Received: 19 December 2020 | Accepted: 31 December 2020

DOI: 10.1002/iub.2453

CRITICAL REVIEW

IUBMB LIFE WILEY

Mitochondrial–nuclear p53 trafficking controls neuronal susceptibility in stroke

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Funding information
Consejería de Educación, Junta de Castilla y León, Grant/Award Numbers: CLU-2017-03, CSI151P20; EU Horizon 2020 Research and Innovation Programme, Grant/Award Number: GA 686009; Ministerio de Ciencia e Innovación, Grant/Award Number: SAF2017-90794-REDT to AA; Junta de Castilla y León; Horizon 2020; European Union; European Regional Development Fund; Instituto de Salud Carlos III, Grant/Award Numbers: PI18/00265, RD16/0019/0018

Abstract

Stroke is a major cause of death and long-term disability in the adult. Neuronal apoptosis plays an essential role in the pathophysiology of ischemic brain damage and impaired functional recovery after stroke. The tumor suppressor protein p53 regulates key cellular processes, including cell cycle arrest, DNA repair, senescence, and apoptosis. Under cellular stress conditions, p53 undergoes post-translational modifications, which control protein localization, stability, and proapoptotic activity. After stroke, p53 rapidly accumulates in the ischemic brain, where it activates neuronal apoptosis through both transcriptional-dependent and -independent programs. Over the last years, subcellular localization of p53 has emerged as an important regulator of ischemia-induced neuronal apoptosis. Upon an ischemic insult, p53 rapidly translocates to the mitochondria and interacts with B-cell lymphoma-2 family proteins, which activate the mitochondrial apoptotic program, with higher efficacy than through its activity as a transcription factor. Moreover, the identification of a human single nucleotide polymorphism at codon 72 of the *Tp53* gene that controls p53 mitochondrial localization and cell susceptibility to apoptosis supports the important role of the p53 mitochondrial program in neuronal survival and functional recovery after stroke. In this article, we review the relevance of mitochondrial and nuclear localization of p53 on neuronal susceptibility to cerebral ischemia and its impact on functional outcome of stroke patients.

Abbreviations: ATM, ataxia-telangiectasia mutated; BAK, B-cell lymphoma-2 antagonist/killer; BAX, B-cell lymphoma-2-associated X-protein; BCL-2, B-cell lymphoma-2; BCL-xL, B-cell lymphoma extralarge; Cdk5, cyclin dependent kinase 5; CRM1, chromosomal region maintenance 1; CTD, C-terminal domain; DAPK1, death-associated protein kinase 1; DBD, DNA-binding domain; DR5/KILLER, death receptor 5; DRAM, DNA damage-regulated autophagy modulator; FAS, FS-7-associated cell surface antigen; FASL, FS-7-associated cell surface antigen ligand; HIF-1α, hypoxia-inducible factor 1-alpha; MCL-1, myeloid cell leukemia 1; MDM2, murine double minute 2; MOM, mitochondrial outer membrane; NES, nuclear export signal; NFkB, nuclear factor kappa-light-chain-enhancer of activated B cells; NOXA, NADPH oxidase activator; OD, oligomerization domain; p53AIP1, p53-regulated apoptosis-inducing protein-1; Peg3/Pw1, paternally expressed gene 3 protein; PIG, p53 inducible gene; PPR, proline-rich region; PUMA, p53-upregulated modulator of apoptosis; SIRT1, silent information regulator 1; SNP, single nucleotide polymorphism; TAD, terminal transactivation domain; TIGAR, p53-induced glycolysis and apoptosis regulator; WRAP53, WD40 encoding RNA antisense to p53.

Irene Sánchez-Morán and Cristina Rodríguez contributed equally to this study.

KEYWORDS

apoptosis, functional recovery, mitochondria, neurons, p53, stroke

1 | INTRODUCTION

Stroke is the result of an acute focal injury of the central nervous system by a vascular pathogenic event and is a major cause of mortality and long-term disability in adults in developed countries. Worldwide, one in four people will have a stroke in their lifetime, and the incidence is expected to increase with the ageing population.[1,2] Most stroke presentations result from the formation of a clot in a cerebral artery (ischemic stroke), leading to severely reduced cerebral blood flow to brain tissue supplied by the affected vessel. In this brain area, named the ischemic core, damage is irreversible, and most neurons die rapidly by necrosis. Surrounding this core is the transition or penumbra zone, where the damage is not irreversible, and neurons mainly undergo delayed apoptosis.[1] Remarkably, in the penumbra, the injury propagates slower, and neurons can be rescued, providing a therapeutical window to prevent brain damage progression.[1] Similarly, stroke resulting from the rupture of cerebral arteries (hemorrhagic stroke) induces neuronal necrosis in the hematoma area and delayed apoptosis in the perihematoma area.[3] Neuronal apoptosis may account for brain damage and the impaired functional recovery of stroke patients.[4,5] Then, it is essential to understand the pathophysiological pathways involved in stroke-induced neuronal apoptosis to identify molecular targets to develop new neuroprotective therapies for stroke.

