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**THE SUBCELLULAR DISTRIBUTION OF THE p53 TUMOUR
SUPPRESSOR, AND ORGANISMAL AGEING #**

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Abstract: The p53 protein, the product of a tumour suppressor gene, is a key regulator of cell growth, differentiation and apoptosis. It is able to induce a transient cell cycle arrest and terminal senescence. Most of its functions are exerted by the transcriptional activation of genes involved in cell cycle control, DNA repair and apoptosis. The activation of p53 is primarily mediated by post-translational modifications that affect its conformation and capacity to bind to several proteins, resulting in its stabilization and enhanced DNA-binding potential. Another way to regulate the biological function of p53 involves changes in its intracellular distribution. This paper presents an overview of the role of p53 in cellular senescence and the regulation of p53 activity by its intracellular distribution.

Key Words: Cell Cycle Arrest, Apoptosis, Nucleolar p53 Sequestration, PML-bodies, Terminal Senescence

**THE ROLE OF p53 IN THE REGULATION OF CELL CYCLE
PROGRESSION AND APOPTOSIS**

Wt p53 phosphoprotein, the product of a tumor suppressor gene, responds to a variety of cellular and environmental stresses inducing either transient cell cycle arrest or apoptosis [1-3]. Thus, it prevents cancer development. However, enhanced wt p53 activity over a prolonged period is also able to induce a terminal cell cycle block in cultured cells, defined as replicative senescence, as well as organismal ageing [4-8]. In normal unstressed cells, the wt p53 protein is maintained at low levels primarily due to the action of mouse or human double

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minute-2 (*mdm-2* or *hdm-2*) protein, its downstream transcriptional target [9-11]. The low basal level of p53 is tightly regulated, and its mode of expression changes during the cell cycle. The p53 phosphoprotein reaches its highest concentration during the G₁ phase of the cell cycle [12]. Mdm-2 controls p53 function in two different ways: via the regulation of p53 transcriptional activity and the intracellular p53 level. Mdm-2, through specific interaction with the transactivation domain of the p53 protein, negatively regulates p53 transcriptional activity. Moreover, *mdm-2* possesses an intrinsic E3 ubiquitin ligase activity and targets p53 for polyubiquitylation and subsequent proteasome-dependent degradation [9, 10]. The importance of *mdm-2* in the regulation of p53 functions during development is reflected in the fact that knock-out embryos die early, before implantation in the uterus [13], whereas the concomitant disruption of the *p53* gene rescues the mice from lethality [14]. Moreover, mice that have been genetically altered to express reduced levels of *mdm-2* are small, hypersensitive to irradiation and lymphopenic [15]. A single nucleotide polymorphism in the MDM-2 promoter attenuates the p53 tumor suppressor pathway and promotes tumor formation in humans [16]. Thus, an autoregulatory feedback loop has been established between p53 and *mdm-2*, in which p53 induces the expression of its own antagonist. The *mdm-2* mediated negative regulation of p53 can be abrogated by human alternative reading frame p14 protein (p14^{ARF}) [17-19]. p14^{ARF}, the product of the *INK4a* gene generated by alternative splicing, binds to *mdm-2*, and through this interaction prevents *mdm-2* mediated degradation of p53.

Under stress conditions, distinct signaling pathways can be activated that directly target p53 for post-translational modifications rendering p53 unsusceptible to *mdm-2* action [20, 21]. The increase in the protein's stability results in nuclear accumulation of the wt p53 protein [20, 21]. Interestingly, phosphorylation also seems to regulate the activity of the *mdm-2* protein [22]. Hyperphosphorylation of serine residues within its central acidic domain is involved in the control of p53 degradation. Mutation of the serine residues abolished or partially reduced the capacity of *mdm-2* to promote p53 degradation, but did not affect ubiquitylation. Since the *mdm-2* mutants completely retained the capacity to act as an ubiquitin ligase *in vivo*, p53 ubiquitylation and degradation can be uncoupled [22].

