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“Mdm2-Tau Complex as a Novel Modulator of the Oncosuppressor Protein P53”

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To my brother Mario

and all my family

Abstract

Cancer and neurodegenerative diseases are unmet medical needs with increasing incidence worldwide. Despite distinct pathogenic characteristics, these two devastating human illnesses share aging as a main risk factor. Aging is characterized by a continuous exposure of the organism to metabolic or environmental stress conditions eventually leading to irreversible consequences. An example is the accumulation of DNA lesions resulting from the large number of daily insults occurring in each cell of our body. To protect the organism, a complex molecular response to DNA damage has evolved. The DNA damage response is orchestrated by the master regulator P53, which stops the cell cycle and induces the DNA repair machinery. However, in case of an irreversible damage to the DNA, P53 is also inducing removal of the damaged cell either by a cell death program or by cellular senescence. DNA damage accumulation and abnormal cell fate decision may contribute to the pathomechanism shared by aging-associated disorders.

Tau is a microtubule-associated protein well-known for its implication in tauopathies, including Alzheimer's disease. These neurodegenerative disorders are characterized by the progressive brain deposition of hyperphosphorylated forms of Tau fibrils, a process correlating with protein toxicity, neuronal cell loss and clinical symptoms. Additional functions of Tau in DNA protection, chromatin remodeling and in cancer emerged recently. Therefore, **the aim of the current thesis project is to prove the hypothesis that a loss-of-function of Tau may contribute to human aging-associated diseases.**

Utilizing Tau-depleted neuroblastoma cells as a model of loss-of-function, I carefully dissected the DNA damage response. This led to the demonstration that cells with depleted Tau decreases P53 stability and activity resulting in an unbalanced induction of cell senescence and reduced cell death. This effect was reversed using an inhibitor of the interaction between P53 and its ubiquitin ligase MDM2. The data suggest thus that Tau may interfere with MDM2-dependent ubiquitination of P53. Indeed, I showed by applying different technologies the existence of a Tau-MDM2 complex in cells and defined which structural domain of each binding partner drives this interaction. Then, I exposed that Tau bound to MDM2 inhibits its P53 ubiquitination. Notably a Tau variant causing frontotemporal dementia has a reduced inhibitory effect. Finally, I report a colocalization of MDM2 with Tau neurofibrillary tangles in Alzheimer's disease brain.

Linking Tau to P53 and cell fate decision in response to a cellular stress represents a tangible breakthrough for the understanding of aging-related human diseases. Taking into consideration the molecular mechanism exposed by the data obtained in my work, the role of the P53-MDM2 axis in neurodegenerative diseases as well as the implication of Tau in the pathogenic process of cancer should be reassessed.

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1. Introduction

1.1 Neurodegeneration

Neurodegenerative diseases are among the major disorders worldwide. Due to the life-span increase and the unmet medical need, it is predicted that the number of individuals affected by these disorders will increase significantly in the next few years. Hebert et al[1] projected a triplication of the number of Alzheimer's disease (AD) patients by 2050, from 45 million to 130 million cases worldwide. Recently, it has been reported that actually 5.7 million of Americans have AD dementia and 11.6 million people have mild cognitive impairment (MCI). The disease incidence is 2 per 1000 individuals 65 to 74 years old and 37 per 1000 individuals over 85 years[2]. The survival after diagnosis is expected to be around 4 to 8 years, depending on different factors like patient's healthcare and lifestyle. These disorders and their increasing incidence is also associated to an important point that cannot be ignored: the Public Health cost; in the United States a cost of $277 billion was estimated in 2018 [3]. The incidence of the disease varies between the sex, in fact woman have an incidence of 21.1% while men only of 11.6%[4].

Neurodegenerative disorders are classified considering different features: anatomical affected brain area (frontotemporal, spinocerebellar or extrapyramidal disorders), primary clinical features (dementia, parkinsonism or motor neuron diseases) or principal molecular abnormality (changes in post-translational modifications or protein aggregation). The most common dementias are amyloidoses, tauopathies, α-synucleinopathies and TDP-43 proteinopathies[5]. In my project, I focus my attention on the study of the protein Tau, as the main protagonist of a group of neurodegenerative disorders known as tauopathies. All tauopathies show pathological Tau accumulation and aggregation in the brain; some examples are Pick's disease (PiD), corticobasal degeneration (CBD), progressive supranuclear palsy (PSP), globular glial tauopathy (GGT) and argyrophilic grain disease (AGD). These disorders are classified as primary and secondary tauopathies. The primary disease is characterized by the fact that Tau protein is mainly responsible for the pathogenesis progression, whereas in the secondary disorders, another protein is involved in the disease progression and/or consequentially it induces changes in the physiological Tau. One of the most well characterized primary tauopathy is frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17), which is due to mutations occurring in the *MAPT* gene encoding for Tau[6]. AD and the inherited prion disease Gerstmann–Sträussler–Scheinker syndrome (GSS) are classified as secondary tauopathies[7]. AD is defined as a secondary tauopathy because its neuropathological diagnosis requires both amyloid deposition and Tau aggregation[8].

Tauopathies are mainly considered as a gain-of-function proteinopathies whereby Tau acquires toxic properties when assuming abnormal conformations and post-translational modifications. However, neuronal deficits may also result from loss-of-function of Tau, e.g., when hyperphosphorylated Tau with reduced microtubule binding capability may lead to altered axonal transport and microtubule disruption[9]. To date, there are no successful treatments to target the cause of tauopathies[10]. Regarding the compensation of neuronal dysfunction some symptomatic

drugs have been approved, like the acetylcholinesterase inhibitors (AChEIs) donepezil, galantamine, rivastigmine[11] for the treatment of AD, like levodopa or dopamine for parkinsonism motor dysfunction. Recent studies also identified new strategies to block Tau accumulation and misfolding, however their efficacy has not been shown yet[10,12]. Indeed, unfortunately we are still far away to discover the exact molecular mechanisms underlying pathogenic Tau dysfunction. Their understanding is required for the identification of specific disease-modifying therapies. There are two non-mutually exclusive models to study the protein Tau in pathology. As a rational for e.g. immunotherapies, the first one is focused on Tau misfolding, oligomerization and fibrils formation, known as gain-of-function, exacerbated by a defect in degradation due to aberrant PTMs[13]. Poorly exploited for new therapies, the second model addresses the loss-of-function of Tau resulting from sequestration of Tau into aggregates and disruption of its physiological function[14]. The risk factors for neurodegenerative diseases are multiple, ranging from genetic factors such as apolipoprotein E allelic variants to environmental factors, as traumatic brain injuries, stressful life and sleep disorders. Currently, early neurodegenerative markers are acquiring more and more relevance when considering preventive therapies or to monitor disease progression before onset of clinical symptoms. AD is diagnosed using neuropsychological clinical tests evaluating cognitive impairments. Clinical diagnosis can be confirmed by wet biomarkers such as phosphorylated forms of Tau in the cerebrospinal fluid. However, all these methods do not allow an early disease diagnosis and unfortunately, they detect the disease already in late phases. In this respect, promising Tau or amyloid PET tracers are becoming available. It is important to discover early neurodegenerative markers that will allow to detect the disease at early phases. To wrap up, this chapter will describe the role of Tau as the main effector of AD, both in its physiological and pathological scenario, even highlighting some new roles of this protein uncovered to date.