The tumor suppressor protein p53 is a tetrameric transcription factor that regulates the expression of many target genes involved in cell homeostasis maintenance, including cell cycle regulation, DNA repair, cell survival, senescence, and apoptosis. Under normal unstress conditions, p53 is very unstable and is maintained at very low levels by proteasomal degradation.[6] The main cellular negative regulator of p53 levels is the E3 ubiquitin ligase murine double minute 2 (MDM2),[7] which controls p53 activity through two different mechanisms. On the one hand, the N-terminal domain of MDM2 interacts with the transactivation domain of p53 and inhibits its transcriptional activity.[7] On the other hand, the E3 activity of MDM2 is dependent on its C-terminal RING finger domain that polyubiquitinates p53 for proteasomal degradation. Lysine residues at the C-terminal domain of p53 are the main sites of MDM2-induced ubiquitination.[8] However, low levels of MDM2 monoubiquitinates p53, driving its chromosomal region maintenance 1 (CRM1)-dependent nuclear export.[9] Finally, MDM2 and p53 operate in a negative feedback loop—p53 transcriptionally upregulates MDM2 expression, which in turn increases p53 proteasome degradation.[10] The related protein MDM4 also inhibits p53 transactivation, although it does not promote p53 degradation.[11]

In response to cellular stress conditions, including DNA damage and ischemia/hypoxia, p53 undergoes posttranslational modifications, which controls protein localization, stability, and activity.[12] These modifications include phosphorylation, ubiquitination, acetylation, methylation, sumoylation, neddylation, *N*-acetylglucosamine acylation, adenosine diphosphate (ADP)-ribosylation, hydroxylation, and β-hydroxybutyrylation.[12] Phosphorylation of p53 is considered a key regulatory event of its proapoptotic function. Upon cellular stress, several kinases phosphorylate p53 at the N-terminal serine and threonine residues that inhibit p53–MDM2 interaction and promote p53 stabilization, which translocate to both the nucleus and the mitochondria.[13] In the nucleus, p53 binds to response elements within the promoter and induces the expression of proapoptotic members of the B-cell lymphoma-2 (BCL-2) protein family, including p53 upregulated modulator of apoptosis (PUMA),[14] nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activator (NOXA),[15] and BCL-2-associated X-protein (BAX).[16] In the mitochondria, p53 activates the proapoptotic proteins BAX and BCL-2 antagonist/killer (BAK)[17] but inactivates the antiapoptotic proteins BCL extralarge (BCL-xL)[4] and BCL-2,[18] which in turn inactivates BAX. Then, mitochondrial localization of p53 represents an important regulatory mechanism of p53 apoptotic function. Interestingly, p53 phosphorylation increases acetylation of C-terminal lysine residues, which inhibits its ubiquitination by MDM2,[19] and then increases its DNA-binding activity that promotes transcriptional activation of PUMA and BAX. Accordingly, deacetylation of p53 mediated by the histone deacetylase-1-containing complex suppresses p53-dependent transcriptional activation and apoptosis.[12]

Although mechanisms responsible for neuronal death after stroke are highly complex and implicate different molecular cascades, several studies evidenced a key role of p53-induced signaling pathways in neurons. The protein rapidly accumulates in the ischemic penumbra and activates the apoptotic pathway of neuronal death through both transcriptional-dependent and -independent mechanisms.[20] Moreover, the subcellular localization of p53 has an important impact on neuronal apoptosis. Here, we review the relevance of mitochondrial and nuclear localization of p53 on neuronal susceptibility to cerebral ischemia and its relevance to the functional recovery of stroke patients.

2 | FUNCTIONAL NUCLEAR AND MITOCHONDRIAL COMPARTMENTATION OF P53

The subcellular localization of p53 plays a key role in the balance between cell apoptosis and survival. Under physiological conditions, synthetized p53 mainly accumulates in the nucleus, and only a small fraction localizes in the cytosol, where the protein is subjected to ubiquitination and proteasomal degradation. In the nucleus, p53 exerts its function as a transcriptional factor by directly binding to target sequences on DNA, typically containing two decameric motifs with the consensus 5′-PuPuPuC(A/T)(T/A)GPyPyPy-3′. However, p53 can also bind to noncanonical DNA sequences and different types of non-B DNA structures, including cruciforms, triplexes, quadruplexes, and left-handed Z DNA.[21] The structure of p53 is organized into five functional domains that includes the N-terminal transactivation domains (TAD1 and TAD2), followed by a proline-rich region (PRR), the core DNA-binding domain (DBD), the oligomerization domain (OD), and the C-terminal domain (CTD), which work in cooperation to facilitate p53 DNA binding and transactivation (Figure 1). Transcriptional activation involves the N-terminal domain,[22] whereas the CTD facilitates DNA binding and promotes conformational changes in the core DBD to stabilize p53–DNA binding.[23] Although the DBD is able to tetramerize and form stables complexes with DNA, the OD promotes these interactions, and mutations in this domain prevent p53–DNA binding and transcriptional activity.[24] Notably, p53 tetramerization and DNA binding cooperativity are necessary for a p53-activated apoptotic pathway.[25] Finally, the PRR also contributes to transcription activation and is essential for p53-mediated apoptosis.[26]

