Chapter 5

Antioxidant Role of p53 and of Its Target TP53INP1

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1. Introduction

Cancer is a complex pathology characterized by aberrant cell proliferation, resistance to induced cell death, and tumoral cell capacity to leave initial tissue and form distant tumors (metastasis). In addition, cancer cells favor angiogenesis which is necessary for tumor survival, progression and dissemination. Genetic events leading to genome instability enable those cell deregulations, in particular gain of oncogenes and loss of tumor suppressors functions observed in all cancer cells. The tumor protein p53 is encoded by the tumor suppressor gene *TP53* which is mutated in more than fifty percent of human tumors, these mutations leading to loss of its tumor suppressive function.

Interestingly, dysfunction of cancer cells is both due to events intrinsic to these cells and to their response to signals generated by normal cells from their environment. In some circumstances, normal cells can even collaborate to neoplasia. This was shown for immune cells, which is paradoxical since they are known to play a crucial anti-tumoral role. Inflammatory immune cells secrete proinflammatory cytokines and chemokines, growth factors, matrix-remodelling proteins, as well as reactive oxygen species (ROS) and reactive nitrogen species (RNS) (collectively called RNOS). Although RNOS actively participate in a diverse array of biological processes including cell proliferation, cell death, and fight against infection, excessive RNOS levels damage cell macromolecular components therefore promoting oncogenesis [1-4]. DNA lesions form either directly when RNOS modify bases or indirectly as a consequence of lipid peroxidation, the resulting products reacting with DNA. DNA lesions may be genotoxic when error-free repair mechanisms fail to remove them leading to mutations. To summarize, clinical and epidemiological investigations have provided evidence supporting the role of RNOS in the etiology of cancer due to both endogenous and exogenous factors. In addition, cancer cells are frequently under persistent oxidative stress, which participates in cancer progression as well as in the selection of resistant cells that are unable to die by apoptosis.

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In this chapter, we will describe the current knowledge on the relationship between p53 and redox, emphazing its complexity since on one hand p53 is regulated by redox and in the other hand p53 regulates cell redox status. We will then review the current knowledge on one of p53 target genes, Tumor Protein 53-Induced Nuclear Protein 1 (TP53INP1), which we have defined as a major actor in p53-driven oxidative stress response, even if the antioxidant role of TP53INP1 at the molecular level is still speculative and remains to decipher. Finally, we will describe some models of genetically engineered mutant mice and experimental inflammation settings which have provided important insights into the link between oxidative stress and cancer.

2. p53 implication in cell redox control

2.1. p53 is a key actor in prevention of cancer development

The p53 protein was discovered in 1979 by different research groups, in particular as interacting with oncogenic viral SV40 Large T antigen (for historical reviews, see [5, 6]). Its name is related to its apparent molecular weight of 53 kDa, which is grossly overestimated (p53 longest isoform is 393 aminoacids long) presumably owing to the presence of a proline-rich region that slows down the migration of the protein in SDS-polyacrylamide gels. Since its discovery, p53 has been the focus of a huge number of investigations. This protein is encoded by the *TP53* gene which is mutated or lost in a large range of human cancers [5, 7]. Loss of p53 function promotes tumor development, featuring p53 as a potent tumor suppressor. Nowadays, alterations in *TP53* are the most universal cancer-driving genetic defects. For this reason, the protein p53 is the most famous tumor suppressor in the field of oncology for basic research scientists as well as clinicians.

Interestingly, p53 was initially reported as a stress factor, highly induced upon stress events, and participating in stress resolution thus elimination of potential protumoral events towards cell homeostasis. In particular, it was shown to be induced in response to DNA damage then named “the guardian of the genome”, an expression that resumes its main physiological function. The DNA-damage response mediated by p53 is also an oncogene-induced barrier against progression of cancer beyond its early stages. p53 is necessary for silencing of mutant thus potentially cancerous cells by all means of tumor suppression, i.e. growth arrest, senescence and apoptosis.

More recent reports emphasize additional role of p53 in basal or low stress (“everyday life” stress) conditions, i.e. distinctly from conditions driving rapid and acute p53 induction in response to high levels of DNA damage. In particular, p53 is shown to be involved in embryonic development and energetic metabolism. In both settings however (acute stress or basal condition), p53 is sensitive and responsive to redox conditions. Thus, p53 is a fascinating multifaceted protein, besides a central player in the redox field.

2.2. Complexity of the p53 world

p53 is complex at many levels. (1) *TP53* gene encodes different p53 isoforms by differential splicing [8]. (2) This gene is the first reported member of a family encompassing three

members: *TP53*, *TP63*, and *TP73*. *TP63* and *TP73* encode also many different protein isoforms. These three members play both overlapping and non-overlapping functions [9-11]. (3) p53 is induced by many different stress conditions, including oxidative stress (Figures 1 and 2). (4) Induction of p53 activity results from different processes, including oxidative modifications (Figures 1 and 2). (5) p53 possesses both transcriptional and non-transcriptional activity (Figure 1). (6) Transcriptional target genes of p53 are numerous, including genes involved in cell redox status regulation (Figure 2). (7) p53 can differently influence cell behaviour upon stress, in particular either cell survival or cell death, depending on stress duration and intensity (Figure 1). And finally, in addition to its key role in stress, p53 is endowed with multiple basal activities (Figure 1). In the following sections, we will focus on the relationship between p53 and the cell redox status at different levels of this complexity, according to informations available in the literature.

