFX7 transcription factor, upregulated by p53, is also involved in the activation of another negative regulator of AKT kinase – a protein known as PIK3IP1 [85]. Thus, p53 uses RFX7 to activate at least two negative regulators of AKT – DDIT4 and PIK3IP1.

LARGE-SCALE DETECTION OF p53-REGULATED GENES

With the advent of transcriptomic methods more than two decades ago, the p53 target genes were identified in large numbers. Usually, the experiment plan was simple, the transcriptomes were compared between p53-proficient and p53-deficient cells exposed to a p53-activating agent. This comparison yielded the list of genes, that were activated or repressed in a p53-dependent manner (directly or indirectly). The mechanism of indirect activation involves the induction by p53 of transcription factors, which directly bind to target genes and activate them. One such factor is the RFX7, which is directly activated by p53. Accumulated RFX7 protein binds to its response element located in many tumor suppressor genes, e.g., *PDCD4*, *PIK3IP1*, *MXD4*, and *PNRC1* [84]. Thus, activation of these genes by p53 is mediated by RFX7. To show that a gene is directly regulated by p53, it must be demonstrated (nowadays usually by chromatin immunoprecipitation) that p53 binds to DNA se-

quences regulating its expression (promoters or enhancers) and that cloned regulatory sequences can be activated in reporter tests (usually using the luciferase gene) by p53. If the p53 response element can be identified it can be mutated and evaluated if p53 can still stimulate its activity. The recently published web-atlas at www.TargetGeneReg.org enables to determine how frequently each gene was found to be regulated by p53 in the 57 studies, which employed the transcriptomic methods to search for p53-regulated genes [18]. Moreover, the tool enables us to examine if p53 binds to the regulatory sequences of the genes. This powerful application helps to identify the most promising candidates for p53 target genes and validate other findings. However, one must keep in mind that many high-throughput transcriptomic studies have been conducted in cell lines (U-2 OS, HCT116 and MCF7) with hyperactive *PPM1D* gene, which codes negative regulator of p53 [86]. Thus, some of the p53 target genes could have been missed in some investigations. Several years ago, researchers tried to agree on the census of p53-target genes. According to Fisher [87], the studies focusing on individual genes yielded 346 genes regulated by p53. The high-throughput studies identified 3509 potential p53 targets. However, these transcriptomic studies revealed limited consistency. Hence, the author counted as high-confidence p53 targets only the genes, which were found activated in at least 3 out of 17 high-throughput data sets. This analysis gave the number of 343 genes. The Gene Ontology (GO) term enrichment analysis performed on these genes yielded GO terms associated with the cell cycle arrest, apoptosis, and metabolism; however, other terms were also enriched, not obviously associated with p53, like cell communication, regulation of cellular protein localization, cell motility, organ regeneration [87]. In the new census, based on 57 high throughput studies, the authors found 3456 genes with high p53 expression scores, which means the genes are positively regulated by p53. This is the number of genes that are activated in a p53-dependent manner in at least a subset of cells in response to at least a subset of stress factors [18]. Thus, the set of genes that may be positively regulated by p53 in at least some conditions is distressingly high (roughly 15% of the human genome!). However, one must keep in mind that the fold-change of expression of many genes was relatively low (e.g., 2). It is hard to imagine that a gene poorly activated by p53 is effective in cell physiology. Thus, the true effectors of p53 are predominantly genes with high fold-change in expression, and their number is much lower than three and a half thousands. It is intuitively obvious that a gene activated 20-fold will have more impact on cell physiology than a gene activated 2-fold, but both count as p53-regulated genes. A remarkably interesting study concerning the census of p53-regulated genes was recently published by Tatavosian *et al.* [88]. They employed human induced pluripotent stem cells (iPSC) and two derived cell lineages differentiated either by all *trans-retinoic* acid (5d-RAI) or by a mixture of agents differentiating cells into cardiomyocytes (CM). These three cell types were exposed for 12h to Nutlin-3a or to the vehicle (DMSO). Next, the authors identified differentially expressed genes (DEGs) upon Nutlin-3a treatment in iPSCs, 5d-RAIs, and CMs. The analysis of RNA-Seq results revealed that only 49 mRNAs were commonly induced by Nutlin-3a in all three cell kinds (one of them is *CDKN1A*), with most mRNAs being induced in a cell type-specific manner. A comparison of DEGs revealed that hundreds of genes were clearly impacted by Nutlin-3 only in iPSCs. Thus, the differentiation state reduces the responsiveness of many genes to activated p53 and strongly modifies the gene expression changes caused by p53 activation. Cellular differentiation prevents the activation of various p53-regulated genes by a process involving epigenetic silencing. Thus, it can by hypothesized that the differentiation tightly packs p53 response elements of many genes into closed chromatin structure. It is also possible that differentiation reduces the expression of many p53 transcriptional co-factors required for the activation of some target genes. Some genes activated by p53 in iPSC are not stimulated in differentiated cells because they are already constitutively upregulated, and activation of p53 does not make any difference [88]. However, it is likely that this work underestimated the census of p53-target genes because Nutlin-3a, even though it is specific p53 activator, does not induce many p53 posttranslational modifications, which are required to activate some p53 target genes [89]. However, the general conclusion is apparently valid – in many cell lines, some p53 target genes are in closed chromatin, and p53 does not have access to them, and in some cell types, the genes are not activated by p53 because they are constitutively induced by other transcription factors.