1.1.1 Tau protein: structural and functional aspects

My field of study focuses on tauopathies, which have as the main actor the Tau protein. In 1975 Weingarten et al[15] described Tau as a microtubule-associated protein belonging to the microtubule associated protein (MAP) family. This heat stable protein is fundamental for the assembly of the microtubules, which is important in physiological conditions for stabilization of the cytoskeleton scaffold and in promoting cellular trafficking[16]. In the brain, Tau mRNAs and proteins are mainly expressed in neurons but their presence is described also in glial cells. In the neuron, the presence of Tau has been principally reported in axons that are rich in microtubules. However, Tau is detected in much lower amounts in somatodendritic compartments, including the plasma membrane, the nucleus and the mitochondria[17]. Tau protein is encoded over 16 exons of the *MAPT* gene localized in the 17q21 chromosome of the human genome[18] (***figure 1***).

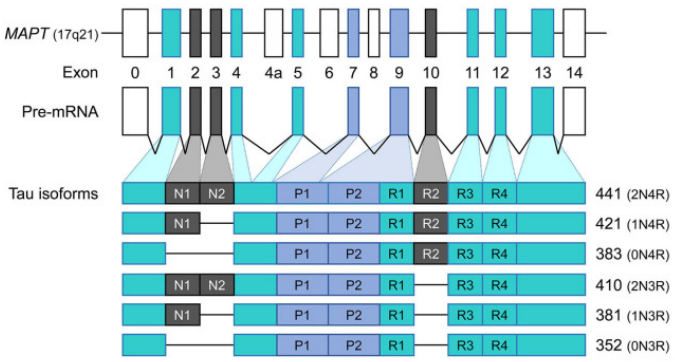

Fig.1 (Kang et al. Frontiers in Neurology_2020)

Schematic structure of the *MAPT* gene with its splicing isoforms present in the human brain

In the human brain, Tau is described to have six isoforms depending on the presence or absence of the exons 2, 3 or 10. This results in Tau isoforms with a number of amino acids ranging between 352 and 441, and molecular weights between 45 and 65 kDa. The ratio between the splicing isoforms changes during the development. In particular, splicing of exon 10 generates the 4R or 3R isoforms. These three to four conserved repeats are crucial for microtubule binding and are located in the positively charged C-terminal part of Tau[19]. The N-terminal part of Tau is the main interactor with other proteins[20]; however, the C-terminus is also involved in protein-protein interactions[21].

Beside its isoforms, Tau is described to be highly susceptible to covalent changes, known as post-translational modifications (PTMs). Tau PTMs can modify the protein functionality from physiological to pathological forms (***figure 2***).

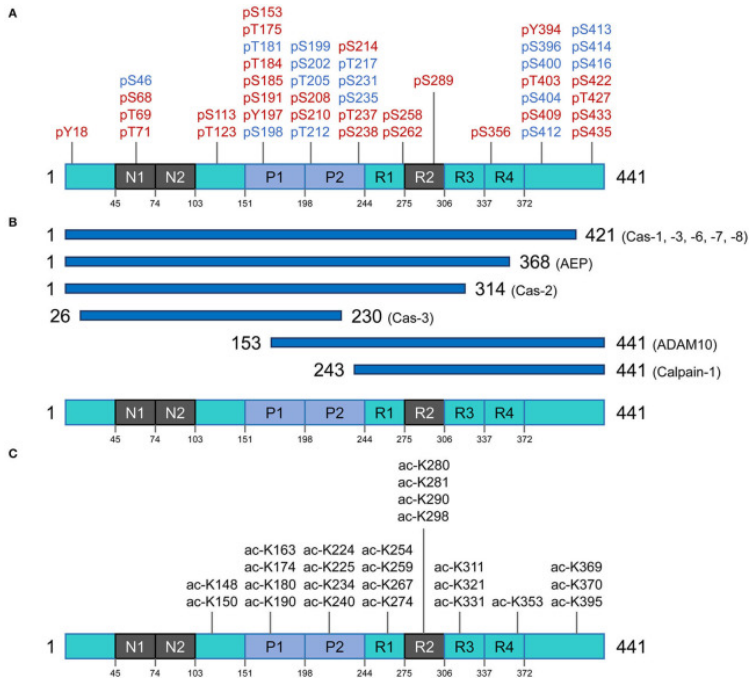

Fig.2 (Kang et al. Frontiers in Neurology_2020)

Graphic map of Post Translational modifications (PTMs) in human Tau

The best characterized and more abundant modification is phosphorylation (***figure 2A***). Tau has theoretically 85 phosphorylation sites, of which about twenty are used normally[22]. In the AD brain, Tau is three times more phosphorylated than in healthy brain, resulting from the use of new sites and increased use of the physiological sites[23]. Tau phosphorylation reflects the dynamic state of the protein, and it correlates with the different functions ranging from microtubules stabilization, axonal polarity, and subcellular localization. It is proposed that increased phosphorylation leads to less interaction with microtubules and the increase in freely moving Tau may result in more protein misfolding and aggregation[24]. In contrast, some phosphorylation sites are believed to have a protective role due to their inhibitory impact on aggregation[25]. However, the majority of the scientific community considered Tau phosphorylation as a trigger event to induce neurodegeneration[26]. Mitogen-activated protein kinase (MAPK), glycogen synthase kinase-3 (GSK-3 with the two isoforms alpha and beta[30]), cyclin-dependent kinase-5 (Cdk5) phosphorylate Tau *in vitro*[27,28]; and Tau phosphorylated at the same sites is found within neurofibrillary tangles (NFTs)[29].