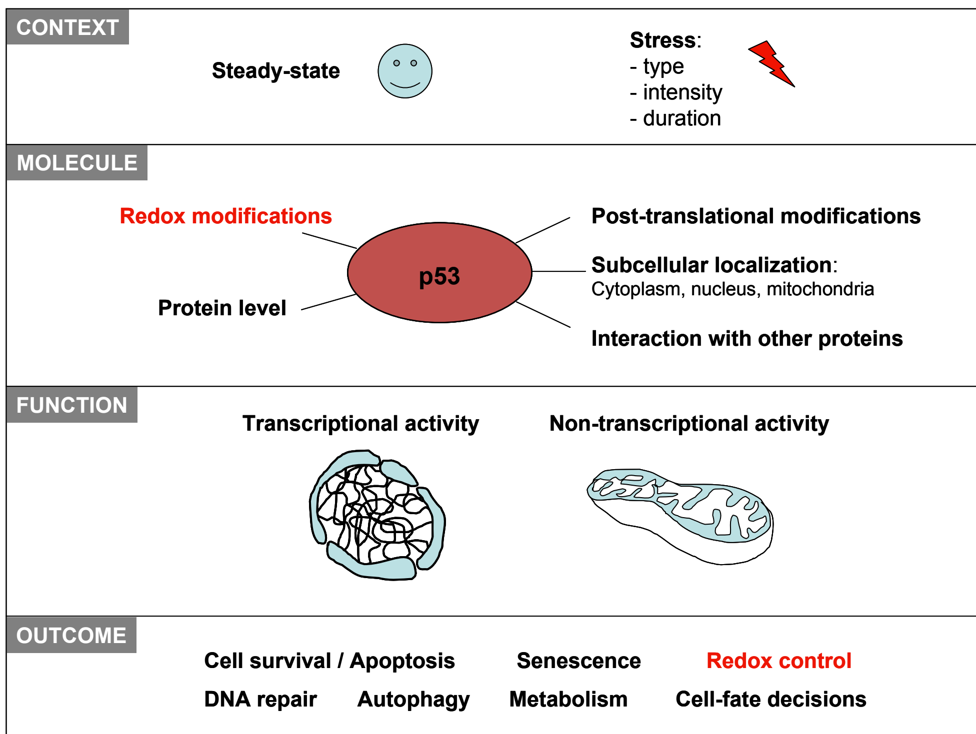

Figure 1. Complexity of p53 at different levels.

2.3. p53 is regulated by redox

p53 is induced by different kinds of stress, either genotoxic (including oxidative lesions induced by ROS and RNS) or non-genotoxic (listed in Figure 2A). This induction relies mostly on structural modifications that turn p53 from dormant to active state via modifications in protein level, subcellular localisation, and interaction with itself

(homotetramer) and other proteins (Figure 1). Dormant state of p53 is mostly due to its interaction with the E3 ubiquitine ligase MDM2 targeting p53 to permanent proteosomal degradation. Upon stress signal, p53 is post-translationally modified then stabilized by loss of interaction with MDM2 thus MDM2-driven degradation [6, 12].

p53 post-translational modifications are very diverse (listed in Figure 2B). They comprise also redox modifications on cysteine and tyrosine residues. Indeed p53 activity can be directly post-translationally modified via thiol redox modulation of critical cysteine residues in its DNA binding domain. The core domain of p53 holds a zinc atom that protects p53 from oxidation and is critical for DNA binding [13]. p53 oxidative modifications were extensively discussed in a recent review [8]. As proposed in this latter, p53 is at the core of a complex network of redox-dependent reactions. In addition, p53 activity can be indirectly modified via thiol redox modulation of kinases which post-translationally affect p53 via phosphorylation. The potential candidates are ATM, LKB1, AMPK, and JNK [14]. In summary, p53 structure can be redox-modified either directly or indirectly via redox-driven induction of kinases activity. Therefore, p53 is a ROS sensor.

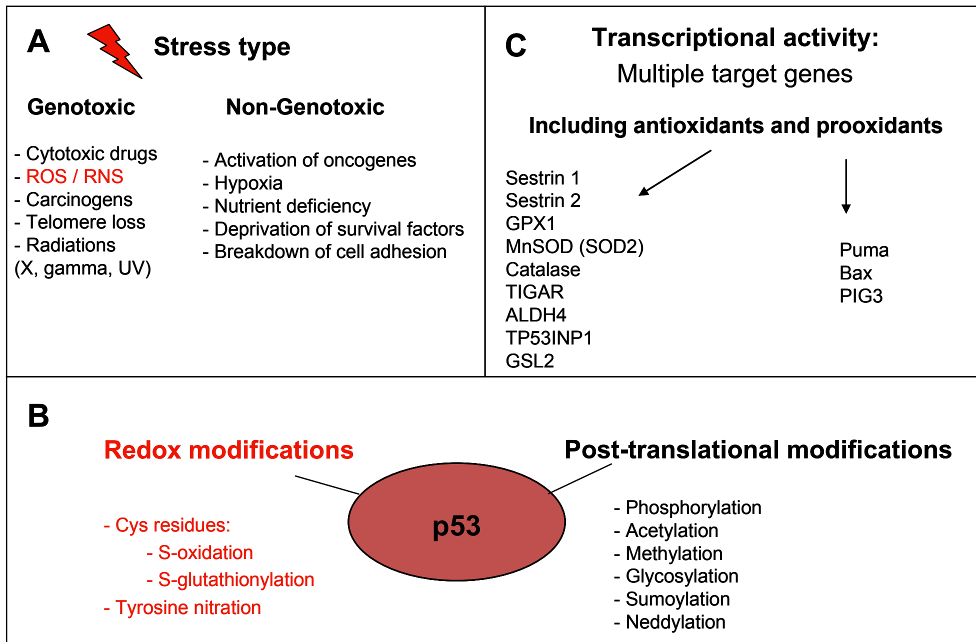

Figure 2. Complexity of p53 with regards to multiple stress inducers (A), multiple post-translational modifications (B), and multiple transcriptional targets involved in redox control (C).

2.4. p53 regulates redox state

The first described molecular activity of p53 was its action as a transcription factor. p53 possesses two amino-terminus transactivation domains and a core DNA binding domain

which can bind tightly to specific DNA sequences [8]. More than one hundred targets of p53 have been well characterized, for which transcription is more often activated. In parallel, p53 was also found to function as a transcriptional repressor. The transcriptional response to p53 induction is highly heterogeneous since it depends on the tissue/cell type and stress context [15]. The proteins encoded by p53-target genes are involved in many different cellular processes, favoring tumor suppression (cell-cycle arrest, senescence, apoptosis) or basal cell homeostasis (energy metabolism, autophagy, differentiation, …) [16].

Among p53 target genes, several play a role in redox control (Figure 2). The link between sestrins family and p53 in redox regulation has been reviewed recently [14]. One of the key functions of sestrins is the regeneration of the peroxiredoxins antioxidant enzymes [17]. Besides this indirect antioxidant action, p53 is known to directly activate the transcription of the antioxidant enzymes GPx1, MnSOD (encoded by *SOD2* gene), and catalase. As such, p53 is endowed with a potent antioxidant activity in parallel with a cell survival outcome. Nevertheless, in conditions of sustained or high intensity stress, this activity can shift to prooxidant with a proapoptotic outcome. Thus dual role of p53 depending on the context was initially demonstrated by Sablina et al. [18]. As these data provided a clue to understand the dual prosurvival versus proapoptotic activities of p53, they were subsequently discussed in several reviews [1, 19].