The other authors used another concept to address the problem of the size of the p53 transcriptional program [67]. They used a multi-omics approach to identify genes whose expression is regulated upon *TP53* activation at various steps, from direct transcriptional targets, to genes regulated at the translational level. They exposed various cancer lines to Nutlin-3a, and they performed ChIP-Seq (to detect p53 binding sites within chromatin), GRO-Seq (global run-on sequencing to identify nascent RNA), RNA-Seq to measure global mRNA abundance and polysomal RNA sequencing to measure mRNA being translated. This allowed the authors to define four classes of genes regulated by p53: i) early, directly activated genes, ii) late with p53 binding site (genes also directly activated), iii) late without p53 binding site (genes activated indirectly), iv) genes activated at the translational level. In HCT116 cells, the number of directly activated genes (group i+ii) was 157, the number of indirectly activated was 573 and the number of translationally activated genes was 472. Similar analyses were conducted on other cancer cell lines of different origin MCF-7 (breast cancer) and SJSA (osteosarcoma). When the individual groups of activated genes were compared between the three cell lines, the highest three-way overlap was between early direct targets (24,8%), lower in late direct targets (4.1%), late indirect targets (3.6%), and the lowest in translational targets (0.2%). When the location of p53 binding sites were compared for four groups of p53-activated genes, the authors concluded that early direct genes reside in chromatin environments that are more permissive to p53 binding. Because p53 exerts tumor suppressive function regardless of cellular context, the authors determined a shared core set of p53 target genes, defined as direct targets in at least one cell line and induced by Nutlin-3a in all three cell lines. 103 genes met these criteria. The core TP53 program includes genes involved in all major known effector pathways, in-

cluding cell cycle arrest (10 genes), apoptosis (14 genes), DNA repair (5), metabolic control (9 genes), and autophagy (4 genes). Unexpectedly, the largest group was composed of genes with other or unknown functions (29 genes). Thus, almost one-third of genes from the p53 core transcriptional program code for proteins with functions, which have not been associated with the known tumor suppressive activities of p53. Because the three cell lines differ in cell fates following treatment with Nutlin-3a (HCT116 – cell cycle arrest, SJSA – apoptosis), suggesting that the genes from the core program are activated regardless of the cell fate selection. Thus, the core program alone is insufficient to specify cell fate choice following treatment with Nutlin-3a. The authors also noticed that the genes from the core program identified in this study were frequently found to be activated in other cancer cell lines, in normal cells, and in response to other stress factors. However, it must be underlined again that this group lacks genes, which belong to the transcriptional program activated by stress factors more potent than Nutlin-3a. Interestingly, this study also confirmed earlier observations that most p53 binding sites do not result in transactivation of the nearest gene. Thus, the binding of p53 near a gene is not enough to activate its expression. To activate some genes, p53 must be released by Nutlin-3a from the inhibitory effect of MDM2 and must be additionally post-translationally modified by stress-activated enzymes to interact with transcriptional co-activators [15].