Up-regulation and transcriptional activation of p53 leads to an elevation of cell cycle inhibitors, such as p21^{waf1}, blocking the progression of the cell cycle [23]. The induction of a cell cycle block at G₁ and G₂ by p53 provides the necessary time for the cell to repair genomic damage before entering the critical stages of DNA synthesis and mitosis. However, in tissues where the stressors generate severe and irreparable damage, p53 can initiate apoptosis, thereby, eliminating damaged cells [24, 25]. A number of pro-apoptotic factors such as bax, caspase-9, APAF-1, PUMA, NOXA or p53AIP-1 have been shown to be under the transcriptional control of the wt p53 protein [25]. Therefore, depending on the cell type and kind of stressors, wt p53 may initiate apoptosis or promote and accelerate the execution of apoptosis at different stages.

Alternatively, wt p53 may mediate a terminal cell cycle arrest called senescence [4, 6, 8]. Senescence observed in cultured cells is irreversible and is accompanied by enhanced p53 activity.

PROMYELOCYTIC LEUKEMIA NUCLEAR BODIES (PML-NBs) ARE FUNCTIONAL MULTIPROTEIN COMPLEXES

Among its numerous discrete regions, the mammalian cell nucleus contains nuclear dots [26]. They were discovered over 50 years ago [27] and called promyelocytic leukemia nuclear bodies (PML-NBs) or alternatively termed nuclear domain 10, Kremer bodies (Kr bodies) or PML oncogenic domains [28, 29]. By immunohistochemical staining, PML-NBs appear as 5 to 30 discrete dot-shaped regions within the nucleus. The number of PML-NBs in the cells, their size and the intensity of the punctate staining by the antibody depends on the cell type and cell cycle status, and markedly increase after viral infection and after the exposure of cells to heat shock, interferons or heavy metals [30]. The PML-NBs recently evoked the major interest of many scientists because they are thought to function in a variety of cell activities such as cell growth control, tumor suppression and apoptosis [31]. PML-NBs have been proposed to be involved in the regulation of nuclear metabolic processes such as DNA replication [30], DNA repair, transcription [31], RNA processing and as nuclear storage depots [32]. However, the ultimate biochemical and molecular function of PML-NBs is currently the object of intense debate. Three classes of PML-NBs can be distinguished on the basis of their dynamic properties in living cells [34]. PML-NBs frequently targeted by viral infections are disrupted in some pathologic conditions e. g. in human acute promyelocytic leukemia (APL). Originally discovered as the targets of autoantibodies present in the sera of primary biliary cirrhosis patients, PML-NBs gained intense attention when their disassembly to a microgranular form in APL was described [33, 34].

PML-NBs consist of a number of functionally distinct proteins [35, 36]. The most important component of PML-NBs is the PML protein [35], a product of the *PML* gene. Proteins present in PML-NBs include, besides PML, Sp100, p53, the Bloom's syndrome gene product (BML), small ubiquitin-related modifier-1 (SUMO-1), Death-associated protein (Daxx), CREB-binding protein (CBP) and many others. Some of the PML-NB components such as PML, pRb, hMre11, p53, HAUSP (USP7), CBP, Sp100 or promyelocytic leukemia zinc finger (PLZF) possessing transcription-modulatory activities are critical regulators of genome stability, cell cycle progression or apoptosis. The PML protein seems to be indispensable for the integrity of PML-NBs [37]. In blast cells from individuals with APL carrying a t(15, 17) translocation, PML-NBs display a more dispersed pattern. This chromosomal translocation fused the retinoic acid receptor α (RAR α) gene to the PML gene on chromosome 15, yielding the fusion protein PML-RAR α [38]. These observations suggested that the disruption of RAR α function was the major cause of APL. According to this line of reasoning, retinoic acid therapy could overcome this pathology. Indeed,

retinoic acid treatment can induce a complete remission of the disease and result in the reformation of PML-NBs [37]. It has been shown that upon retinoic acid treatment, the PML-RAR α fusion protein was proteolytically degraded, resulting in the reformation of PML-NBs and the differentiation of leukemic cells. However, more recent data showed that the picture is more complicated because APL is associated with four different gene rearrangements (for review; see [38]).