In our laboratory, we identified a new target of p53 involved in oxidative stress response named TP53INP1. We recently demonstrated that TP53INP1 is able to mediate the antioxidant function of p53 (see part 2).

Besides its direct impact on the regulation of gene expression in the nucleus, p53 was found to possess non-transcriptional biochemical activities. These are very diverse and can be exerted both in the cytoplasm and the nucleus [20]. In particular, p53 influences mitochondrial functions such as apoptosis and respiration which is the most prominent source of ROS. p53 was shown to indirectly promote mitochondrial functions and inhibit glycolysis [21-23]. The consequence of this promotion of oxidative phosphorylation is a decrease in oxidative stress and thus prevention of DNA damage. In addition, by inhibiting glycolysis, p53 can prevent the Warburg effect which is one of the features of cancer cells [24].

2.5. Clinical issues

The central role of p53 in human cancer makes it a target for cancer therapy development. This task is hindered by the fact that p53 is neither a cell surface protein nor an enzyme which are targetable by antibodies or inhibitors. Efforts have been undertaken in developing p53 gene therapy and restoring p53 activity [6]. Restoration of wild type p53 expression triggers elimination of tumors *in vivo*. Interestingly, some of the small molecules which are able to reactivate mutant p53 and induce apoptosis share the ability to target thiols and affect the redox state of p53 [25]. There is no doubt that the future in the p53 and cancer field is restoration of p53 tumor suppressive activity. This endeavor benefits from basic research on deciphering the diversity of p53 activities and regulation modes at the molecular level, in particular as a main ROS sensor and actor in the redox equilibrium.

3. TP53INP1 antioxidant role

3.1. Characterization of TP53INP1

TP53INP1 (also known as TEAP, SIP, and p53DINP1) is a p53 target gene that encodes the TP53INP1 protein. It was first described by Carrier et al. as an acidic protein of unknown function in the mouse thymus, suspected to be an important factor in thymocyte maturation [26]. In parallel, *TP53INP1* was identified by Tomasini et al. as a stress response gene highly induced during acute pancreatitis in the mouse [27]. Afterward, TP53INP1 was shown to be involved in a large panel of cellular processes, like apoptosis, cell cycle regulation, cellular adhesion and migration, ROS regulation, and autophagy (see below) [28-32].

TP53INP1 gene is localized in the human chromosome 8q22 [33], and is expressed ubiquitously in the whole organism, but with differences in the expression level between organs. Basal levels of TP53INP1 are high in thymus, heart and testis ; low in lung, skeletal muscle, kidney, colon, spinal cord, bone marrow, pancreas and stomach, and very low in brain [27, 34]. The sub-cellular localization of TP53INP1 is nucleo-cytoplasmic, but upon ectopic over-expression the protein accumulate in the nucleus of the cell, more precisely in sub-nuclear structures called the promyelocytic leukaemia protein nuclear bodies (PML-NBs) [35]. More recently, we showed that TP53INP1 is also localized in autophagosomes into the cytoplasm [28], but the addressing mechanism to the different cellular compartments remains to be elucidated.

TP53INP1 gene encodes two isoforms, TP53INP1α and TP53INP1β (18 and 27 kDa, respectively), resulting from the alternative splicing of the transcript [27, 36]. The two proteins are identical in sequence, except the additional C-terminal part in TP53INP1β. They don’t show any known motif, apart from a sequence rich in proline, glutamic acid, serine and threonine residues, the PEST region, which is characteristic of short half-lives proteins, and a LIR (LC3-interacting region) which allows the interaction between TP53INP1 and LC3 within the autophagosomes [28]. To date, any difference between the cellular effects of both isoforms has been identified.

3.2. *TP53INP1* is a target gene of p53

Tomasini et al. showed induction of *TP53INP1* expression in response to adriamycin or hydrogen peroxide (H_2O_2) treatment, in NHF (normal human fibroblasts) but not in cell lines where p53 is mutated or deleted: HeLa (derived from a cervix adenocarcinoma), H358 cells (derived from a lung adenocarcinoma), BxPC-3 cells (derived from a pancreatic adenocarcinoma) and SW480 and HT29 cells (both derived from a colorectal adenocarcinoma) [34]. Moreover, *TP53INP1* expression is induced by wild type p53 expression, but not by a mutated form of p53. A p53-response element site is found at position -1329 of the *TP53INP1* promoter.

TP53INP1 was in parallel identified as a p53 target gene by Okamura et al. in 2001 [36]. These authors used a differential display approach to isolate p53-inducible transcripts, in

cell line expressing wild-type or mutated p53, and they identified *TP53INP1* among other p53 targets. Furthermore, they observed an induction of *TP53INP1* following γ-irradiation in p53+/+ MEF but not in p53-/- MEF. Finally, they found a p53 binding site of 20 nucleotides in the intron 2 of *TP53INP1*, which matches the consensus p53 binding site by 85%. This p53 binding site was confirmed by electrophoretic-mobility shift assay and luciferase reporter assays.

Altogether, these data clearly indicate that *TP53INP1* is a target gene of p53.

3.3. TP53INP1 is implicated in p53-driven response to stress

In turn, TP53INP1 is able to activate the transcriptional activity of p53, therefore being implicated in a positive feedback loop with p53.

How TP53INP1 activates the p53 response to stress? The phosphorylation of p53 on its serine 46 (Ser-46) seems to play a key role in the activation of p53-driven apoptosis by TP53INP1. It was well referenced that Ser-46 phosphorylation of p53 and induction of p53AIP1 are essential features to DNA damage response [37, 38]. Okamura et al. showed that co-expression of p53 and TP53INP1 enhances p53 Ser-46 phosphorylation, induces p53AIP1 and strongly increases apoptotic cell death, as observed by flow cytometry and Terminal deoxynucleotidyltransferase-mediated dUTP Nick End-Labeling (TUNEL) [36]. Moreover, inhibition of TP53INP1 expression by antisens oligonucleotides represses p53AIP1 expression. These observations suggest that TP53INP1 activates p53 protein toward activation of apoptosis by regulating phosphorylation at Ser-46, and that modified version of p53 activates transcription of apoptosis-inducing genes such as *p53AIP1*. Using a kinase *in-vitro* assay with immunoprecipitated TP53INP1 and p53, Okamura et al. suggested that TP53INP1 interacts with a specific p53 Ser-46 kinase.