ATYPICAL TARGETS OF p53

Recently, we published a preliminary study reporting our RNA-Seq screening for candidate p53 target genes in lung cancer cell line A549 exposed to actinomycin D and Nutlin-3a (A+N) [90]. These two substances activate p53 by various mechanisms – actinomycin D induces nucleolar stress and activates some kinases phosphorylating p53, while Nutlin-3a prevents MDM2-p53 binding, which, on the one hand, stabilizes p53 by preventing its polyubiquitination and degradation, but on the other hand, it may prevent the concealment of p53 transcription activating domain by MDM2. Actinomycin D and Nutlin-3a synergize in the activation of p53 and in stimulating the expression of at least some of its target genes [89,91]. In the screening study, we identified 500 genes upregulated at least 2-fold by actinomycin D and Nutlin-3a. The GO term the most significantly enriched in the group of genes upregulated by A + N was "DNA damage response, signal transduction by p53 class mediator". Our data allowed us to pick out various genes that are strongly stimulated by A+N and find out if they were considered p53 targets in other high-throughput investigations. Another GO term significantly enriched in the group of genes upregulated by A+N was "Regulation of axonogenesis." One of these genes, *SEMA3B,* according to the online database prepared by Fisher *et al*., has a high p53 expression score and its promoter and enhancer regions are bound by p53 [18]. *SEMA3B* was also identified as a p53 target in the study by Ochi *et al.* [92]. Thus, this is a gene directly regulated by p53 in various stress conditions. The protein coded by this gene is a secreted member of the semaphorin family, inducing apoptosis of tumor cells applied as a soluble ligand. SEMA3B induces apoptosis at least in part by antagonizing pro-survival, Akt kinase-dependent signaling pathway [93]. Another gene activated by A+N and involved in the regulation of axonogenesis is *NTN1,* coding for a protein named netrin-1. *NTN1* has a high p53 expression score [18]. Research indicates that netrin-1 is upregulated by chemotherapeutic agents in a p53-dependent manner [94]. This finding is surprising because netrin-1 is an antiapoptotic ligand overexpressed in several aggressive cancers where it inhibits cell death. Interference of its binding to cognate receptors by a monoclonal neutralizing anti-netrin-1 antibody actively induces apoptosis and tumor growth inhibition ([95] and refs therein). Why p53 activates the expression of such prooncogenic protein remains a puzzle. Another gene with a high p53 expression score, activated by A+N and involved in the regulation of axon guidance, is *PLXNB3*, which codes for plexin B3 protein, which is a functional receptor for semaphoring 5A [96]. This signaling axis is protumorogenic [97], which again is surprising for a gene positively regulated by p53. However, there is a peculiarity about *PLXNB3* regulation by p53. The cells with a rare Li-Fraumeni p53 missense mutation (p.G334R) affecting the tetramerization domain showed normal upregulation of "classical" p53 target genes, but defective activation of other p53 targets, including *PCLO, PLTP* and *PLXNB3.* This is like a negative photo image of the mouse models, which are not cancer-prone even if they cannot activate "classical" p53 target genes. The structural analysis of the mutant p53 tetramer demonstrated that it is thermally unstable [98]. Thus, for the upregulation of some target genes, the tetramer of p53 molecules must be in optimal shape. *PLXNB3* is one of such genes. The fact that upregulation of *PLXNB3* is defective in cells from cancer prone individuals may suggest that it belongs to antitumor, p53-regulated set of genes. P53 also participates in the regulation of DRAXIN [99]. It is also involved in the regulation of axonogenesis and is a negative regulator of the Wnt signaling pathway [100]. This negative regulator of the Wnt pathway may mediate the anticancer activity of p53. However, the role of DRAXIN outside the nervous system is not well known.

Another gene strongly upregulated by A+N is *PANK1*. It has a high p53 expression score; its promoter and enhancer are bound by p53 [18] and it was found to be regulated by p53 [101, 102]. *PANK1* catalyzes the rate-limiting step of coenzyme A biosynthesis. This indicates that by promoting the formation of this crucial coenzyme, p53 also modulates cellular metabolism. The role of PANK1 in carcinogenesis remained more elusive. Research suggests that overexpression of PANK1 inhibits the proliferation, growth, invasion and tumorigenicity of hepatocellular carcinoma cells. PANK1 cooperates with CK1α to phosphorylate N-terminal serine and threonine residues in β-catenin, a protein inhibiting signaling through the Wnt/β-catenin pathway, which is hyperactive in hepatocellular carcinomas [103]. PANK1 was found to influence the so-called metabolic reprogramming, which involves restrained glycolysis and enhanced fatty acid oxidation in people on high-fat diet (HFD). The expression of p53 in the liver was elevated in mice on HFD. PANK1 was up-regulated in the liver tissue of mice on HFD and participated in metabolic reprogramming induced by palmitate. The p53 activation enhanced the fatty acid oxidation and gluconeogenesis but suppressed glycolysis. This metabolic reprogramming induced by p53 was PANK1 de-

pendent [104]. Thus, p53 induces metabolic reprogramming at least partially through activation of PANK1.