THE ROLE OF PML IN THE REGULATION OF p53 ACTIVITY

As indicated above, PML-NBs function as a scaffold for assembling several different proteins, some of which are involved in cell cycle regulation, apoptosis and senescence [39]. The NBs also facilitate various post-translational modifications among these proteins. Due to p53 being a crucial molecule in several of the most important pathways within the cell, the interactions with and the modifications of p53 have been investigated relatively well, especially within the last decade. Some of the post-translational modifications to which p53 is subjected take place within the PML-NBs [39]. These modifications stabilize or destabilize p53, some enhancing and others decreasing the transactivational capabilities of the tumor suppressor protein. Obviously, the exact context and the balance of the incoming signals decide in which direction the action of p53 is driven.

Most importantly, the tumor suppressor protein PML itself acts as a transcriptional coactivator of p53 by creating the optimal environment for the assembly of p53 and CBP/p300 in the PML-NBs. This interaction promotes the acetylation and stabilization of p53 [40, 41]. Upon γ -irradiation, p53 is acetylated by CBP/p300 and in PML^{-/-} cells, acetylation of p53 after DNA damage is significantly impaired [40]. Deacetylation and destabilization of p53 is performed by histone deacetylase 1 (HDAC1) [42]. A protein shown to activate the functions of p53 by way of phosphorylation is HIPK-2 (homeodomain-interacting protein kinase-2) [43, 44]. Despite the finding that HIPK-2, together with the highly homologous kinase HIPK-3, is able to form subnuclear structures called HIPK domains, it can also localize to the PML-NBs; its phosphorylation of p53 at serine-46 and the subsequent enhancement of p53-dependent transcriptional activity relies on this spatial distribution [45].

A pathway leading to the degradation of p53 via the proteasome is a modification by which ubiquitin moieties are covalently attached to the protein. In normal cells, mdm-2 is the primary E3 ubiquitin ligase for p53 [9, 10]. The ubiquitin residues are bound to the target protein through a isopeptide linkage, and thus far 6 lysine residues in the C-terminus of p53 (K370, K372, K373, K381, K382, K386) have been described to be modified via this bondage [46, 47]. As a consequence of DNA damage, p53 is phosphorylated at several sites [48], thereby preventing its binding to mdm-2 and its ubiquitylation by the protein. Additionally, mdm-2 can be phosphorylated after cellular stress via oncogenic stimulation. This phosphorylation is performed by p14^{arf} (ARF), and it makes mdm-2 incapable of modifying other proteins [49].

Recently, a 135 kDa protein designated HAUSP (herpes-virus-associated ubiquitin specific protease) was found; it is able to directly bind to p53 and to remove the ubiquityl moieties from the protein *in vivo* and *in vitro* [50]. This was also the first report of a mammalian ubiquitin-specific processing protease that directly binds and de-ubiquitylates a cellular target [50, 51]. This reaction takes place in the NBs and prevents the degradation of the tumor suppressor protein. Additionally, it was shown that overexpression of HAUSP induces an elevated p53 level, and that the rate of p53-dependent transcriptional activity was also increased as indicated by the higher level of p21, a protein the expression of which is known to be induced by p53. It was also shown that p53 stabilization through HAUSP, by counteracting the effects of mdm-2, leads to the inhibition of cell growth or the induction of apoptosis [50], depending on the status of the cell. The authors also showed that the stabilizing activity of HAUSP on p53 depends on its de-ubiquitylating activity, since a point mutant of the protein with impaired enzymatic activity could still bind p53 but had lost its specific p53-stabilizing properties. These new findings are especially interesting in light of the fact that the stabilization of p53 through the ARF pathway was found to be dispensable for some types of oncogenic activation [52].