The p53 protein contains two nuclear export signals (NESs), with one located at the N-terminal (TAD)[27] and one in the OD[28] that synergistically collaborate with each other to control the nuclear–cytosol shuttle. There is also a nuclear localization signal located between the DBD and the OD. Stress-induced p53 phosphorylation decreases p53–MDM2 interaction, leading to protein accumulation and tetramerization, which conceals OD NES and then retains p53 in the nucleus.[28] In addition, the TAD NES contains serine residues phosphorylated after stress, particularly serine 15, which results in NES inhibition and collaborates with p53 export blockage.[27] Moreover,

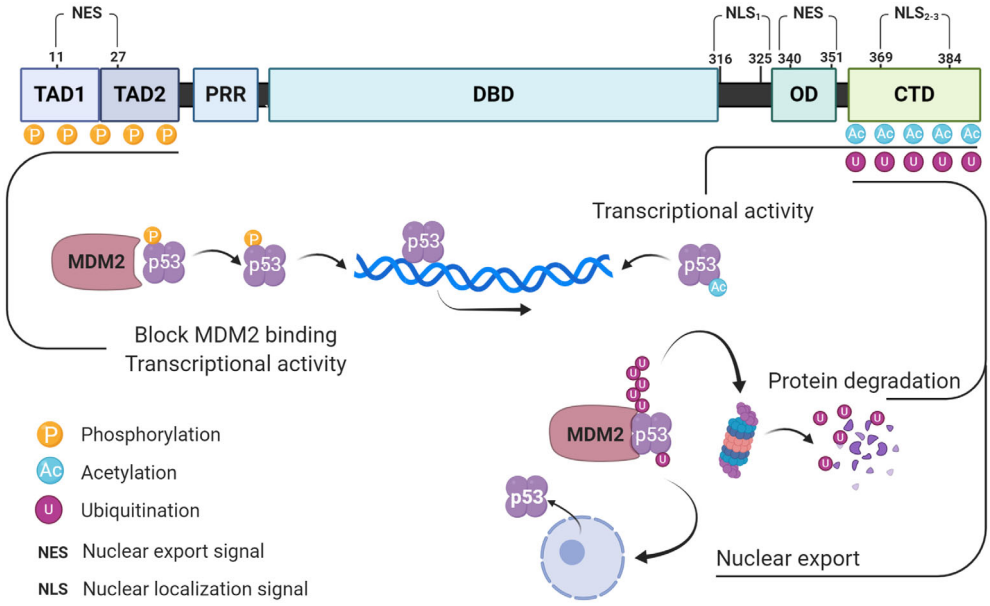

FIGURE 1 Overview of p53 structure. p53 is organized into five functional domains: the N-terminal transactivation domains (TAD1 and TAD2, residues 20–40 and 40–60) followed by a proline-rich region (PRR, residues 60–90), essential for p53-mediated apoptosis; the sequence-specific DNA-binding domain core (DBD, residues 100–300); the oligomerization domain (OD); and the C-terminal domain (CTD, residues 363–393), which regulates DNA–DBD interactions and p53 transactivation. Major posttranslational modifications are indicated. Phosphorylation on the N-terminal serine and threonine residues blocks murine double minute 2 (MDM2)–p53 interaction, promoting p53 stability. Moreover, these modifications induce p53 interaction with transcriptional cofactors, enhancing p53 transactivation. p53 phosphorylation in N-terminal boosts lysine acetylation in the C-terminal region, which increase p53 stability and transcriptional activity. The same lysine residues are targeted for MDM2 induced poly- or monoubiquitination, triggering either protein degradation or nuclear export, respectively

phosphorylation also results in p300-induced acetylation of lysine residues in CTD to promote p53 stabilization[19] (Figure 1). All these enchained events facilitate p53 DNA binding, which increases transcriptional activation of proapoptotic targets PUMA, NOXA, and BAX but inhibits the expression of antiapoptotic BCL-2 and myeloid cell leukemia 1 (MCL-1).[12] Furthermore, PUMA induces the release of cytosolic p53 from BCL-xL,[29] leading to the activation of BAX and BAK, which oligomerize and form pores in the mitochondrial outer membrane (MOM), resulting in cytochrome c release and the onset of apoptotic cell death.[30]

Although the transcriptional activity of p53 has been considered its main function, p53 also functions as a nontranscriptional effector of apoptosis by interacting with mitochondrial proteins. Upon an apoptotic insult, cytosolic p53 translocates to the mitochondria, where it interacts with BCL-2 family proteins to rapidly activate MOM permeabilization.[17] Moreover, FOXO3a promotes CRM1-dependent p53 nuclear export and induces p53 mitochondrial localization.[31] In the mitochondria, p53 interacts with antiapoptotic proteins BCL-xL, BCL-2, and MCL1, disrupting the complex with proapoptotic proteins BAX and BAK.[32] Furthermore, p53 can directly bind and activate BAX[33] and BAK,[34] leading to MOM permeabilization. Under oxidative stress conditions, p53 also interacts with cyclophilin D and triggers mitochondrial permeability transition pore opening and release of cytochrome c independent of BAX or BAK activation.[35]

The causal relationship between stress-induced specific posttranslational modifications and mitochondrial targeting of p53 is still controversial. Some evidence failed to identify different phosphorylation and acetylation patterns of nuclear and mitochondrial p53 pools after genotoxic stress.[36] In contrast, other studies found that modifications, including acetylation of lysine 120[37] and phosphorylation on serine 46,[38] specifically promote p53 translocation to the mitochondria upon DNA damage.