Several proteins were shown to have a kinase activity on the Ser-46 of p53 and to promotes p53-dependent apoptosis: the homeodomain-interacting protein kinase-2 (HIPK2) [39, 40], the p38 MAPK [38], and the protein kinase C delta (PKCδ) [41]. Tomasini et al. demonstrated a direct interaction between TP53INP1, p53, and HIPK2 by GST-pulldown and co-immunoprecipitation assays [35]. Moreover, TP53INP1 co-localizes with HIPK2 and p53 in PML-NB, which are described to be the site where HIPK2 binds to p53 and phosphorylates its Ser-46. Using luciferase-reporter assays, the authors showed that TP53INP1 and HIPK2 can regulate the p53 activity on genes involved in cell cycle regulation (*Mdm2* and *p21*) and apoptosis (*Pig3* and *Bax*). Flow cytometry and TUNEL experiments confirmed the cellular effect of this transcriptional regulation on cell cycle arrest in G1 phase and on apoptosis. Another study demonstrated that TP53INP1 co-immunoprecipitates also with PKCδ, which can phosphorylate p53 on Ser-46 in response to DNA damage [41]. This work also showed that PKCδ is able to modulate the expression level of TP53INP1, confirming the implication of this kinase in p53 activation through TP53INP1.

Our molecular model is summarized in Figure 3. During a cellular stress, *TP53INP1* transcription is induced by p53. In response, TP53INP1 is able to bind different kinases

(HIPK2, PKCδ) in PML-NB, forming a multiproteic complex which can recruit p53. Those kinases will phosphorylate p53 on its Ser-46, and this phosphorylation will trigger transcriptional activity of p53 on its targets: *p53AIP1*, *Mdm2*, *p21*, *Pig3*, *Bax*. This cascade will lead to G1 cell cycle arrest or apoptotic cell death in response to severe cellular stress.

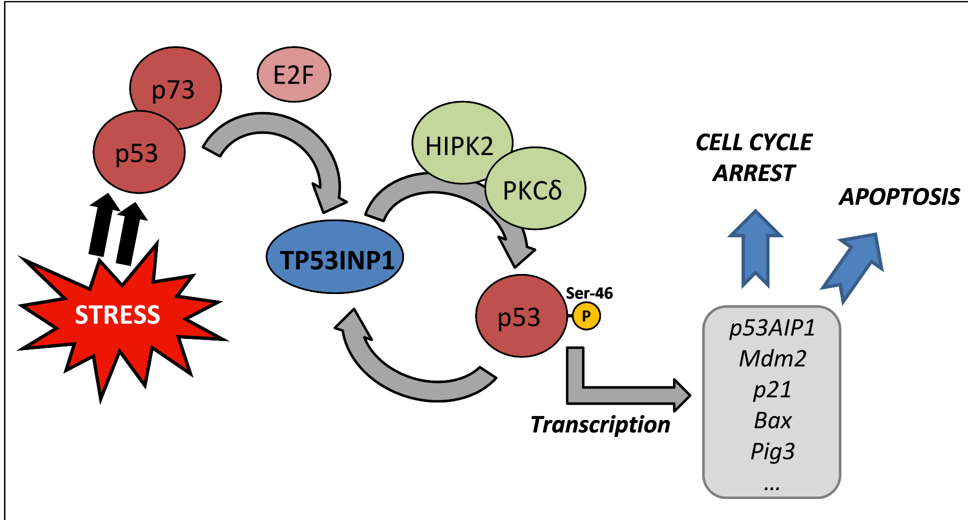

Figure 3. Molecular model of p53-TP53INP1 functional interactions (positive feedback loop).

Study of TP53INP1 induction in MCF7 cells (expressing wild type p53) treated with several stress (γ-irradiation, UV radiation, adriamycin) showed that stress-triggered DNA double-strand breaks strongly induced TP53INP1 within 4h, whereas TP53INP1 is induced more slowly and to a lesser extent by UV radiation [36]. By contrast, p53 was induced similarly by both stresses. Moreover, DNA damage-induced cell death and cell cycle arrest (upon γ-irradiation and adriamycin treatment) were strongly decreased after inhibition of TP53INP1 expression by oligonucleotide antisens, whereas antisens had no effect on UV radiation-induced cell death. Those observations led Okamura and coll. to suggest that at least two different p53-dependent mechanisms are involved in TP53INP1 induction.

As described in the first part, p53 is a tightly regulated protein maintained at low levels under normal conditions. In response to stress, p53 is activated mainly by complex post-translational modifications, changes in protein-protein interaction and sub-cellular re-localization. This activation leads to transcription of several genes which will trigger a large panel of cellular processes, like cell cycle arrest, apoptosis, autophagy, DNA repair, senescence, or redox state regulation. To control this broad variety of mechanisms, the transcriptional activity of p53 is highly dependent on the promoter context and on the type of stimulus. All the presented data suggest that TP53INP1 is one of the p53 co-factors involved in such a regulation.

TP53INP1 is also regulated by E2F, which directly binds to its promoter, like other pro-apoptotic p53 co-factors (ASPP1, ASPP2, JMY) [42]. Moreover, E2F1 induces phosphorylation of p53 on Ser-46 through TP53INP1 and this modification is important for E2F1-p53 cooperation in apoptosis.