Tau is subjected also to proteolytic cleavages (***figure*** 2B). This modification can produce fragments that can differ from the full-length protein in microtubule stabilization activity, cellular localization, interaction activity and solubility. In fact, Tau seems to be a target of many endoproteases and in pathological conditions these undegraded fragments increase their propensity to form aggregates escaping degradation through the proteasomal or the autophagic pathways[31]. Indeed, some fragments of Tau characterized by different size accumulate in disease. A well-known example is the Tau fragment produced by the caspases 1, 3, 6, 7 and 8 when cleaving at residue 421. This fragment shows an increased tendency to aggregate compare to the full-length protein and they are more abundant in NFTs[32].

Another important modification of Tau is acetylation: Tau has 20 Lys residues that can be targeted by acetylation[33]. By neutralizing the repulsion of the positively-charged Lys residues, acetylation favors parallel stacking of β-sheets and hence it promotes Tau fibril formation[34]. Moreover, it has been reported that Tau can auto-acetylate itself through catalytic Cys residues present in its microtubule binding domain[35]. Acetylation of Tau is also found to affect the degradation of the protein, inhibiting the ubiquitination of Lys residues, and causing a slow rate of protein turnover[36]. Tau acetylation is associated also with reduced microtubule binding and promotion of aggregation. Beyond these PTMs, others can play a role in aggregation like sumoylation and methylation through participating in electrostatic and hydrophobic interactions[37]. To summarize, Tau has well known roles in microtubules stabilization and cytoskeleton dynamics. Importantly, all these processes are tightly regulated inside the cell by other factors such as Tau interaction with other proteins/structures or proper Tau degradation and regulation mediated for instance trough PTMs. All these factors can finally lead to the disruption of the existing equilibrium between physiology and pathology.

1.1.2 Pathological Tau: tauopathies

The cells of our body have precise quality control systems maintaining their homeostasis when exposed to environmental changes. However, every day, each cell is exposed to thousand insults that can affect this balance. Some proteins are more sensitive to changes than others, thereby leading to pathologies. Tau is described as a fickle protein. This feature can be due either to germline mutations, like in the case of FTDP-17 or to sporadic causes, like environmental factors or DNA damage accumulation during aging[33]. Tau lacks the "structure-function paradigm", thus a three-dimensional structure associated to a specific activity. Tau is classified as an intrinsic disordered protein. This feature is typical of proteins able to change very rapidly their conformation and to act as a scaffold bringing binding partners in vicinity. Tau presents high water solubility and heat stability, which counteracts the association of high protein flexibility with an increased predisposition to misfolding[38].

Although neurodegenerative disorders are often defined as protein misfolding diseases, tauopathies are actually linked to the conversion of Tau from a normally intrinsic disordered monomeric protein into highly ordered β-sheet-rich multimeric paired helical filaments (PHFs), which consequently deposit in NFTs[39] (***figure 3***).

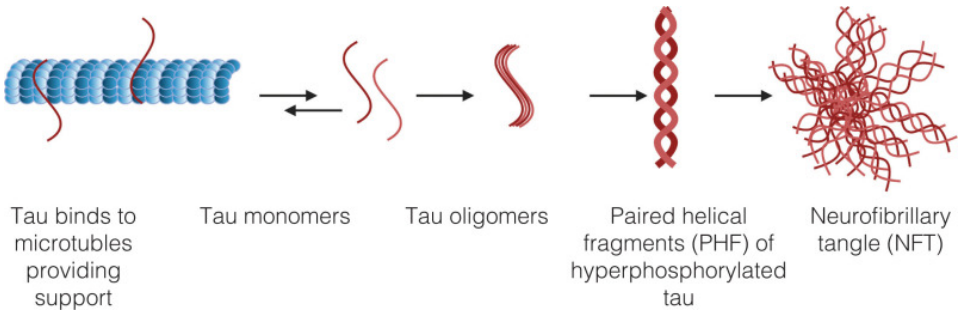

Fig.3 (Sheppard et al. Alzheimer disease: drug discovery_2020)
Model of microtubule-associated protein Tau aggregation

A big area of research is to identify the different molecular mechanisms and proteins modifications associated with toxicity in the CNS. One of them are PTMs such as phosphorylation, acetylation and SUMOylation which regulate the association of Tau to microtubules, a status that is believed to safeguard Tau from aggregating[40]. The alteration of the affinity of Tau for microtubules may be due to the decrease of positive net charges of its microtubule binding domain upon Ser or Thr phosphorylation or Lys acetylation[41]. These modifications, possibly together with the increase propensity in oligomerization and fibrils formation, may explain why hyperphosphorylated Tau is the primary component of NFTs. Several studies identified kinases and phosphatases that regulate Tau phosphorylation in physiological conditions, and showed that an imbalance between the two brings phospho-Tau in a pathological conformation[42]. Another PTM responsible for the pathogenesis of Tau is acetylation. Tau has its auto-acetylase activity but its main acetylase is p300/CREB binding protein HAT[35]. Acetylation occurs at the same Lys residues that are also ubiquitinated. So, if acetylation happens together with phosphorylation, the negative effects of this

latter on Tau are increased by blocking its degradation. Importantly some studies have also reported that hyper-acetylation versus hypo-acetylation of specific sites of Tau can affect the clearance of NFTs[43]. In neurites the level of Tau ubiquitinated and phosphorylated is found at comparable level. While Tau ubiquitination is fundamental for its targeting to proteasomal degradation, a high level of ubiquitination of Tau is found also in the early and intermediated stages of the disease, possibly as an attempt to eliminate accumulating forms of Tau[39].

PHFs are made of twisted ribbons or straight Tau filaments with diameters between 2.1 and 15 nm[44]. Recent advances describe a new feature of Tau, which seems to be able to compact itself in a specific disease-associated manner defined as strain. Indeed, brains from patients suffering from the same tauopathy display the same Tau strain that may differ from other tauopathy strains[45]. In most cases Tau goes from a disordered unfolded monomeric state to a highly ordered fibrillary structure[46]. In a healthy cell, there are a large number of chaperon proteins that act as controllers of an equilibrated proteome by facilitating protein folding or protecting partially misfolded or monomeric complex members from degradation[47]. Tau is shown to be degraded principally in two ways, the autophagy-lysosomal pathway and the ubiquitin proteasome system (UPS)[48]. A compromised degradation system can increase the local protein concentration thus favoring its potentially toxic aggregation and for this reason the degradation machinery may be useful as therapeutic target.