Studies on mitochondrial-targeted p53 proteins and mutated p53 peptides that fail to localize into the nucleus demonstrate a rapid and direct effect of p53 on MOM permeabilization and cytochrome c release independent of p53 transactivation activity.[17,33] In this context, apoptosis induced by the nontranscriptional program of p53 is rapid and occurs prior to the activation of the transcriptional pathway.[33] Finally, the identification of a human single nucleotide polymorphism (SNP) at codon 72 of *Tp53* gene that controls p53 mitochondrial localization and cell susceptibility to apoptosis strongly supports the key function of the p53 nontranscriptional pathway in cell death,[4] which may represent a new target for therapeutic intervention.

3 | AN OVERVIEW OF P53 IN STROKE

Stroke results in a dramatic decrease in oxygen and glucose delivery to a brain region, leading to adenosine triphosphate (ATP) depletion and the onset of a rapid cascade of interconnected events including, but not limited to, mitochondrial dysfunction, oxidative stress, excitotoxicity, and inflammation. All these events promote brain edema, blood–brain barrier disruption, and neuronal cell death,[39] which exacerbate brain damage and the impaired functional recovery of stroke patients.[4,5]

Given the central role of p53 in cellular stress response and cell death,[12] the protein has emerged as a key factor in the pathophysiology of stroke.[5,20,30] Numerous in vitro and in vivo studies have evidenced the function of p53 in neuronal apoptosis and its accumulation in apoptotic lesions after cerebral ischemia. Furthermore, genetic depletion and pharmacological inhibition of p53 prevents neuronal damage and improves functional recovery after stroke.[4,40–42] Interestingly, the neuroprotective role of pifithrin-μ, a specific inhibitor of p53 mitochondrial translocation, is higher than that of pifithrin-α, which inhibits p53 transcriptional activity, demonstrating the higher efficacy of the mitochondrial proapoptotic pathway versus the transcriptional route of p53 in cerebral ischemia[5,40,41] (Figure 2).

After the onset of ischemia, p53 is rapidly activated by phosphorylation in its N-terminal region.[12] In this context, the activation of protein kinases triggered by DNA damage or excitotoxicity after ischemia[39] promotes p53 phosphorylation at key residues and then disturbs the p53–MDM2 interaction, which results in p53 stabilization and determines its nuclear and mitochondrial trafficking and proapoptotic activity.[13] In this situation, calcium overload in response to excitotoxicity activates death-associated protein kinase 1 that phosphorylates p53 at serine 20, leading to neuronal death.[43] Cyclin-dependent kinase 5 (Cdk5) is also activated in response to glutamate-mediated neurotoxicity in postmitotic neurons[44,45] and phosphorylates different substates, including p53 at serine 15, linking excitotoxicity and neuronal death.[46] Moreover, Cdk5 inhibition attenuates p53-dependent apoptosis after cerebral ischemia.[47] Finally, ischemia-induced DNA damage and oxidative stress also induces p53 phosphorylation at serine 15 through a mechanism dependent on protein kinase ataxia-telangiectasia mutated activation.[48]

The effects of p53 activation in stroke mainly depend on both the area of infarction and the subcellular location of the protein. Within the ischemic core, which is the most severely affected area by the sudden interruption of blood flow, cells die rapidly by necrosis. In response to ischemia-induced oxidative stress, p53