In addition to the role of TP53INP1 in the regulation of the p53-dependent response to stress, Tomasini et al. showed a p53-independent action of TP53INP1 [43]. This independency was initially suggested by the observation that TP53INP1 is induced in p53-/- mice during acute pancreatitis, and that TP53INP1 over-expression is able to trigger G1 cell cycle arrest in p53 deleted or mutated cell lines. The mechanistic explanation was provided by the demonstration that TP53INP1 is a target of p73. p73 belongs to the p53 family. It also encodes a nuclear transcription factor which shares structural and functional homologies with p53. Many isoforms of p73 exist, which result from alternative splicing and from differences in the initiation of transcription by different promoters. Some isoforms share functional similarities with p53 [11]. p73 is also known to be able to activate p53 target genes and to induce cell cycle arrest and apoptotic cell death. Tomasini et al. showed that p73α and β isoforms induce *TP53INP1* [43]. p73 binds directly to the promoter of TP53INP1, as demonstrated by CAT-reporter assays. Similarly to its action on p53, TP53INP1 then modifies the transcriptional activity of p73 and stimulates G1 cell cycle arrest and pro-apoptotic functions. Nevertheless, the ability of TP53INP1 to stimulate the activity of p53 is slightly higher than that observed with p73.

3.4. Induction of TP53INP1 in response to genotoxic and oxidative stress

As described above, TP53INP1 is a stress response protein. The expression of this gene is induced by a large panel of cellular stresses.

- *In vivo*, TP53INP1 is induced in pancreatic acinar cells in a mouse model of acute pancreatitis (intraperitoneal injection of caerulein). TP53INP1 expression is rapidly induced within 3h after induction, with a maximum at 9h. mRNA level then decreases to reach control values 15h after induction [27]. TP53INP1 expression is also induced during chronic pancreatitis [44]. Moreover TP53INP1 expression is highly increased in the thymus of mice upon *in vivo* treatment by inducers of thymocyte oxidative stress and death, *i.e.*, whole-body γ-irradiation or dexamethasome (corticoid analog) intraperitoneal injection [32].
- *In vitro*, TP53INP1 is described to be quickly and strongly induced by different cell stress agents: adriamycin, UV irradiation, γ-irradiation, heat shock, methyl methanesulfonate, ethanol, cisplatin, and oxidants such H_2O_2 [27, 36, 43]. Different levels and kinetic of TP53INP1 expression were observed by authors in response to each of these treatment, leading them to suggest different pathways of TP53INP1 activation.

TP53INP1 is also induced by oncogenic stress (mutated Ras^{V12D} and viral E1A protein). Tomasini et al. suggested that this induction occurs through the activation of p53-dependent mechanisms in response to cell transformation [27]. Hershko et al. explain the E1A-induced expression of TP53INP1 by the disruption of RB/E2F complex by E1A, leading to

deregulation of E2F activity, resulting in activation of TP53INP1 [42]. Therefore, TP53INP1 seems to be involved in all major stress pathways, induced both by genotoxic stress and oxidative stress, suggesting that this gene plays a central role in cellular response to damage.

3.5. Chronic oxidative stress in TP53INP1-deficient mice

First evidences of exacerbated oxidative stress in absence of TP53INP1 have been demonstrated *in vivo* thanks to TP53INP1-deficient mice [31]. Indeed, once a part of mechanistic implicating TP53INP1 had been elucidated, it became important to know which phenotype would be observed in an *in vivo* murine model. Mice with inactivated *Trp53inp1* gene (Knock-out mice) were generated in our team by homologous recombination on a mixed 129/Sv x C57BL/6 background [31]. Knock-out mice were then backcrossed on the C57BL/6 parental genetic background for nine generations [32]. The main phenotypes of TP53INP1-deficient mice were shown to be independent of the genetic background (unpublished data).

As TP53INP1 is induced by stress including oxidative stress, we postulated that this protein could be involved in cell redox homeostasis. Oxidative stress arises from an imbalance between oxidants and antioxidants in favor of the former, leading to an overload of ROS and RNS as described in the Introduction. To get further insights into the physiological role of TP53INP1 during oxidative stress, we first evaluated in TP53INP1 KO and WT mice the level of small anti-oxidant molecules such as plasmatic ascorbate (vitamin C), and lipid peroxide content as reflect of the total antioxidant capacity of the body. As altered ascorbic acid status has been reported in the mucosa [45] and plasma [46] in Inflammatory Bowel Diseases patients, measurements were carried out in colon and plasma of mice. ESR (Electron Spin Resonance) spectroscopy analyses demonstrated that TP53INP1 deficiency is associated with decreased ascorbate levels and increased lipid peroxide content in plasma [31]. Data obtained on colons of mice during colitis further confirmed these results as TP53INP1 KO mice displayed more colonic ROS than their WT counterparts. Interestingly, oxidative stress in the colon and plasma was also observed at basal state i.e. in the absence of induced colitis. This was the first demonstration of a chronic oxidative stress in TP53INP1-deficient mice.

Additional proofs supporting this observation came just four years later by studies achieved in our lab by N'guessan et al. by the use of DCF-DA (2',7'-dichlorofluorescein diacetate) which is a cell permeable dye oxidized and retained within cell in DCF fluorescent probe [32]. Staining on total thymocytes of mice challenged or not with whole-body γ-irradiation (6 Grays) showed that absence of TP53INP1 increased ROS levels in the latter. This was further validated by ESR spectroscopy in thymocytes as well as in blood samples. Once it was proven that there was a deregulated redox status in TP53INP1 KO mice, it was important to assess whether this deregulation was linked with an overall deficit in antioxidant defenses, as demonstrated previously in the colon [31]. We confirmed that thymocytes, blood and different organs of TP53INP1-deficient mice (colon, intestine, spleen) were strongly depleted in ascorbate and glutathione [32]. Others organs have also been tested but displayed different ascorbate and glutathione profiles. No difference was seen in

pancreas, and interestingly, in liver and thymus, levels of vitamin C were higher at basal state in KO mice. Our data suggest a higher de novo production of ascorbate in TP53INP1-deficient liver that could be due to a higher need owing to higher ROS level in TP53INP1 -/- mice. Regarding thymus, which displays a different pattern of oxidative defenses compared to thymocytes, we suggest a higher provision of ascorbate in TP53INP1-deficient thymus, further suggesting a protection of thymus against oxidative stress. Nevertheless, in spite of this protective microenvironment, irradiation stress induces a higher production of ROS in deficient thymocytes compared to WT. Taken together, our data demonstrate a profound dysregulation of antioxidant balances in the absence of TP53INP1.