Moreover, an aspect with a high clinical relevance is the transcellular propagation of the pathogenic forms of Tau, with a consequent spread of the disease. This process is also defined as prion-like because it is characterized by a pathogenic Tau conformation able to propagate like a sort of molecular stamp on physiological Tau forms. Pathological Tau involved in the transmission can have different biochemical forms like soluble monomeric forms, soluble oligomers or insoluble fibrils[49]. Several studies have shown that smallest oligomers have more facility to be transmitted[50]. A recent research of Kayed et al[51] have shown that cortical neurons are able to uptake the different Tau species through distinct pathways. Other studies have demonstrated that Tau modification, like the phosphorylation status, can affect its transmission, hence this feature can be used to track the disease progression and severity[52]. To further explore this aspect, Pedrioli et al[53] have looked in the possible involvement of extracellular vesicles (EVs). For the first time, they showed that a profibrillogenic form of Tau transported through EVs is able to interact with endogenous Tau of the host cell in an acidic compartment, outlining the contribution of autophagy in the spreading of the pathology[53]. All these studies defined many possible targets for a therapeutic intervention. However, we still lack an understanding of the precise molecular mechanisms transforming e.g., a microtubule-associated Tau monomer into self-propagating pathogenic Tau multimers. This possibly explains the lack of efficacious drugs targeting this pathway. Likewise, how these multimeric Tau species exert their toxicity on neurons is also poorly understood and the possible contribution of a loss-of-function may need more attention.

1.1.3 A new role of Tau: nuclear protection

To date, multiple roles have been attributed to Tau, which is now considered as a multifunctional protein. The importance of Tau in the regulation of microtubules dynamics, axonal transport, maturation and elongation is well characterized. Also, synaptic vesicles release and the activity of NDMDA and AMPA receptors are regulated by the interaction of pre- and post- synaptic Tau with synaptic proteins. In the recent years, Tau has been detected in nucleus, where it has a protective role on DNA[54,55]. Interestingly nuclear Tau is found also in control and AD patients, and even if the specific isoform of Tau in this compartment is not yet clarified, it may miss exon 2 and 3[56]. Phosphorylation seems to have an important impact in the behavior of Tau in the nucleus, especially in its intranuclear localization[57]. Some reports indicate the presence of both phosphorylated and unphosphorylated forms of Tau in the nucleus[58,59], although it appears that the majority is non phosphorylated[60] when using the Tau-1 antibody, which in our hands display nuclear immunoreactivity also in Tau-KO cells. However, DNA damage is associated to hyperphosphorylated nuclear Tau[61]. Several groups have suggested that Tau may have a possible role in chromatin function and/or organization in neuronal, non-neuronal and cancer cells, but the mechanisms are not yet fully characterized[62,63]. Similarly to its ability to bind to microtubules, Tau binding to DNA is drastically reduced in presence of phosphorylation[64]. It is proposed that Tau binding to DNA is able to protect against DNA breakage[65]. In support to this hypothesis, some studies showed that Tau, in primary cortical neurons, displays a function similar to the one of the heat shock protein 70 (HSP70)[54]. This chaperone-like behavior is validated by Rossi et al[66], who showed that in fibroblasts and peripheral blood lymphocytes derived from patients affected by FTD and carrying the P301L mutation, there is the presence of several chromosomal mutations, from aneuploidy to rearrangements. Furthermore, chromosome mis-segregation and aneuploidy are also described in mice knocked-out for Tau[67]. Additionally, Tau may be also involved in mechanisms of DNA repair[68], although this remains controversial[66]. Tau is also involved in modulation of gene expression. This is expected to occur through its binding between its proline-rich and microtubule binding domains and the AT-rich minor groove of the DNA[64]. Normally the minor groove has the function to alter the DNA conformation, causing it to unwind. This facilitate the formation of multi protein-DNA complexes, which can enhance or inhibit gene transcription[69]. Since now it is reported that some genes are significantly upregulated after Tau depletion like *smarce1*, *calbindin*, *lsm12 homologue* or *map1lc3a*[70]. The functions of nuclear Tau previously described can be altered by amino acid mutations, abnormal Tau phosphorylation and oxidative stress. The impact of phosphorylation on nuclear Tau is more complex. On one hand, there is evidence showing that an abnormal phosphorylation reduces nuclear Tau translocation[71] and subsequently decreases its ability to bind and protect DNA[72]. This effect can be considered as a loss-of-function of Tau. This contrasts with the gain-of-toxic function for hyperphosphorylated Tau associated to an increase of nuclear stress and disrupted heterochromatin organization[73,62]. In our laboratory, through sensors targeting the different compartments of cells, the presence of Tau within the nucleus was confirmed[61]. Spectrometry analysis revealed that nuclear Tau is more

phosphorylated at T_{181} and S_{404} when compared to cytosolic Tau. Interestingly in presence of a DNA damage induced by Etoposide, an increase of nuclear Tau was accompanied by a decrease in the phosphorylation of T_{181}[61]. Since this phospho-T_{181} is used as a disease biomarker in cerebrospinal fluid, these data propose a molecular mechanism linking Tau hyperphosphorylation, DNA protection and the neurodegenerative process. However, this protective role of Tau in the nucleus remains controversial, as an additional study of 2022 has confirmed the presence of phospho-Tau within the nucleus, where it seems associated with p53-dependent apoptosis and nuclear dispersion, which are characteristics of neurodegeneration[74]. Rico et al[75] have demonstrated an interaction between 4R Tau and histones H3 and H4, with as a consequence the stabilization of condensed chromatin and heterochromatin. Since the binding occurs only with the unmodified histones, these data suggested a general role of Tau in the nucleus in preventing chromatin remodeling[75]. Notably, in some tauopathies, Tau is also present in aggregates even in the nucleus, however this needs further investigation[76]. These new findings about the role of Tau in the nucleus confirm the importance to investigate the multiple function of Tau in view of the possible contribution of loss-of-function in disease. However, it should be noted that *MAPT* knocked-out mice develop normally without major cellular defects but with reduced microtubule stability and slightly less axogenesis[77]. One possible explanation for this very mild phenotype despite the multifunctional activity of Tau is the possible redundance with other MAP proteins, in particular MAP2. Alternatively, the contribution of Tau-depletion to disease may require exposure of the organism to various forms of stress during aging.