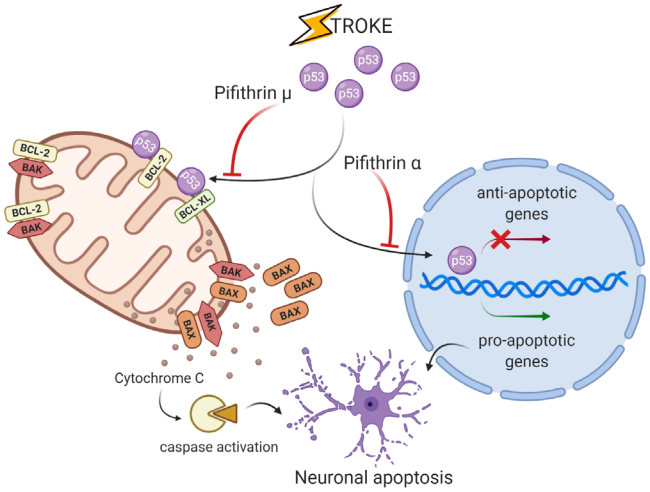

FIGURE 2 p53 activates both transcriptional and mitochondrial apoptotic programs after stroke. Once p53 is activated after cerebral ischemia, it rapidly translocates to the outer mitochondrial membrane, where it directly binds, and inactivates, the antiapoptotic proteins B-cell lymphoma-2 (BCL-2) and B-cell lymphoma extra-large (BCL-xL), leading to B-cell lymphoma-2 associated X-protein (BAX) and B-cell lymphoma-2 antagonist/killer (BAK) oligomerization. The pores in the mitochondrial outer membrane trigger cytochrome c release to the cytosol, unleashing caspase cascade and neuronal death. Moreover, p53 also localizes into the nucleus, where it binds to the p53-responsive elements located at target gene promoters, where it initiates its transcriptional apoptotic program, activating proapoptotic genes such us BAX, p53 upregulated modulator of apoptosis (PUMA), NADPH oxidase activator (NOXA), and BID or inhibiting the expression of BCL-2 and myeloid cell leukemia 1 (MCL-1) antiapoptotic genes. Pharmacological treatment with p53 inhibitors, pifithrin-α and pifithrin-μ, prevents protein nuclear and mitochondrial translocation, respectively. Although both treatments are efficient, pifithrin-μ shows greater neuroprotective effects

accumulates in the mitochondria and promotes mitochondrial permeability transition pore opening by direct interaction with cyclophilin D, which trigger necrosis.[35] The activation of nuclear p53 has also been related to ischemia-induced autophagy via the transactivation of two p53 target genes, DNA damage-regulated autophagy modulator and p53-induced glycolysis and apoptosis regulator.[49] However, the role of p53 in the penumbra apoptosis is by far the most evident and studied.[5] Once p53 is activated after the onset of ischemia, two different but interconnected apoptotic programs are triggered according to the subcellular location of p53.

The transcriptional apoptotic program includes the activation of genes induced by nuclear p53, such as the BCL-2 family protein members. Among them, the proapoptotic proteins BAX, PUMA, NOXA, and BH3 interacting domain death agonist (BID) are upregulated in response to ischemia and activates the intrinsic neuronal apoptotic pathway, as mentioned.[30] The extrinsic apoptotic pathway is also regulated by p53 via transactivation of receptors such as death receptor 5, FS-7-associated cell surface antigen (FAS), and FAS ligand.[50] Furthermore, p53-transactivated genes after ischemia include p53-regulated apoptosis-inducing protein-1, p53-inducible gene (PIG), and SIVA, all target genes involved in p53-dependent apoptosis. P53AIP1 and PIG are translocated into the mitochondria where they are linked to mitochondrial dysfunction and oxidative stress. SIVA has a direct role in ischemia by establishing interaction with proteins of the tumor necrosis factor receptor family, as well as antiapoptotic BCL-2 family proteins.[51] Paternally expressed gene 3 protein (Peg3/Pw1) is also involved in p53-mediated cell death in brain ischemia/hypoxia, acting as a mediator between p53 and BAX mitochondrial translocation in neuronal death.[52]

During cerebral ischemia, nuclear p53 function is, in turn, regulated by other transcription factors. Hence, the signaling pathway involving notch/hypoxia-inducible factor 1-alpha (HIF-1α)/nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB)/p53 promotes neuronal death after ischemic stroke conditions.[51] Notch contributes to the pathogenesis of ischemic stroke by promoting both the stability of p53 and its transcriptional activity.[53] Moreover, stabilization of p53 is HIF-1α dependent and increases the expression of p53 target genes, such as p21.[54] The ischemic insult also stabilizes the binding of p53 to its transcriptional coactivator p300, thus obstructing the p300/NFkB interaction and promoting the transcription of p53 proapoptotic genes and blocking the transcription of NFkB-dependent antiapoptotic genes such as BCL-2, BCL-xL, manganese superoxide dismutase, or inhibitors of apoptosis protein.[50]

The nontranscriptional mitochondrial apoptotic program occurs in the early stages after the stroke. The translocation of p53 to mitochondria is rapid, occurring within the first hour after ischemic damage, and precedes changes in mitochondrial membrane potential, cytochrome c release, and procaspase-3 activation.[30,55] In response to brain ischemia, p53 translocates to the surface of the outer mitochondrial membrane and forms complexes with BCL-2 and BCL-xL and also directly with BAX and BAK,[4] triggering MOM permeabilization and cytochrome c release[17,30] (Figure 2).

The consensus signal for mitochondrial translocation is still unknown, but it is somehow regulated by post-translational modifications. Briefly, upon ischemic brain injury, the prolyl isomerase Pin1 triggers p53 cis/trans isomerization, which favors its monoubiquitination by MDM2 and therefore p53 translocation.[17] Furthermore, p53 acetylation is not necessary for p53 mitochondrial

translocation but enhances p53 mitochondrial effects. In fact, p53 deacetylation has a neuroprotective role in cerebral ischemic damage. Silent information regulator 1 (SIRT1) deacetylase is associated with low BAX levels and BCL-2 stability, preserving mitochondrial function and preventing cell death during ischemic stroke.[56] Therefore, mitochondria are central integrators and transducers of proapoptotic signals, forming the nexus between the nonspecific inducer and the final execution phase of apoptosis.