3.6. Chronic oxidative stress in TP53INP1-deficient MEFs *in vitro*

In order to study more in depth and more easily the impact of TP53INP1 in the regulation of cellular redox status, primary Mouse Embryonic Fibroblasts (MEFs) were prepared from TP53INP1 WT and KO mice. Cano et al. demonstrated that what was observed *in vivo* could be transposed *in vitro*: MEFs deficient for TP53INP1 exhibited higher DCF staining thus higher ROS level than WT cells when challenged during 1h with 50 μM H_2O_2 treatment (after 3 or 10h recovery) but also at basal state. DCF is a general oxidant indicator rather than a specific marker for H_2O_2 [47]. Further experiments demonstrated that TP53INP1 deficiency provoked more particularly H_2O_2 accumulation linked with abnormal extracellular release of H_2O_2-derived free radicals after H_2O_2 challenge [30]. These results represented the first report of TP53INP1 cell-intrinsic antioxidant function.

Same series of experiments was carried out on E1A-Ras^{V12D} transformed MEFs exposed to γ-irradiation (10 Grays) which is at the origin of a global oxidant stress [32]. The fact that ROS content was different between TP53INP1 WT and KO MEFs 24h after irradiation underscored dysfunction of ROS regulation in deficient cells. By contrast with primary MEFs, no significant difference was seen at basal state. Treatment with antioxidant NAC (N-acetylcysteine, a precursor of glutathione) significantly reduced ROS level in both genotypes. Interestingly, other anti-oxidants such as Trolox (a water-soluble vitamin E derivative) and Ebselen (organo-selenium compound possessing βantioxidant properties) were able to decrease ROS content in WT but not in TP53INP1-deficient cells. As neither Trolox nor Ebselen can correct a defect in glutathione and regarding our *in vivo* results related to glutathione deficiency, we can propose that loss of glutathione in TP53INP1-/- cells is the important factor in sensitizing these cells to oxidative stress. Whether this loss is the cause or consequence of chronic oxidative stress in TP53INP1-deficient animals and cells deserves further investigation.

3.7. Apoptosis, cell cycle arrest, proliferation: redox-linked TP53INP1 tumor suppressor role

3.7.1. Tumor suppressor role upon ectopic over-expression

The elucidation of the TP53INP1 mechanistic led us to assess the role of this protein in the cellular context. Tomasini et al. in 2001 first demonstrated that over-expression of exogenous

TP53INP1 α and β in COS7 cells induced cell death via an apoptotic pathway [27]. Further works in our lab demonstrated that TP53INP1s and HIPK2 regulate the p53 transcriptional activity on genes involved in apoptosis (*Pig3 and Bax*), consistent with 2001 Tomasini's works, but also on genes involved in cell cycle regulation (*Mdm2 and p21*). Flow cytometry analysis on HEK 293T cells transfected with TP53INP1 α or β did revealed a G1 cell cycle arrest in presence of TP53INP1. p21, as a cell cycle inhibitor, could be one of the molecules involved in the increase in G1 phase arrest. These preliminary data on cell-death resistance and replicative potential are reminiscent of hallmarks of cancer depicted by Hanahan and Weinberg [24] and pinpointed first tracks of implication of TP53INP1 in tumor suppression.

3.7.2. Tumor suppressor role assessed in TP53INP1-deficient models, in relation with redox status

Then, investigations have been performed to try to validate this hypothesis. We first showed that TP53INP1 was lost in human pancreatic and gastric cancer and that its restoration inhibited tumor development [48, 49]. In TP53INP1-deficient mice, we put in place three different models of induced tumorigenesis. i/ First model consisted in injection of transformed E1A-Ras^{V12D} MEFs in nude mice. TP53INP1-deficient MEFs revealed more aggressive than WTs [48]. ii/ In parallel, we developed a genetic model by crossing mice deficient for TP53INP1 with p53 KO mice: p53 heterozygous mice displayed an accelerated tumor development in absence of TP53INP1 and majority of tumor revealed to be lymphoma [30]. iii/ Last model consisted in induction of colorectal tumors by injection of carcinogen AOM (Azoxymethane) followed by a chronic colonic inflammation provoked by 3 ingestion cycles of DSS (Dextran Sulfate Sodium) assuring promotion of tumoral cells initiated by AOM. Our results clearly showed that TP53INP1 -/- mice were far more sensitive to development of induced colorectal tumors compared to WT [31]. All models strongly suggested an anti-tumoral role of TP53INP1.

As mentioned in the introduction, ROS have a promoting role in tumor initiation and promotion. As ROS regulation is impaired in absence of TP53INP1, this could at least partially explain its tumor suppressor role. To evaluate this supposed link, ROS implication has been considered in the two last tumorigenesis mouse models. Notably, in the absence of TP53INP1, oxidative stress-related lymphoma incidence was markedly increased in p53+/- mice (model ii), and oxidative stress-associated carcinogenesis in the colon was promoted (model iii). Altogether, these data showed that chronic oxidative stress in the absence of TP53INP1 played a crucial role in facilitating tumorigenesis.

To go more in depth in the link between TP53INP1, ROS and tumor suppression, experiments have been performed in MEFs cells and thymocytes *ex-vivo*. Cano et al. demonstrated that TP53INP1-deficient primary MEFs proliferated more rapidly than WT cells. This feature, known to promote cancer progression is to be put in correlation with G1 cell cycle arrest observed by Tomasini et al. in presence of TP53INP1. NAC treatment abolished differences observed between WT and KO cells, linking increased proliferation in absence of TP53INP1 with ROS. Against all expectations, N'Guessan et al. showed that

TP53INP1-deficient cells were more sensitive to induced death than WT. These differences could be abolished by supplementing media with NAC, showing that oxidative stress, which is a feature of TP53INP1-deficient cells, is responsible for their sensitivity to induced apoptosis. Thus, although this observation is explained by a higher level of excess of ROS in those cells, these results seemed contradictory with what have been published previously demonstrating a proapoptotic role of TP53INP1 consistent with a tumor suppressor role. To reconcile these apparently contradictory observations, we postulated that TP53INP1 is protective against cancer by a proapoptotic activity upon strong stress, but that its absence impairs stress resolution and sensitizes cells to induced cell death by a lack of a prosurvival activity. We then hypothesized that this lack relies on a deficit of autophagy in TP53INP1 -/- cells. We recently demonstrated that TP53INP1 is indeed involved in autophagy [28]. Autophagy would then represent a protective process for cell against stress.

On the whole, we clearly demonstrated at cellular level the anti-tumoral role of TP53INP1 related with its function as antioxidant regulator.