Another type of post-translational modification of proteins in the NBs is one very similar to ubiquitylation, namely, SUMOylation. This type of covalent modification is accomplished via the attachment of the small protein SUMO-1 (small ubiquitin-related modifier-1), alternatively designated PIC1, onto the target protein. Most importantly, PML itself is SUMOylated and this modification is an obligatory prerequisite for the proper formation of PML-NBs [53]. PML is SUMOylated on its lysine residues 65, 160 and 490 [53, 54]. The authors also suggested that PML has to be SUMOylated in order to localize to the NBs, and then it is capable of recruiting additional NB components. Interestingly, NBs are re-formed when PML^{-/-} cells are transfected with a wt PML form but not when the PML lacks the lysine residues usually modified by SUMO [54]. SUMO-1 protease-1 (SuPr-1) is the specific protease capable of cleaving off SUMO-1 moieties from PML; this removal leads to destabilization of PML-NBs [55]. Also, p53 can be SUMOylated and mdm-2 is capable of accomplishing this modification. Most importantly, only one lysine residue in p53 can be SUMOylated, namely K386, the most C-terminal lysine, which can also be ubiquitylated [56]. SUMOylated p53 is stabilized and seems to have a stronger transcriptional activity [56]. The cleavage of SUMO moieties from p53 can be performed by SSP3, a SUMO-specific cysteine isopeptidase.

Recently, a new type of post-translational protein modification with a strong resemblance to both ubiquitylation and SUMOylation was found [57-59]. By NEDDylation, the small protein Nedd8/Rub1 is covalently bound to a lysine residue of the acceptor protein. Among the first mammalian proteins shown to be modified by Nedd8 were p53 and mdm-2 [60]. In mammalian cells, an impact of modification by Nedd8 could first be shown for protein degradation via the cullin ubiquitin ligases [61]. Nedd8 does not generate polymeric chains like

ubiquitin but it is a necessary prerequisite for protein degradation mediated by SCF ubiquitin ligases [62]. Interestingly, mdm-2 was shown to be the protein responsible for the attachment of Nedd8 to p53 [60], and additionally, for its E3 ligase function towards p53 regarding ubiquitylation and SUMOylation. The action of mdm-2 can be reversed by Nedd8-specific protease (NEDP1). At steady state, only a small amount of p53 is NEDDylated, and it has been shown that the depletion of mdm-2 by RNAi leads to a further decrease in the level of NEDDylated p53. Of the 6 lysine residues of p53 which can be ubiquitylated, only 3 can be NEDDylated (K370, K372, K373), and a specific p53 molecule can be ubiquitylated and NEDDylated at the same time [60]. Unlike in the case of ubiquitylation, an inhibitory effect of the phosphorylation of p53 on NEDDylation has not been found. The biological consequence of the NEDDylation of p53 seems to be a reduction of its transcriptional activity, also illustrated by the fact that a NEDDylation-deficient p53 mutant possesses an increased level of transcriptional activity [60]. It remains to be elucidated whether NEDDylation is used to lower the global transcriptional activity of the tumor suppressor or whether it is a tool to downregulate the activation of a specific subset of p53 responsive genes. Interestingly, in contrast to ubiquitylation, the NEDDylation of p53 has no negative effect on the stability of the protein, but only on its transcriptional activity.

The transcriptional regulator and Fas-binding protein death-associated protein (DAXX) which could also be located at the PML-NBs is another protein that can bind p53 [63-65]. DAXX negatively regulates the transcriptional and apoptotic activities of p53 [66], and it has been shown that DAXX knock-out mice die early in embryogenesis due to excessive apoptosis [67], a feature common to mdm-2 knock-out mice. Most importantly, DAXX can only bind p53 when the C-terminal lysine residues are not modified via acetylation [66] and, possibly, also other post-translational modifications that change the positively charged environment at the C-terminus of the tumor suppressor. Therefore, in cells lacking in mdm-2, where p53 is readily acetylated, DAXX is not able to exert its anti-apoptotic properties on p53 [66].