The fine-tuning of p53 and MDM2 protein levels is precisely one of the key regulation points to afford attenuation of p53-mediated apoptosis after ischemia. Moreover, the p53–MDM2 interaction plays an essential role in neuroprotection exerted by ischemic preconditioning. The increased MDM2 protein levels observed after ischemic preconditioning (sublethal ischemia) lead to p53 cytosolic destabilization and prevent p53-mediated apoptosis after subsequent ischemia.[57] On the contrary, treatment with the specific inhibitor Nutlin-3a that disrupts p53-MDM2 binding negatively affects neuronal survival after ischemia.[42] Furthermore, the MDM2–p53 interaction has been found to be neuroprotective in stroke patients and a reliable predictor of functional outcome after stroke. A functional polymorphism in the *Mdm2* intronic promoter, consisting of a T > G nucleotide change at position 309 (rs2279744), determines MDM2 protein expression and attenuates p53 proapoptotic activity. Thus, patients harboring the SNP309G allele possess higher MDM2 protein levels and better functional outcome after ischemic or hemorrhagic stroke.[42]

A p53-related gene, *Wrap53* (WD40 encoding RNA antisense to p53), is also determinant in p53 abundance after DNA damage. It encodes both a regulatory RNA and the scaffold WD40 protein WRAP53. The regulatory RNA functions as a natural p53-antisense transcript, and it is required for p53 mRNA stabilization to enhance p53 transcription after DNA damage.[58] Interestingly, the role of the WRAP53 protein in ischemia is also related to DNA damage and counteracts apoptosis caused by ischemia-induced p53 stabilization. WRAP53 cellular localization is essential for its prosurvival function. While mitochondrial sequestration circumvents the WRAP53 neuroprotective role in ischemic neurons, nuclear WRAP53 drives the repair of neuronal DNA breaks after ischemic damage, determining neuronal survival and better functional outcome of stroke patients.[59]

Although neurons are the most sensitive cells to ischemia-induced brain damage, the entire neurovascular unit, including glial and endothelial cells, may also be affected. In this context, p53 stabilization regulates endothelial apoptosis. Moreover, endothelial cell survival is essential to improve neovascularization, brain repair, and functional outcome of stroke patients in a p53-dependent manner.[60] In relation with glial cells, astrocytes are more resistant to ischemic damage than neurons, mainly due to their metabolic and antioxidant characteristics,[61] but little is known about the mechanisms involving the activation of apoptosis in astrocytes after ischemia. Although some authors suggest that this process may be mediated by the activation of p53,[62] future studies will be necessary to confirm these results. The p53 upregulation observed in neurons, glia, and endothelial cells not only causes the abovementioned cell injuries but also activates microglia. After cerebral ischemia, p53 transactivates miR145, miR155, and miR34a and promotes microglia's proinflammatory function,[63] while p53 absence leads to the alternative activation of anti-inflammatory effects during neuroinflammation, which is associated with tissue repair.[64] Therefore, the understanding of mechanisms that mediate p53 regulation and brain injury after stroke may help us to identify targets for neuroprotective treatments.

4 | INTERACTION OF P53 WITH THE MITOCHONDRIA REGULATES NEURONAL DEATH AND FUNCTIONAL OUTCOME IN STROKE

The regulation of neuronal death in the ischemic penumbra and perihematoma areas is essential for the different functional recovery of patients after ischemic and hemorrhagic stroke, respectively.[42] In this context, p53 plays an important role as it activates transcriptional and mitochondrial proapoptotic pathways, as detailed in previous sections, that should be modulated in order to minimize neuronal death and, consequently, the ischemic damage progression. Pharmacological inhibition of p53 is indeed neuroprotective against ischemia,[4,5,40,41] but the efficacy is higher in case of the mitochondrial proapoptotic pathway. The mitochondrial effect of p53 occurs early, within the first 30–60 min after ischemia; then, it represents a major threat to cell survival compared to the transcriptional pathway.[5] Pifithrin-μ inhibits the association of p53 with the mitochondria by reducing its affinity for apoptotic proteins BCL-xL and BCL-2, without affecting the transcriptional activity of p53. Consequently, pifithrin-μ shows a greater neuroprotective efficacy, revealed by a decrease in the release of cytochrome c and Smac/Diablo, the activation of caspase-3, and the expression of other proapoptotic proteins NOXA and PUMA in the ischemic brain.[65] In contrast, the use of pifithrin-α[40] caused neuroprotection through suppression of the transcriptional effect of p53,[4] although its effect is less potent,

which again highlights the importance of the mitochondrial pathway in cell survival after an ischemic insult.