Table 1 recapitulates the state of knowledge regarding the impact of TP53INP1 on cellular processes in the settings of gain of function (ectopic over-expression) and loss of function (deficient cells and mice).

Mechanisms regulated by TP53INP1	Loss of function	Gain of function
Proliferation	↗	↘
Cell death by apoptosis	↗	↗
Autophagy	↘	↗
Intracellular ROS level	↗	↘
Level of anti-oxidant small molecules	↘	

Table 1. Impact of TP53INP1 deficiency (Loss of function) or over-expression (Gain of function) in different cell processes.

3.8. Role of TP53INP1 on redox status can be p53-dependent but also p53-independent

As mentioned above, p53 plays its tumor suppressor role mainly via transcriptional induction of target genes involved in cell cycle, apoptosis, and regulation of cell redox status. p53 antioxidant function is dependent on its transcriptional activity and proceeds by sequential induction of antioxidant targets. However, none of the known p53 targets were able to fully recapitulate the p53-mediated antioxidant response in the p53-deficient cells. As a target of p53, TP53INP1 could be a major actor in p53-driven oxidative stress response.

Interestingly, we demonstrated that TP53INP1 absence confers increased thymocyte death sensitivity both in a context of p53-dependent cell death (irradiation, and etoposide treatment), and in a p53-independent cell death context (dexamethasone) [32]. Consistent with this, quantitative RT-PCR experiments did not show any difference in the induction of expression of *Bax, Puma, Noxa* and *Bim* (pro-apoptotic target of p53) between TP53INP1 WT and KO thymocytes. For those reasons, we propose that death sensitivity in the absence of TP53INP1 does not exclusively depend on p53 transcriptional activity. In the same manner, TP53INP1 action over ROS could be, at least in part, independent of p53. To test this hypothesis, Cano et al. performed transduction experiments to reintroduce expression of TP53INP1 α or β, and/or p53 in p53 KO primary MEFs. TP53INP1 restoration induces a decrease of ROS level in p53-deficient cells (Table 1). Level of ROS was even lower than after restoration of p53 alone. TP53INP1 antioxidant effect was even unchanged after cotransduction of p53 along with TP53INP1. Altogether, these data show that ectopic expression of TP53INP1 in p53-deficient cells is sufficient to restore a normal redox status, defining TP53INP1 as a major actor in p53-driven oxidative stress response. Therefore, once TP53INP1 is induced upon oxidative stress, it seems to play its antioxidant function independently of p53.

3.9. Hypotheses on TP53INP1 antioxidant function

Figures 4 and 5 schematically recapitulate the state of knowledge regarding TP53INP1 activities. Figure 4 illustrates the dual (dependency/independency) relationship between TP53INP1 and p53. In low stress conditions, moderate amount of TP53INP1 located mostly in cytoplasm is involved in autophagy, and in consequence favors cell survival. By contrast, in high stress conditions, high levels of TP53INP1 would induce apoptosis both by promoting autophagy-dependent cell death in the cytoplasm and p53-driven cell death in nucleus.

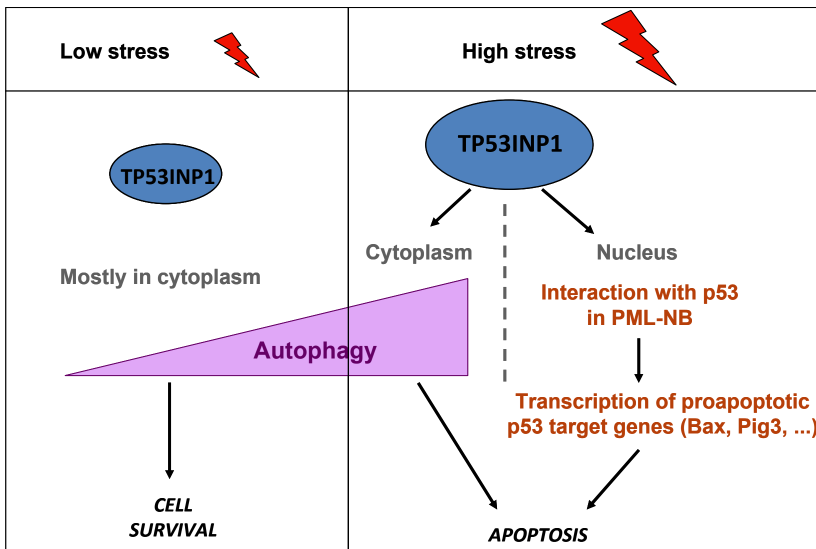

Figure 4. Model of p53-dependent and -independent TP53INP1 activities.

Figure 5 takes Figure 4 forward by adding the setting of TP53INP1 absence observed in tumors and in experimental TP53INP1-deficient mice. Absence of TP53INP1 is associated with ROS increase which promotes cancer initiation and progression (Figure 5, left). Deficient cells lack the redox control activity of TP53INP1 which is schematically shown here as a direct activity but that can be an indirect effect. Furthermore, deficient cells lack the tumor suppressive pro-apoptotic activity of TP53INP1 which is induced during high stress situation (Figure 5, right).

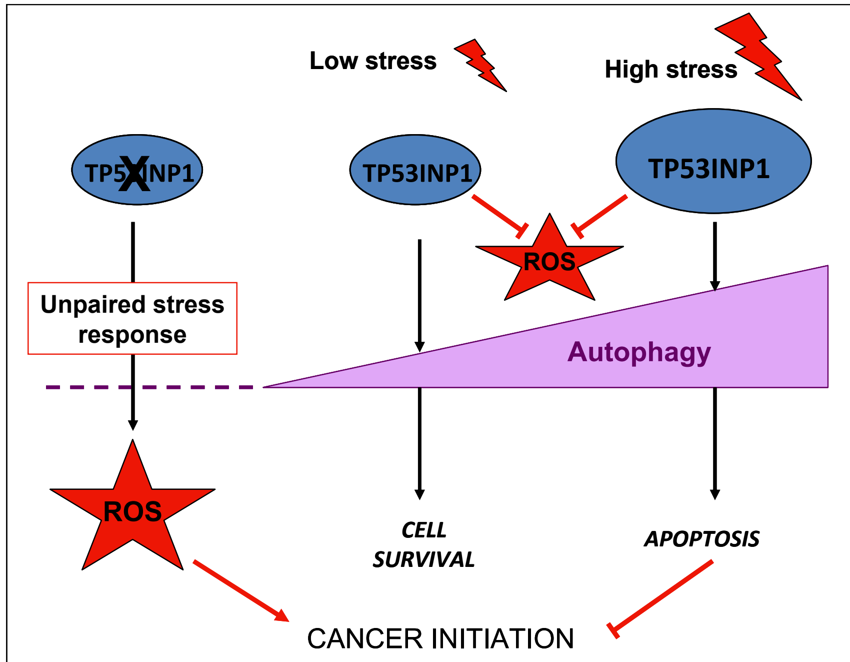

Figure 5. Model recapitulating anti-tumor activities of TP53INP1 and the consequences of its absence in tumor cells.