A protein that targets p53 and PML and turned out to be of paramount significance for the negative regulation of senescence is the oncoprotein E7 from the human high-risk papillomaviruses 16 or 18 [68]. This specific interaction was investigated only very recently and the effect of the E7 protein might have been hidden by other neoplastic activities of the oncoprotein that were known about for a long time. Interestingly, the viral E7 protein was thus far linked to the degradation of the Rb family of proteins [69, 70], whereas the E6 protein was known to promote the degradation of p53 [71]. PML, p53 and CBP can form a senescence-promoting complex that can be targeted by the E7 protein, thereby inhibiting the transcriptional activity of p53 [68]. The viral E7 protein is also able to prevent the acetylation of p53, and this leads to the destabilization and decreased activity of the protein [68].

As shown by this overview of various interactions of p53 and other proteins that constitute the PML-NBs, the regulation of cell cycle progression, apoptosis and

senescence executed by the tumor suppressor proteins p53 and PML is very complex and sometimes confusing. However, the significance of those interactions in tumor development is of utmost importance, and it is therefore a paramount goal to elucidate all the facets of these biological pathways.

THE ACCUMULATION OF THE p53 PROTEIN IN THE NUCLEOLUS

The nucleolus plays a dual role in the regulation of the biological function of p53. The nucleolar sequestration of p53 can contribute to its (re)-activation by preventing accelerated ubiquitylation and the subsequent degradation of the p53 protein. Remarkably, the inhibition of proteasome activity in normal human fibroblasts results in an increase in the level of intracellular p53 protein that accumulates in nucleoli in co-localization with nucleolin [72]. In nucleoli, wt p53 undergoes complex formation with a few proteins such as nucleolin, nucleophosmin, topoisomerase I and poly(ADP-ribose) polymerase-1 (PARP-1) [73]. Nucleophosmin regulates the stability and transcriptional activity of p53 [74, 75]. Under some stress conditions such as ionizing radiation or heat shock, translocation of nucleolin from the nucleolus to the nucleoplasm was observed. The relocation was promoted by the formation of p53-nucleolin complexes [74]. Nucleophosmin binds to wt p53 protein and results in an increase in the stability and transcriptional activation of the p53 protein in response to different types of stress stimuli [73]. The nucleolar sequestration of p53 seems to be one of the mechanisms regulating the reactivation of the p53 protein in cisplatin-treated human cervix carcinoma HeLa cells [76].

On the other hand, the accumulation of mdm-2 in nucleoli may indirectly affect the stability of p53 by spatial separation of the negative p53 regulator from its target. It has been observed that p14^{ARF} sequesters mdm-2 in the nucleoli, thereby physically separating mdm-2 and p53 in different subcellular compartments [77]. However, it has also been reported that the relocation of mdm-2 to the nucleolus is not necessary for p14^{ARF}-mediated stabilization of p53 [78]. Thus, the nucleolus is involved directly in the mediation of p53 function or indirectly by the control of the localization of p53 interplayers.

THE SIGNIFICANCE OF p53 IN ORGANISMAL AGEING

The tumor suppressor protein p53 has been known for many years to be one of the central molecules in DNA damage response, apoptosis and cell cycle regulation. p53 and the other members of the p53 family have also been shown to play an important role in development. Recently, it was possible to establish that p53 has an important function for p53 in organismal ageing [79]. Studies *in vivo* with the goal of clarifying the impact of p53 on cellular and organismal ageing were conducted via the inactivation of the *p53* gene, but proved cumbersome due to the fact that p53 knock-out mice show severe genomic instability and are highly susceptible to cancer development. Very frequently, they die at a young age from neoplastic lesions and are therefore not suitable

for ageing experiments [80]. By contrast, transgenic mice expressing elevated levels of p53 die at an early embryonic stage due to an enhanced rate of apoptosis resulting in severe developmental defects.