The impact of genetic susceptibility to apoptosis, and specifically p53-related mutations, has also proven to be crucial in stroke prognosis.[4,30,42,59,66] Several SNP and sequence variations at the human *Tp53* gene have been described so far. Most of these variations occur in non-coding sequences, with no known biological relevance, with exceptions such as the well-characterized intronic *Ins16bp* SNP (rs7878362), which consists of a 16 base pair insertion in intron 3.[67] This SNP has been associated with increased risk of several types of cancer and a decrease in apoptotic and DNA repair capacity in immortalized cell lines.[68] Although there is no evidence linking the *Ins16bp* SNP to reduced neuronal apoptosis in stroke,[4] a functional polymorphism in its close proximity, the coding *Arg72Pro* SNP (rs1042522) in exon 4 of *Tp53*, has indeed proven to modulate neuronal apoptosis and functional prognosis after stroke.[4,66]

The *Arg72Pro* SNP is located in a region that encodes the proline-rich domain in the p53 protein, which is critical for its role in apoptosis.[26] The substitution of arginine (CGC) to proline (CCC) at codon 72 altered the electrophoretic mobility and affected the structure of the proline-rich domain, suggesting that two different variants might produce functionally different proteins.[69] In fact, the arginine variant of p53 (Arg72-p53) initiates apoptosis with faster kinetics and suppresses cancer development more efficiently than the proline (Pro72-p53) one.[69] The higher efficiency of Arg72-p53 to induce apoptosis is the result of its transport to the mitochondria, where it directly binds to and inactivates BCL-xL.[4] As a consequence, there is a release of cytochrome c that promotes caspase-9 activation and cell apoptosis. These events will be detrimental to neuronal survival and brain repair after ischemia[60] and, as recently shown, will be closely linked to the prognosis of stroke patients.[4,60] Thus, the *Arg/Arg* genotype strongly associates with increased neuronal[4] and endothelial cell death[60] and poor functional outcome, while patients harboring the *Pro* allele showed significantly better functional recovery after stroke. Hence, the *Arg72Pro* SNP of the *Tp53* gene controls neuronal susceptibility to ischemia-induced apoptosis and dictates the functional outcome of patients with stroke, regardless of whether the origin is ischemic or hemorrhagic.[30]

The *Arg72Pro* SNP conditions the subcellular location of protein p53. So, it will somehow modify the stability of the protein, its transcriptional activity, and proteasomal degradation.[68] This fact is important in ischemic situations, which promote p53 stabilization and subsequent p53-dependent apoptosis. However, this is also relevant to induce neuroprotection and ischemic tolerance in preconditioning models.[66] While ischemic

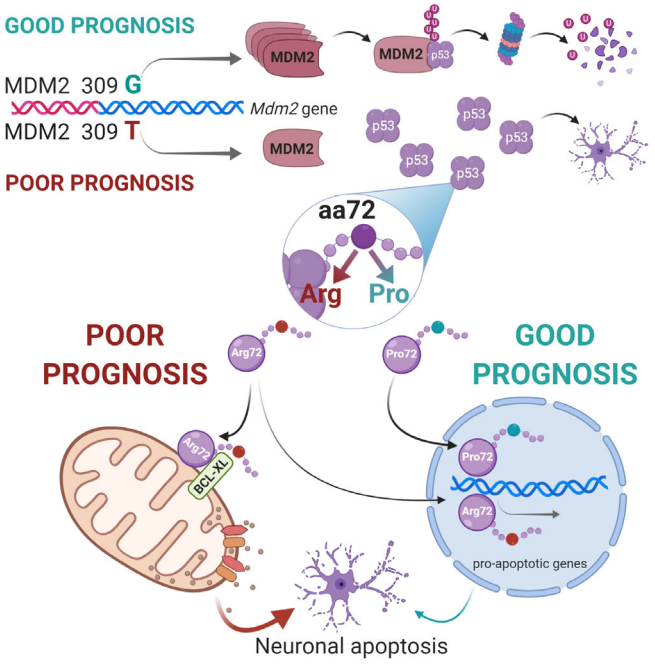

FIGURE 3 The human *p53 Arg72Pro* and *Mdm2* 309 T > G polymorphisms modulate neuronal susceptibility to ischemia-induced apoptosis and condition functional outcome in stroke. Murine double minute 2 (MDM2), the main negative regulator of p53, presents a functional SNP309T > G in its gene promoter that modulates MDM2 protein expression. Patients harboring the *G* allele show higher MDM2 levels, which promote p53 proteasomal degradation and cell survival. Conversely, stroke patients presenting *T/T* genotype express less MDM2 and suffer poor functional recovery. The *Arg72Pro* single nucleotide polymorphism (SNP) located in the proline-rich region of p53 is crucial for p53-mediated apoptosis. Both Arg72-p53 and Pro72-p53 polymorphic variants transactivate proapoptotic p53-targeted genes. However, the Arg72 variant rapidly translocates to the mitochondria, inducing neuronal death more efficiently than the Pro72 variant. Thus, *Pro* allele is associated with better functional outcome of stroke patients

preconditioning prevented nuclear and cytosolic stabilization of Pro72-p53, this effect was not observed in mitochondrial Arg72-p53-expressing neurons, which suffer neuronal apoptosis and caspase-3 activation. Likewise, *Arg72Pro* SNP was recently evidenced as a noninvasive biomarker to predict functional prognosis and ischemic tolerance in patients who have experienced a transient ischemic attack.[66]