1.1.4 Aging: cell death as a hallmark of neurodegeneration and cancer

Neurodegeneration and cancer are among the most frequent disorders nowadays due to increased longevity and stressful life. One common and well-established risk factor of these disorders is in fact aging. Aging is a chronic phenomenon which leads to a functional deterioration of the human body. It can be considered natural, due to the irreversible running of time coupled to the exposure of external insults such as air pollution, UV light, radioactivity, poor alimentation and hygiene, which also promote frequent disorders such as cancer, neurodegeneration, diabetes and cardiovascular diseases[78]. In particular, aging is characterized by a permanent biological decay[79]. Some well-known features are telomere shortening, DNA damage accumulation, cellular replication decline, mitochondrial dysfunction and impaired protein and organelle accumulation[80 81]. Aging affects the cells at the mechano-biological level by affecting nuclear transport, cellular spreading and cytoskeleton formation (***figure 4***)[82]. These characteristics of an aging cell can be paramount as biomarkers of its progression e.g., when searching for drugs against aging-related disorders. The phenomenon of aging may affect in a different manner the tissues of the human body.

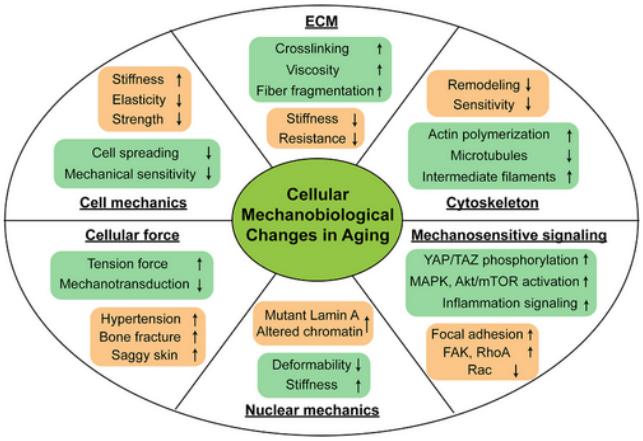

Fig.4 (Bajpai et al. Annals of the New York academy of science_2021)
Map of biomarkers in Aging

Mattson et al[83] described an association between brain aging and the development of AD, PD and stroke with compromised bioenergetics, neuroplasticity and Ca^{2+} homeostasis. These changes may result in an inflammation state. At a behavioral level aging brings along a progressive decline in learning and memory, sensory perception and motor coordination[84].

Another disease where aging is the major risk factor is cancer. The National Cancer Institute has reported that 54% of all cancer diagnoses between 2012 and 2016 in the USA affected people above the age of 65, with an average age of death at around 72[85]. This statistic strongly corroborates the link between aging and tumorigenesis[86]. This nexus can also arise to a new research focus, more on longevity than on cancer mechanisms. An example is given by a leader in the study between aging and cancer: Haim Cohen. Cohen et al[87] demonstrated that SIRT6 is a protein deacetylase that regulates longevity in mammals and many other processes like DNA repair, stress response and apoptosis. In fact, a depletion of this protein is known to accelerate aging and to promote cancer and can be counterbalanced by its overexpression that extends lifespan.

Aging is associated with protein dysfunction, altered homeostasis and progressive deterioration of the cells, eventually leading to cell senescence or cell death[88]. In this section, I will focus on two types of cell fates: apoptosis and senescence and their link with human disease. The reason is because these are the main functional outcomes of a DNA Damage Response (DDR) when the cell fails to repair the DNA lesion. The term “apoptosis” was first proposed by Kerr in 1972[89], who distinguished between apoptosis and necrosis. Differently from necrosis, apoptosis is somewhat a routinely used, healthy programmed self-sacrifice following an active decision taken for the greater benefits of the organism following internal or external stimuli [90]. Apoptotic cell death prevents an inflammatory response because - instead of an uncontrolled release of the cell content following the rupture of the plasma membrane - its main characteristic is the formation of apoptotic bodies, which are then engulfed by phagocytes in a process named efferocytosis[91]. Morphologically the cells undergoing apoptosis show blebbing, shrinkage, and fragmentation of nucleus and genetic material. Another peculiarity is the pyknosis, the irreversible condensation of chromatin, which happens in the early stage of apoptosis. The end of the apoptotic process is marked by phagocytosis

of all the structured formed and recycling of the biological building blocks[92]. Two types of apoptotic programs are described: the extrinsic and the intrinsic pathways. The extrinsic pathway refers to a receptor-mediated initiation of apoptosis e.g., with the activation of TNF and FAS receptors, where a binding with an external ligand initiates the release of death signal in the intracellular space[93]. The intrinsic pathway is characterized by non-receptor-mediated initiation and mitochondria regulation. In this case the signal directly acts at the intracellular level by governing mitochondrial outer membrane permeabilization[94] with the consequent dissipation of the membrane potential[95]. Important regulators of apoptosis are the members of the Bcl-2 protein families, which are classified as pro-apoptotic: Bax and BH3 family, or anti-apoptotic/pro-survival: Bcl2 family. 25 genes in the Bcl-2 family were identified and they are described to control apoptosis. A key player in the cell cycle machinery: p53, initiates apoptosis by e.g., down-regulating members of the Bcl-2 family[91], or by directly inducing the transcription of Bax[101]. Furthermore, recently research has shown that also the endoplasmic reticulum (ER) is involved in apoptosis with the alteration of the calcium homeostasis[96]. Apoptosis occurs mainly through the action of a type of cysteine proteases known as caspases that act as initiators or effectors of the process[97]. Caspase-8 and -9 are the initiator while caspase-3 is the main effector, accompanied by caspase-6 and -7[98]. Many researchers highlighted the importance of apoptosis in the self-defense mechanism of the immune system[99]. However, a clearance of the dying apoptotic cells by phagocytosis is also fundamental to avoid developing apoptotic-dependent pathologies[100]. Apoptosis is fundamental as controller of fidelity and quality of proliferation. While positive genetic mutations are the basis of evolution, cells with extensive genetic errors or cellular damage are subjected to apoptosis. Alteration in apoptosis have been associated with a wide spectrum of disorders including autoimmune, neurodegenerative, infectious, cardiovascular and oncological diseases. A deficiency in apoptosis may lead to non-controlled increase of the replicative ability of the cells and to cancer[102]. On the contrary, an increase of apoptosis is linked to certain pathological conditions. In AIDS, the human immunodeficiency virus infects the T cells of the immune system, which increases expression of FAS receptors and the induction of cell death[103]. In AD, PD and Huntington's disease (HD) an increase of neuronal apoptosis is described. For example Tau-mediated apoptosis induced by the exposure to toxic β-amyloid peptides characterizes AD[104]. To conclude, a dysregulation of the apoptotic pathway and impaired clearance of apoptotic cells are both linked to many human diseases, making them attractive targets for drug discovery[105]. As an example, Bcl-2 family members are intriguing drug targets because their abnormal expression can cause many diseases[106]. Some tumors, like Follicular Lymphoma, are associated to an increased expression of Bcl-2. Topoisomerase inhibitors, antimetabolic agents and alkylating agents are cytotoxic drugs used to induce apoptosis in tumor cells[104]. In contrast, blocking the apoptotic process is regarded as a possible approach to treat pathologies like AD, transplantation rejection or the consequences of cerebral ischemia[107].