I mentioned that p53 indirectly activates various genes by inducing the expression of RFX7 transcription factor, which activates its downstream targets [85]. When we analyzed the functions of genes upregulated by A+N, we noticed genes, which code for transcription regulators. One of them is ZNF425 [90]. It is activated by p53 in 22 out of 57 high-throughput studies, and both its promoter and enhancer are bound by this protein [18]. Thus, *ZNF425* appears likely to be a direct target of p53, mediating the regulation of its transcriptional program. Unexpectedly, the ZNF425 gene and protein are not well understood. Currently, PubMed lists only three papers dealing with this protein. This transcription regulator is transiently expressed during embryonal development; however, its overexpression has been linked to inhibiting mitogen-activated protein kinase (MAPK) signaling. Mechanistically, ZNF425 is a repressor inhibiting transcription from serum response elements (SRE) [105]. Thus, p53 appears to inhibit promitogenic MAPK signaling in part by activating the ZNF425 transcriptional repressor. Thus, p53 inhibits genes not only by indirectly activating DREAM repressive complex but also by stimulating expression of transcriptional repressors.

An interesting gene, *NUPR1* has not been identified as a p53 target in any individual study but is strongly activated by A+N. It appears as a plausible target of p53 because it was frequently observed as a gene upregulated by p53 (25/57 studies), and its promoter can be bound by p53 [18]. It is a well-studied, small, unstructured, basic protein, which is associated with the functioning of p53 because NUPR1 appears to bind with p53 and p300 during the activation of the promoter of the gene for p21 protein [106]. *NUPR1* is an unexpected target for a tumor suppressor because it shows various pro-carcinogenic functions [107]. This gene is induced by various stress factors like oxidative stress and endoplasmic reticulum stress. It may contribute to drug resistance of cancer cells by promoting various defense mechanisms. NUPR1 controls redox homeostasis and protects mitochondria [108]. If p53 activates the expression of NUPR1, it may create a positive feedback loop in activation of specific p53-target genes, e.g., the gene for p21 protein. Moreover, by activating *NUPR1*, p53 may exert a strong antioxidant defense. It may be beneficial, considering that p53 promotes oxidative phosphorylation, which is a considerable source of reactive oxygen species.

Another intriguing gene potentially activated by p53 is *SLC4A11*. It is strongly upregulated by A+N, frequently found as a p53 target in transcriptomic studies (43/57) and its promoter is bound by p53 [18]. The gene is best known for causing Congenital hereditary endothelial dystrophy (CHED), a disorder of the corneal endothelium characterized by nonprogressive bilateral corneal edema and opacification. The gene codes the membrane transport protein with unsettled function, but it is a plausible transporter for sodium, proton, borate, ammonia or even water [109]. Some experiments suggest that SLC4A11 is a proton transporter activated by high pH and NH_3. Ammonia is produced by cells primarily as a by-product of amino acid metabolism, particularly glutamine catabolism. Experiments using cell lines from Slc4a11 knockout mice indicate that this protein facilitates the use of glutamine in the citric acid cycle and reduces ammonia-related oxidative stress. This predicts the decreased oxygen consumption and ATP production in Slc4a11-deficient mice. Moreover, SLC4A11 activated by ammonia, by regulating inner mitochondrial membrane permeability, reduces superoxide production (reviewed by Bonanno *et al.* [110]). Thus, one can hypothesize that by activating *SLC4A11* p53 promotes glutamine catabolism and, at the same time, reduces the production of reactive oxygen species.