Mitochondrial–nuclear p53 trafficking is then essential to determine neuronal apoptosis and, thus, brain damage after ischemia. The identification of the *Arg72Pro* SNP that controls p53 mitochondrial localization and cell susceptibility to apoptosis strongly supports the key function of p53 in this context, positioning itself as a new target for therapeutic intervention. However, the influence

of genetic variants on p53 regulatory genes should not be underestimated either due to their significance in stroke prognosis. Thus, SNPs in the gene encoding the p53 regulator MDM2 have proven to have a key effect on neuronal death and functional outcome after stroke.[42] In particular, the SNP309T > G polymorphism (rs2279744) in the *Mdm2* intronic promoter determines MDM2 protein expression and controls p53-mediated apoptosis. Furthermore, patients harboring the *G* allele in the *Mdm2* promoter had higher MDM2 protein levels and showed better functional outcome after stroke than those harboring the *T/T* genotype. On the other hand, the *T/T* genotype was associated with large infarct volume in ischemic stroke and increased lesion volume in patients with intracerebral hemorrhage.[42] Different mechanisms but the same consequences were observed when the *Wrap53* gene, in close proximity to p53, is mutated. A nonsynonymous SNP in codon 68 of the first coding exon of *Wrap53* c.202C > G (rs2287499) promotes WRAP53 accumulation in the nucleus, leading to DNA repair after ischemia.[59] Substitution of arginine (Arg68) for glycine (Gly68) affects protein function and location within the cell. These results confirm, once again, that controlling neuronal susceptibility to ischemia-induced apoptosis promotes better functional recovery after stroke as patients carrying the SNP showed less infarct volume and better prognosis[59] (Figure 3). The study of these mechanisms underlying neuronal death by apoptosis will therefore help to better manage patients in order to provide them with better functional recovery after stroke and might explain why patients who initially show similar symptoms and lesion volumes may improve dramatically or worsen after stroke. In short, these results demonstrate p53 as a promising therapeutic target to be considered in the pathology of stroke.

5 | CONCLUSION AND PERSPECTIVE

Neurons are particularly sensitive to ischemia and will respond by activating cell death mechanisms after stroke. The survival rate is directly proportional to the time elapsed from the activation of these signaling cascades that conclude with the release of effector caspases and consequent cell death. Thus, activation of the mitochondrial proapoptotic pathway, which is much faster than the transcriptional pathway, will decrease the chances of rescuing affected neurons from the damaged brain after stroke, which is detrimental to the functional outcome of patients. The role played by p53 in this aspect is fundamental as it is a main regulator of neuronal death. The relevance also lies in the ability of p53 to translocate to the mitochondria to activate the apoptotic pathway within the first hour after ischemia. Therefore, any strategy that effectively diminishes the stabilization of p53 and its migration into the mitochondria would alleviate neuronal loss in ischemic brain regions. Indeed, the use of pharmacological inhibitors designed to prevent the association of p53 to mitochondria has already shown a neuroprotective effect in animal models of stroke. Unfortunately, currently, there is no evidence of a successful translation of these findings to the clinical practice. Although initial testing of small molecule activators of p53, such as Nutlin-3a and analogs, has been completed in clinical trials for cancer therapy, no clinical studies have been conducted with compounds designed to block p53 function. The involvement of p53 in numerous and important cellular processes makes the translation of p53 inhibitors into clinical practice difficult. In this context, pifithrin-μ, which inhibits p53 binding to mitochondria without affecting p53-dependent transactivation, would be a promising approach to develop new therapies involving p53 inhibition. However, pifithrin-μ also interacts with the heat-shock protein 70 family of proteins, which causes toxicity and reduces its potential use in stroke patients. It is certainly a field that could be implemented in the coming years with the synthesis of more specific and less toxic compounds, which may lead to promising therapies aimed at minimizing brain damage after stroke. The complex mechanism involved in p53 translocation from cytosol to mitochondria, where it binds to the mitochondrial BCL-xL, still remains unknown. Although it has been described to be regulated by post-translational modifications, the precise phosphorylation profile of p53 responsible for protein translocation remains obscure. The understanding of regulatory mechanisms underlying p53-mediated neuronal death and the discovery of these important yet controversial details will bring positive consequences not only in the context of stroke but also in neurodegenerative diseases such as Alzheimer's, Parkinson's disease, or amyotrophic lateral sclerosis. Finally, the analysis of functional polymorphisms indicative of increased proapoptotic activity of p53 in neurons might be a good target linked to the assessment of rehabilitation needs and a feasible strategy to improve functional recovery of stroke patients.

ACKNOWLEDGEMENTS

This work was funded by the Instituto de Salud Carlos III (PI18/00265, RD16/0019/0018), European Regional Development Fund (FEDER), Ministerio de Ciencia en Innovación (SAF2017-90794-REDT), European Union's Horizon 2020 Research and Innovation Programme (Grant Agreement 686009 to AA), and Junta de Castilla y León (CSI151P20 and CLU-2017-03). Figures were created with BioRender.com.

CONFLICT OF INTEREST

The authors declare no potential conflict of interest.

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How to cite this article: Almeida A, Sánchez-Morán I, Rodríguez C. Mitochondrial–nuclear p53 trafficking controls neuronal susceptibility in stroke. *IUBMB Life*. 2021;73:582–591. https://doi.org/10.1002/iub.2453