Cellular senescence is another possible cell fate in the presence of unrepaired DNA damage. Senescence is a stable cell cycle arrest that occurs in diploid cells, normally subjected to different

stresses that limit their proliferative life span. The first description was done by Hayflick and Moorhead in 1960[108], when they observed that human fibroblast could reach a maximum number of cell divisions before arresting their growth. This phenomenon, known as "Hayflick limit", is caused by a progressive shortening of telomers upon each cell division. Indeed, it can be considered as a physiological response in order to prevent the accumulation of genomic instability and the accumulation of DNA damage[108]. This is known as replicative senescence. However, there is an other type of senescence which is unrelated from telomere shortening, known as premature senescence[109]. This senescence response is rapidly activated after a certain insult, such as genotoxic or metabolic shock. Despite the involvement in different pathological conditions, senescence is physiologically important during embryogenesis, tissue remodeling and repair[110]. The difficulties in studying cellular senescence are based on the fact that senescence is not characterized by universal and specific hallmarks, but rather by a number of non-exclusive biomarkers. The principal one is the cell cycle arrest, which correlates with increased amounts of cell cycle inhibitors, including p16INK4a, p21CIP1 and p27. Moreover, also p19ARF, p53 and PAI-1 are increased in senescent cells. Another well-characterized indicator of senescence is the increased activity of senescence-associated β-galactosidase (SA-β-gal) relating to decreased lysosomal functioning[111]. Other features associated with senescence are altered cell size with a more smoothed shape, heterochromatin foci formation, accumulation of lipofuscin, DNA damage foci, loss of lamin B1, distension of satellites, expression of embryonic chondrocyte-expressed 1 (DEC1) and decoy death receptor 2 (DCR2), upregulation of some microRNAs and secretion of a large number of factors including growth factors, cytokines, chemokines and proteases, known as senescence-associated secretory phenotype (SASP). Despite this large number of markers, the search for new ones that could have a prognostic potential in aging and cancer is rising[112]. One of the characteristic of senescence is the metabolism: the cells remain metabolically active and continue to produce and secrete a plethora of factors that can affect the microenvironment in different ways[113]. At the metabolic level, fundamental for induction of senescence is the depletion of the catabolic enzyme glycogen phosphorylase, which in turn leads to an accumulation of glycogen. Growing literature on the metabolic state of senescence also reports an increase both in glucose consumption and lactate production[114]. Aging is a process shared by multicellular organisms and it can be defined as a progressive loss in tissue and organ functions. All the senescence markers listed above also characterize aging-linked pathologies in mammals and indeed cellular senescence may play a critical role[115]. Recent data generated by several laboratories underline that in the human brain the presence of senescent cells, in particular non-neuronal cells, correlates with neurodegeneration. Astrocytes in the frontal cortex of AD patients express higher amounts of p16, γ-H2AX and other senescent markers compared to the age-matched control samples. Although their contribution to disease is disputed[116], the elimination of senescent cells is sufficient to delay cognitive impairments in a mouse model of tauopathy[117]. Senescent cells are thus a novel important target for pharmacological intervention in AD and other neurodegenerative diseases. Cellular senescence has also a fundamental role in different cancer stages such as tumor initiation, establishment and

escape[118]. Senescence stops proliferation and blocks the transition from benign to malign tumor, and it is thus considered a physiological tumor-suppressive mechanism, referred to as "oncogene-induced senescence" when activated e.g., by HER2, EGFR and PI3K[109]. Since DNA damage can induce apoptosis and senescence, many genotoxic drugs were developed for clinical use, such as docetaxel, bleomycin, doxorubicin, vincristine, etoposide and cisplatin[119,120]. However, SASP has a double-face antitumorigenic or tumor-promoting function. SASP can recruit and activate immune cells, whereby senescent tumor cells are then eliminated degraded by macrophages, natural killer cells, and T-lymphocytes. Alternatively, SASP can act detrimental when increasing the level of inflammation in the surrounding tissue[121]. For this reason, some groups are searching for senolytic drugs able to target and eliminate senescence cells as potential therapies for neurodegenerative disorders, some forms of cancer and other adverse human conditions.

1.2 The novel link between neurodegeneration and cancer

Although considered unrelated diseases, neurodegeneration and cancer share aging as a main risk factor. Cancer is characterized by hallmarks such as uncontrol proliferation, resistance to cell death, invasion and metastasis, possibly linked to altered cellular energy, genome instability and inflammation. Almost 20% of human cancers are caused by chronic inflammation related to infection, exposure to irritants, etc[122]. On the flip side, neurodegeneration is characterized by neuronal dysfunction and increased cell loss. These opposed hallmarks can reinforce the observation of the inverse comorbidities (***Figure 5***).

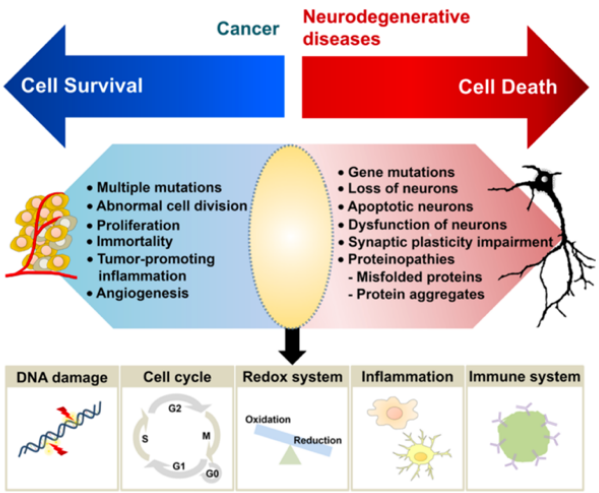

Fig.5 (Seo et al. Cellular and Molecular life sciences_2020)
Hallmarks of neurodegeneration and cancer diseases

Some examples of inverse comorbidity are known. A history of smoking related cancer has a protective impact against AD and the incidence of lung cancer is lower for AD patients[123]. For amyotrophic lateral sclerosis (ALS) , symptomatic patients are more protected against cancer, but cancer does not affect ALS onset[124]. However, a positive correlation is found between PD and melanoma[125] as well as cancer and stroke, osteoarthritis and non-neurodegenerative dementia.