The p53 directly activates another gene coding for an enzyme involved in nitrogen metabolism – ASS1 [111]. It is strongly upregulated by A+N and was frequently found (28/57) to be p53-target in transcriptomic studies. Coding for argininosuccinate synthase 1, it participates in the synthesis of amino acid arginine from citrulline and aspartate. Argininosuccinate is directly transformed into arginine by argininosuccinate lyase. Thus, cells can make their own arginine and do not have to rely on external supply. However, in various cancer cells, this enzyme is missing due to hypermethylation of its gene promoter. This makes tumor cells dependent on the supplementation of this amino acid. What are the benefits of suppressing ASS1 in cancer cells? The leading hypothesis is that depletion of this enzyme may accelerate cellular growth by causing an increase in available aspartate, which is needed to make pyrimidines for nucleotide synthesis. Multiple studies have shown aspartate to be a key limiting metabolite for the growth of cancer cells (reviewed by Rogers and Van Tine [112], 2019). Thus, the activation of ASS1 by p53 may decrease the pool of available aspartate for nucleotide synthesis of cancer cells. However, the researchers who discovered the regulation of ASS1 by p53 suggested another (complementary?) mechanism, namely that ASS1 is a repressor of Akt kinase [111].

Another gene strongly activated by A+N [90] with a high p53 expression score [18] is *MAPKBP1*. Originally named Jun N-terminal kinase (JNK)-binding protein, it appears to facilitate the specific and efficient activation of the JNK signaling pathways [113]. It is also involved in the regulation of NF-kappaB signaling [114]. Except for the first reports on function, nothing is known about the role of this protein. JNK kinases have a plethora of functions involving the regulation of cell growth and apoptosis [115]. It is possible that MAPKBP1 protein upregulated by p53 guides JNK toward specific targets and induces an extremely specific cellular response, but it is only speculation. The role of MAPKBP1 upregulated by p53 deserves more attention.

The aforementioned genes are usually not well-publicized p53 target genes, but their functions are at least partially known. However, there are p53 target genes, even the ones belonging to the p53 core transcriptional program [67], whose functions are not known. One such gene is *KLHL30*. It is strongly upregulated (almost 200-fold) by actinomycin D and Nutlin-3a co-treatment [90]. *KLHL30* was found to be a p53 target in 24 out of 57 high-throughput studies, and its enhancer is bound by p53 [18]. However, the function of this gene is totally obscure. According to a recently pub-

lished report, *KLHL30* is an essential regulator for myoblast differentiation [116]. Considering that this gene is strongly upregulated in different cancer cell lines in response to various stress factors, one can guess that its biological function exceeds myoblast differentiation.

Very interesting gene belonging to the p53 core transcriptional program with a poorly defined function is *CYSRT1* (C9orf169) [67]. *CYSRT1* is strongly activated by actinomycin D and Nutlin-3a (10-fold) [90], has a high p53 expression score and p53 protein is bound to both its promoter and enhancer [18]. As of this writing, there are two papers in PubMed dealing with this gene/protein. According to one report, CYSRT1 protein is an interacting partner of protein members of LCE group. Late cornified envelope (LCE) proteins are small cationic epidermal proteins with antimicrobial properties. Like LCEs, CYSRT1 appears to have antibacterial activity against *Pseudomonas aeruginosa* [117]. Interestingly, we found five LCE genes (*LCE1B, LCE1C, LCE1D, LCE1E, LCE1F*) strongly upregulated (more than 1000-fold) in A549 lung cancer cell line exposed to actinomycin D and Nutlin-3a [90]. The calculated fold-change was extremely high because the expression of these genes in control cells was near zero. The *LCE1* genes are activated in a p53-dependent manner (Fig. 2) [118]. Thus, p53 can strongly upregulate the genes with antibacterial properties whereby it can protect the epithelium of the lung and other epithelia. Our unpublished data also showed that actinomycin D and Nutlin-3a strongly upregulate *LCE1* genes in a cell line derived from melanoma and in another cell line from lung cancer. This antibacterial activity of p53 is poorly known. For instance, the PubMed search for "late cornified envelope and p53" yields only two papers.