Fortunately, elucidation concerning the significance of the tumor suppressor protein on organismal ageing came from 2 variants of p53 mutant mice. One mutant mouse line expressed a temperature-sensitive (ts) p53 variant with an alanine to valine substitution at position 135 of the protein. Depending on the ambient temperature, the p53^{I35Val} mutant protein adopts the wild type conformation (32°C) or a mutant conformation (37°C). Importantly, the mice only showed the hallmarks of premature ageing in tissues that were localized to regions of the animal (e.g. skin) exposed to a lower ambient temperature [81]. These surprising effects can be explained by the properties of this specific p53 variant that adopts the wt conformation in the cooler parts of the animal body close to the surface, while adopting the non-functional mutant conformation in the core regions of the animal with a higher temperature.

The other mouse strain possessed a shortened *p53* gene, termed p53 m, which only expressed the 5 exons coding for the N-terminal part of the protein and was missing the 6 exons encoding the C-terminal part of the protein [81, 82]. The expressed tumor suppressor protein was truncated and showed a significantly prolonged half-life. p53 m is capable of increasing the transcriptional activation and growth suppression activity of its wt counterpart. In these mice, a strong decrease in the number of neoplastic transformations was visible. However, this beneficial effect was counteracted by the induction of accelerated ageing. The mutant mice had early ageing-associated phenotypes and their life span was reduced to approximately 80% of their wt littermates. Interestingly, the onset of premature ageing became evident solely in animals older than 1 year. Recently, a short p53 variant was detected in various organs and tissues; it displays a strong resemblance to p53 m and also leads to elevated p53 stability and activity [83].

Why does an increased level of p53 activity lead to premature ageing and a reduced life span in mammalian organisms? The data from the literature clearly implies that enhanced p53 activity leads to an elevated induction of apoptosis and cell cycle arrest. This in turn seems to exhaust the organism's capability to renew certain tissues, most of all organs and tissues with a high turnover rate that rely on the capacity of stem cells to replace lost cells. It seems that the incapability of an organism to pursue this cellular homeostasis leads to the described negative effects commonly summarized under the term ageing.

Recently, new data was published illustrating that excess levels of wild-type p53 can, under specific circumstances, protect mice against cancer and ageing. This line of evidence gives rise to a model where increased levels of p53 may protect organisms against neoplastic transformations without the unwanted collateral effects of premature ageing [84]. A Spanish group recently generated novel transgenic mice, called "super p53" [84]. Like normal mice, the super p53 mice produced wild-type p53 from their two naturally occurring alleles, and additionally possessed one or two copies of a wt *p53* gene, inserted as transgene

in form of a large genomic fragment. Importantly, the additional p53 gene copies were expressed from their natural promoter. This guarantees that the transgenically expressed p53 was regulated in the same pattern as the endogenous p53 gene. The super p53 mice were more sensitive to DNA damage because higher levels of p53 led to an enhanced rate of apoptosis and they were also more efficiently protected from chemically induced cancers. However, in contrast to the assumptions originating from the traits of the mutant p53 m animals, these super p53 mice did not display any signs of accelerated ageing. One explanation for the absence of the negative side effects of premature ageing in “super” p53 mice may be the fact that the transgenically expressed p53 was regulated from its natural promoter; therefore, this p53 was appropriately expressed. This data provides a ray of hope that the protection of cells against cancer might be possible by introducing the properly regulated tumor suppressor gene into stem cells.

In conclusion, these findings clearly demonstrate that the p53 protein plays a central role in organismal ageing; additionally, it has key importance in DNA damage response and cell cycle regulation. Nevertheless, very thorough investigations will be needed to eventually elucidate the plethora of pathways involved in ageing.

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