The effects on comorbidity may be explained by the influence of environmental factors, drug treatments[126] or genetic variations[127]. Interestingly, genes up-regulated in cancer are often down-regulated in neurodegeneration and vice versa.

The shared functional pathways spark the interest upon a possible link between their etiopathogenesis and the properties of molecules involved. Recently, Ibanèz et al[128] confirmed this by a meta-analysis of microarray gene expression datasets comparing diseased and healthy control samples. The analysis was performed on three neurological disorders (AD, PD and schizophrenia) and three cancers (colorectal, prostate and lung). The hypothesis is that the expression of certain genes would impact in an opposite manner the risk to develop CNS disorders versus cancer. Indeed as an example the key regulator of cell division PIN1 protein is typically overexpressed in cancers and it is considered a therapeutic drug target. In contrast PIN1 is depleted in AD and mouse knocked-out for this gene presents neurodegenerative phenotypes. Overall, 74 genes were found to be simultaneously downregulated in three CNS disorders and upregulated in three cancer types. Some of these genes are involved in lipid biogenesis such as *ACLY* and *MECR* and other are transcription factors such as *NME2* and *TFCP2*, for which a genetic association with AD is debated. Conversely, 19 genes were found upregulated in the three cancer types and downregulated in neurodegeneration. Among these genes there are many involved in the signal transduction like *TNFRSF1A*, *CDKN1A*, *NFKBIA*, *PTH1R*. Interestingly, some genetic components modulate the risk in the same direction. One example is *ATM*, important for DNA repair. *ATM* mutation causes ataxia-telangiectasia, a neurodegenerative disorder and, in the meantime, it is associated with a high risk of cancer[129].

Notable in the context of my thesis are also the data obtained by the pathway analysis by GSEA, in particular regarding the P53 signaling pathway[128]. P53 is well-known to be de-regulated in cancer, however recent studies reported a role of P53 also in CNS disorders. In contrast to cancer, in neurodegeneration the level and activity of P53 are increased[130]. In the AD brain, increased P53 is associated with an increased level of apoptosis detected by P53-dependent apoptosis-related proteins such as Bcl-2 and Caspase-3[131]. Even if genetic mutations of P53 in neurodegeneration have not been reported, some functionally compromised variants of P53 or with aberrant conformations have been observed in AD samples. SH-SY5Y cells overexpressing APP present unfolded P53, which is associated with a lack of P53 pro-apoptotic activity and an impairment in responding to cytotoxic injury[132]. However, a defective P53 pathway, would also lead to the accumulation of potentially pathogenic DNA damages, which may result in a dysfunctional neuron. Beside the action of the molecules and genes in investigating the relationship between the two pathologies, recent studies have shed light on another important point: the crosstalk between cancer and neuronal cells in the brain. Malignant glioma cells can secrete an excess of glutamate, which generates a toxic microenvironment with the consequent excitotoxicity, cell death and neurodegeneration. This effect can be reduced by blocking the NMDA glutamate receptor with antagonists like memantine. These findings are further supportive of a positive correlation between brain tumors and neurodegeneration[133]. An additional therapeutic intervention could target the

TIAF1/WWOX/P53 axis, with proteins involved in both cancer and AD. Remarkably, this triad is found to suppress cancer progression, and at the same time it causes protein aggregation and neurodegeneration, due to the functional antagonism between P53 and WWOX[134]. As discussed previously, mutations in the *MAPT* gene encoding for the protein Tau can cause inherited FTDP-17. However, *MAPT* mutations may also increase the risk of cancer, Cimini et al[135] reported evidences that lymphoblastoid cell lines from patients bringing P301L Tau mutation shown less P53 stabilization and less apoptosis, leading to an increase of cancer aggressiveness. Tau is also recognized as a prognostic marker of cancer and a modifier of some chemotherapeutics drugs. In fact, thanks to its ability to bind microtubules, Tau can compete with chemotherapeutics such as Taxanes, which block cell division by binding to tubulin. This is confirmed by studies showing that when Tau is less expressed, the response of the cells to Taxanes is improved; in particular in ovarian, gastric, prostate and non-small cell lung cancer[136]. Overall, a positive correlation between Tau expression and survival is found in breast, kidney, lung carcinoma as well as in pediatric neuroblastoma and glioma. Inversely, a negative correlation regards ovarian, prostate and colorectal cancer[136].

Further in-depth investigations into the cellular and molecular mechanisms related to inverse or direct correlation between cancer and neurodegeneration together with epidemiological studies for gene mutations, will assist in the development of additional biomarkers and new therapeutics. Despite the great social impact of these studies, the unresolved challenge lies in understanding the biology behind the fact that the comorbidity is not general between these two illnesses, but it varies depending on the type of neurodegenerative disorder or of cancer.

1.3 The DNA Damage Response (DDR) Pathway

To date it is not known if the process of aging is due to a unique causal mechanism or is grounded in multiple sources. However, there is strong evidence that DNA damage affects most, if not all, aspects of the aging phenotype, making it a potentially primary driver. It has been already known since 1940 that rodents exposed to irradiation exhibit multiple symptoms of premature aging[137] and the first proposal that DNA damage can be the driver of cellular aging was reported in 1960[138]. This also suggest the possibility of pharmaceutical intervention to influence aging by targeting the molecular mechanisms regulating DNA damage and repair. We are continuously exposed to environmental stress factors. It is estimated that up to 10^5 DNA lesions occur in an active mammalian cell on a daily basis[139]. DNA damage can result from exogenous insults such as UV radiation from the sun, chemicals, X-rays, chemotherapy or from endogenous ones like oxygen radicals, advanced glycation end products, aldehydes, etc. Damage to the DNA can have several consequences: genome instability, telomere dysfunction, epigenetic alterations and compromised mitochondrial function. An elaborated network of highly sophisticated DNA damage response (DDR) and DNA repair system counteract the time- and exposure-dependent erosion of the genetic information arising when DNA lesions cannot be repaired and accumulate[140]. Cells carrying unresolved DNA lesions have to be somehow eliminated in order to protect the organism, and this

occurs as discussed above through apoptosis or senescence. The molecular mechanisms orchestrating and executing the DDR are thus crucial in health and disease.