P53 may also regulate inflammatory response by modulating the metabolism of arachidonic acid (Fig. 2) – a molecule modified by various enzymes, e.g., by cyclooxygenases and lipooxygenases, giving rise to such regulators of inflammation as prostaglandins, prostacyclins, thromboxanes and leukotrienes. A gene involved in arachidonic acid metabolism, according to KEGG (Kyoto Encyclopedia of Genes and Genomes; pathway hsa00590) and activated by p53 is *GPX1. GPX1* codes for glutathione peroxidase, which is a major antioxidant protein [119]. P53 activates two genes, *ALOX5* and *ALOX12B*, which code for enzymes transforming arachidonic acid into other molecules like leukotriene (ALOX5) or the compounds responsible for the formation of skin barrier (ALOX12B) [120, 121]. Interestingly, the ability of p53 to upregulate *ALOX5* and *ALOX12B,* thereby promoting the transformation of arachidonic acid to other compounds, is utilized in selective killing of cancer cells with mutant p53 by a molecule known as niclosamide. It promotes arachidonic acid-dependent mitochondrial apoptotic cell death. By reducing the concentration of arachidonic acid by ALOX5 and ALOX12B, the cells with wild-type p53 avoid niclosamide-induced apoptosis [121]. Another gene activated by p53 and associated with the metabolism of arachidonic acid is *CYP4F3* [122]. This enzyme performs ω-hydroxylation of arachidonic acid and leukotriene, which is important in the regulation of inflammation and carcinogenesis [123].

Another poorly publicized function of p53 is the regulation of plasma membrane permeability to various ions and other molecules. For instance, *CLCA2*, activated by p53 [124], codes for calcium-activated chloride channel regulator, which can inhibit epithelial to mesenchymal transition of cancer cells [125]. In our experience, it is one of the most strongly (almost 3000-fold) activated genes in cells exposed to actinomycin D and Nutlin-3a [90]. Another gene regulated by p53 – *SCN3B* codes for sodium channel subunit beta [126]. The role of SCN3B in p53-dependent cellular physiology is obscure. According to one report, it promotes p53-dependent apoptosis [126], and according to another report, it may be an element of the negative-feedback loop in the p53 signaling pathway [127]. Even though the expression of another gene, *GABRD,* regulating the transport of molecules across plasma membrane was not reported to be p53-dependent in any individual report, the gene was found to be activated by p53 in 12 high-throughput studies, and the enhancer of the gene is bound by p53 [18]. The gene codes the delta subunit of gamma-aminobutyric acid (GABA) type A receptor. GABA is the major inhibitory neurotransmitter in the mammalian brain. By a mechanism that is unknown, increased expression of *GABRD* gene was related to the poor prognosis of patients with colon cancer [128]. The functional associations between *TP53* and *GABRD* genes are intriguing and need more attention. FXYD3 protein regulates the activity of the sodium/potassium-transporting ATPase. Like GABRD, it was not associated with p53 by any individual study but was frequently found as p53-activated gene high-throughput studies and has an enhancer bound by p53 [18]. In our experience, it is strongly (more than 200-fold) activated by actinomycin D and Nutlin-3a [90]. Studies indicate that FXYD3 may be related to cancer development [129], but again, the details of its impact on cancer biology remain obscure.

In this review I mentioned some experimental approaches employed to study p53 functions. I summarized them in Table 1 together with their advantages and drawbacks.

HIGHLIGHTS AND CONCLUDING REMARKS

The functions of genes activated by p53 and their number indicate that this protein is able to modulate every aspect of physiology at the level of cells, tissues and probably the whole system. This pleiotropy might suggest that p53 is indispensable for viability of an organism, however it is not the case because individuals with mutations of both p53 alleles (mice as well as humans) can complete normal embryonal development. However, mice and humans with homozygous p53 mutations show enormously elevated risk of cancer.

It is universally accepted that promoting cell cycle arrest and apoptosis are important functions of p53. Surprisingly, the animal experiments suggest that the p53-induced apoptosis or cell cycle arrest do not play crucial role in protection against cancer. It is not known if the same conclusion can be drawn regarding humans. However, due to obvious ethical reasons we cannot test this experimentally. Interestingly, p53 inhibits the division in virtually all cell types, while

Table 1. Selected experimental approaches employed in studies of p53 functions – their advantages and drawbacks.