The question whether TP53INP1 would be a direct ROS-detoxifying enzyme, or a co-transcription factor of genes implicated in ROS elimination remains unclear for the moment. The possibility that TP53INP1 is a ROS-sensor is high since both TP53INP1 isoforms are rich in cysteine residues. Both isoforms could therefore be post-translationally redox-modified, which would result in modifications of both their physical interaction with partners and their subcellular localization.

We have other propositions, implying the p53 tumor suppressor gene homologue p73 notably. As mentioned above, Tomasini et al. demonstrated in 2005 that *TP53INP1* gene is a transcriptional target of p73, and that in turn, TP53INP1 modulates p73-induced cell cycle arrest and apoptosis by modulating p73 transcriptional activity, independently of p53. Several authors showed that p73 was induced in response to oxidative stress and was implicated in oxidative cellular response [50, 51]. TP53INP1 could regulate redox status by activating p73 and thus transcription of target genes implicated against oxidative stress.

Finally, as mentioned above in Figure 4 and 5, the implication of TP53INP1 in autophagy could indirectly be the way of its antioxidant activity. Indeed, macroautophagy is a catabolic process removing malfunctioning organelles responsible for ROS generation and oxidative stress. Thus TP53INP1 could be involved in redox level regulation via its participation in autophagy.

4. Mouse models of oxidative stress and cancer

4.1. p53 mouse models

Mouse models targeting the *Trp53* gene (encoding p53 in mice) have provided a wealth of information regarding p53 function. Mice in which *Trp53* was inactivated by homologous recombination (p53-null mice) apparently develop normally. However, some reports showed that a fraction of p53-deficient embryos display exencephaly and die *in utero* [52]. Additionally, absence of p53 in Mdm2-deficient mice rescues these latter from embryonic lethality which is probably related to the absence of p53 degradation. Altogether, these observations show that p53 plays an important role during embryonic development, which must be kept under control by Mdm2.

The *Trp53*-deficient mice are remarkable since they are prone to develop a variety of tumors during the first six months of life, independently of their genetic background. This emphases the crucial role of p53 as a tumor suppressor [53-55]. One hundred percent of null (p53 -/-) mice die during the first months of age, developing mainly T-cell type lymphomas, while heterozygous p53-deficient mice (p53 +/-) develop cancers at later age and lower incidence, with a broader panel of tumor types than p53-null mice. Interestingly, p53 deficiency is associated with an increase in intracellular ROS and with excessive oxidation of DNA and linked genomic instability, showing an antioxidant role for p53 [18]. Strikingly, long-term dietary supplementation with NAC completely prevents lymphoma development in p53-null mice, suggesting that their permanent oxidative stress is the primary cause of lymphoma carcinogenesis. Conversely, deficiency in TP53INP1, which we have defined as a major actor in p53-driven oxidative stress response (see above), decreases p53 +/- mice viability by exacerbating chronic oxidative stress in those mice and favoring lymphoma development.

Reciprocally, different models of p53 transgenic mice have been generated, most of them showing an impact on life-span, either an increase or a decrease [56]. These observations illustrate the role of p53 in regulating organismal aging, related to its impact on redox control either as an antioxidant or a pro-oxidant [52].

4.2. Antioxidant enzymes mouse models

Mouse models of oxidative stress were recently reviewed, illustrating several cases where inactivation of one antioxidant enzyme promotes cancer development [57, 58]. In addition, these reviews underscore other transcription factors than p53, such as JunD, FoxOs, Bmi1, and HIF-2α, also involved in the modulation of antioxidant enzymes expression. Deficiency

of one of these transcription factors also favors oxidative stress and redox-driven tumorigenesis. Finally, deficiency in ATM, a sensor of DNA damage and involved in the DNA damage response upstream from p53, is also an oxidative stress-associated tumor prone mouse model.

4.3. Inflammation and cancer

As mentioned in the introduction, RNOS are found at high levels in inflammatory sites, participating in elimination of the inflammation cause (infection or wound). However, RNOS can be harmful depending on duration or intensity of inflammation. Indeed, chronic inflammation was demonstrated to be a risk for cancer development. For examples, *Helicobacter pilori* chronic gastritis increases the risk of gastric cancer, Hepatitis viruses infection favors liver cancer development, pancreatitis promotes pancreatic cancer, and Inflammatory Bowel Diseases increase the risk to develop colon cancer [59-62]. Experimental models of inflammation-associated cancer are widely used both in basic and applied research. For example, the murine AOM/DSS colitis-associated colorectal carcinogenesis protocol rely on a single injection of procarcinogen AOM inducing tumor initiation, followed by repeated cycles of DSS ingestion mimicking chronic colitis thus promoting colorectal tumors [63, 64]. Hence, this mouse model represents an excellent preclinical system to both characterize the molecular events required for tumor formation at inflammation sites, and assess the ability of agents to inhibit this process.

5. Conclusion

In this chapter, we recapitulate the state of knowledge regarding p53 antioxidant role, which rely on different activities, mainly transcriptional induction of antioxidant molecules and control of energetic metabolism. Furthermore, we resume the identification of p53-target TP53INP1 as a main actor in p53-driven redox control. Antioxidant activity of TP53INP1 at the molecular level is still elusive. We propose several hypotheses which deserve being studied further. Finally, we underscore the interest of mouse mutant mice endowed with a chronic oxidative stress, such as p53 and TP53INP1 deficient mice. These mice provide plenty of basic knowledge, and can be used as preclinical models in cancer research.

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