Method	Application	Advantages	Drawbacks
Ectopic expression of p53 from plasmid vectors in cells cultured *in vitro*	Early method to study p53 function in cells. Employed to examine effects of p53 on cell transformation. Currently, frequently used for testing the influence of p53 on co-transfected gene regulatory elements cloned from candidate p53-target genes.	Simplicity and low cost.	Usually no regulation of p53 expression, which is generally higher than physiological. Transfection does not deliver p53 expression plasmid to most cells.
Ectopic expression of p53 using viral vectors in cells cultured *in vitro*	Expression of p53 in the majority (all) of cells growing in culture.	Expression of p53 virtually in all exposed cells. Some vectors contain regulated promoters, hence p53 expression is closer to physiological.	In the past, the preparation of these vectors was complicated. Introduction of viral sequences to cells could activate the innate immunity. Observation of p53 function in a given cell type. The role of p53 in regulation of interaction between different cell types is not studied.
The p53 knockout in cultured cell lines	The knockout of p53 expression in the majority (or all) of cells growing in culture, usually performed by a variant of CRISPR/Cas9 technology.	Easy way of generating p53-deficient cells and observation of how p53 impacts the cell autonomous effects.	Possible off-target effect of CRISPR/Cas9. Frequently it is necessary to perform time-consuming selection of p53 knockout clones.
The p53 knockout in animals	Observation of the physiological effects of p53 knockout in every cell of an animal. Variants of the method allow for gene knockout in selected tissues.	Investigation of the role of p53 in the physiological setting of the living animal.	Animal strain-specific effects can occur. The p53 functions differently in mice and in humans – not all observations in mice can be extrapolated to humans. Animals are kept in sterile conditions – the role of p53 in immunity can be underestimated.
The p53 gene knockin in animals	Replacement of wild-type p53 gene sequence with the sequence containing the desired mutation.	Observation of the molecular and physiological consequences of specific mutations in p53 gene in a living animal.	Conclusions drawn from animal models cannot be entirely extrapolated to humans.
RNA-Seq of p53- proficient and deficient cells	Identifications of genes positively or negatively regulated by p53 (directly or indirectly).	High throughput detection of p53-regulated genes in particular cell line and in selected stress conditions.	Conclusions are valid for particular cell type and stress conditions. Nutlin-3a, used as p53 activator, is not able to stimulate many p53 target genes. Some frequently used cell lines (e.g. HCT116, MCF7) express hyperactive, negative regulator of p53 – PPM1D.
ChIP-Seq (sequencing of DNA from immunoprecipitated chromatin)	Identification of DNA loci occupied by p53 in the studied cell line and stress conditions.	Helps to understand transcriptional regulation on genome-wide scale. High throughput identification of p53 binding sites. Can detect p53 binding sites within non-canonical sequences. May suggest potential p53-target genes not identified by other methods.	Results critically depend on the quality of antibody used for immunoprecipitation. Conclusions limited to particular cell type and stress conditions. P53 bound in proximity of a gene in many cases does not regulate its activity.

apoptosis is induced only in a subset of cell lineages after very strong stress. Thus, the inhibition of cell division appears as the most conspicuous role of p53. The p53 function, which is related to the cell cycle arrest is the inhibition of protein translation and the reduction of glucose utilization by glycolysis. Moreover, p53 triggers other modifications of metabolism, which promote cellular quiescence. P53 impacts on the cellular redox homeostasis by promoting expression of both pro-oxidant and anti-oxidant genes. Another important function of p53 is the regulation its own activity by positive and negative feedback loops. Poorly studied, but probably important role of p53 is the modification of extracellular matrix. Very important skill of p53 is the protection against viruses. For this reason many of them encode proteins, which inactivate p53. On the other hand some viruses exploit the activation of p53 for their own purposes. Apparently, there is fierce arms race going on between p53 and viruses. This hints at another role of p53, which is the regulation of immunity. The high throughput studies and other experiments show, that p53 activates many immuni-

ty-related genes. Their major purpose is to promote killing of infected or incipient cancer cells by the immune system. In my opinion, we may expect new and exciting findings in this area of research. The important question is - what functions of p53 play significant role in the protection against cancer? I have presented several examples of genes, which according to the transcriptomic studies are very likely targets of p53 but their functions are unknown. This suggests that we may still miss very important pieces of p53 puzzle.

More than 40 years of research on p53 gene and protein yielded an enormous amount of information. The role of p53 as a regulator of the cell cycle and activator of apoptosis is well-established. This protein also participates in the regulation of other forms of cell demise, like ferroptosis [130] and autophagy [131]. All these processes can be studied in cell culture *in vitro*. However, judging by the genes activated by p53, this protein also participates in functions, that involve extensive communications between various cell types, as in immunity [132]. These processes are more difficult to study in cell culture, and mouse studies will not always provide information relevant to humans due to significant differences in the functioning of p53 between both species. Thus, p53 still holds many mysteries, which are revealed only with the advent of more sophisticated experimental models.

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