1.3.1 DDR cascade

The DDR is an evolutionary conserved signaling cascade that senses and responds to DNA double-strand breaks by organizing down-stream cellular events, spanning from cell cycle arrest to damage repair or cell disposal. In higher organisms, the DDR prevents neoplastic transformation by preserving the information contained in the genome, by regulating cell fate decisions, and by ensuring the removal of irreversibly damaged cells. In the recent years, a paracrine element induced by the DDR was also described. These extracellular DDR signals can be subdivided in two types: those that act rapidly within few hours from the damage and those, such as the senescence-associated secretory phenotype, which occurs after multiple days[141]. Overall, DDR-mediated extracellular signals are fundamental in order to protect the surrounding tissue, to boost the local immune response, and as positive feedback into the senescent cells. As already highlighted, the DDR is essential in the maintenance of genome stability, via the initiation and coordination of DNA repair mechanisms with appropriate cell cycle arrest checkpoints[142]. The responses of a cell vary among the different classes of DNA lesions. However, they occur generally by a common program. Some lesions are subjected to direct protein-mediated repair, whereas the majority are repaired by a sequence of catalytic events mediated by multiple proteins. Histone modifications and chromatin remodeling are essential steps in DNA repair[143]. One important example is the role of histone H2AX. ATM/ATR and DNAPK phosphorylate H2AX at Ser 139 at the chromatin damage site. This modification is essential for the recruitment of additional DDR factors and chromatin-modifying components, as shown in ***Figure 6***. Key elements of DDR in mammalian cells are the protein kinases ATM and ATR, which are recruited and activated at the site of the DNA damage[141]. ATM and ATR are known as transducers in the DDR cascade. The best described target of ATM and ATR are the protein kinases CHK1 and CHK2. These kinases, together with ATM and ATR, are the transducers responsible to inhibit cyclin-dependent kinases (CDKs) by various mechanisms, some of which are mediated by activation of the P53 transcription factor[144]. By blocking CDKs, the cell is able to slow down or arrest the cell cycle in G1-S phase, in turn this is important to increase the time available for DNA repair before the DNA replication re-start. In the meantime, ATM and ATR are also responsible for the recruitment of DNA-repair proteins at transcriptional and post-transcriptional level. The activation of DNA-repair proteins occurs by modulating their PTMs, like phosphorylation, SUMOylation, acetylation or ubiquitylation[145].

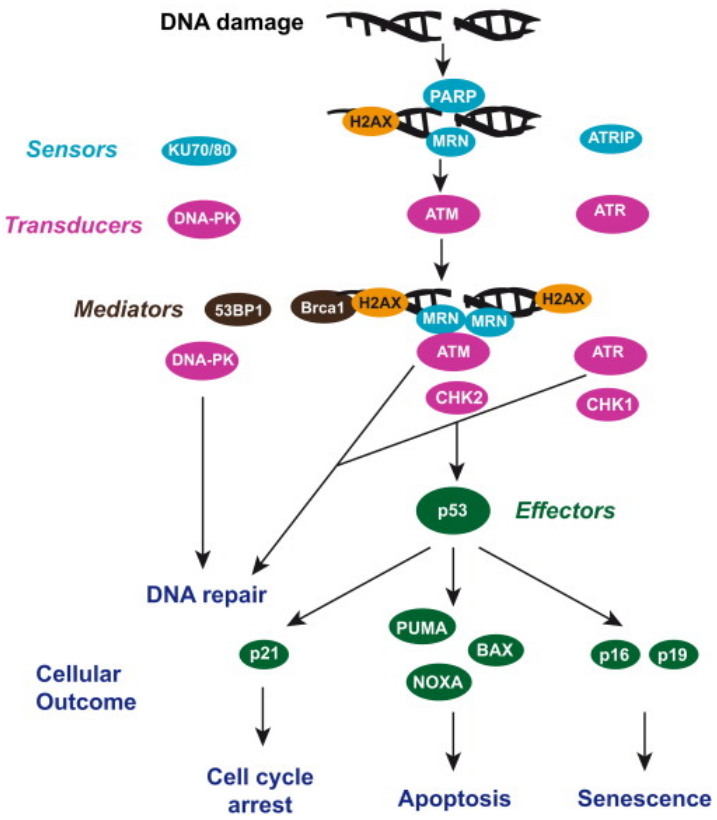

Fig.6 (Blanpain et al. Cell_2010)

Model of DDR cascade

A successful DNA repair induces the cell to re-enter in a normal cell cycle. In contrast, an irreparable DNA damage and the chronic DDR signaling trigger a permanent cell cycle arrest (senescence) or a programmed cell death (apoptosis). The DDR is influenced by different DNA insults and by the cell type. Indeed, under physiological conditions, some DNA-repair pathways are downregulated upon cellular differentiation. Moreover, also non-dividing cells have different DDR signals. For example, in terminally differentiated neurons and macrophages a new type of repair is described, termed differentiation-associated repair, in which both transcribed and not-transcribed DNA strands are repaired but not-transcribed loci are repaired poorly or not[146]. On the other hand, stem cells, because of their importance for tissue homeostasis and renewal, rely heavily on the DDR. We can conclude that a tight regulation of the DNA damage pathway within the cells is necessary, and its deregulation can cause many adverse human conditions. The main cause of cancer is genome instability[147]. For example, mismatch repair defects cause microsatellite instability that predisposes to colorectal and endometrial carcinomas[148]. Furthermore, chromosomal instability is observed in most sporadic solid tumors, where it can be linked to the shortening of the telomers[149]. At later stage of cancer progression, chronic hypoxia might contribute to deregulate the DDR pathway and to genomic instability[150]. Aberrant cell proliferation, the main hallmark of cancer, can also activate the DDR. Notably, the DDR has a protective role when activated in the early stage of neoplastic regions[151]. Some evidence exists for a link between neurodegeneration and DDR. The first cause is linked to the high use of mitochondrial respiration by the neurons, which in turn

produced oxygen-species that can damage the mitochondrial and nuclear DNA[152]. Another reason why the DNA damage is so diffuse in the CNS is the limited capacity to replace the terminally differentiated neurons in adulthood. The accumulation of DNA damage in both cancer and neurodegeneration, in addition to other disorders, increase the interest to study the differences of the DDR between disease and healthy cells to develop specific DNA-damaging therapies. Specific markers of an active DDR may also find use for early diagnosis. In cancer, the mode of action of radiotherapy and chemotherapy is to generate irreversible DNA damage to actively dividing cells. Nevertheless, the very effective DNA repair mechanisms in glioma stem cells render them less sensitive to radiation treatment[153]. In contrast, many cancer cells show impairment of at least one step of the DDR, so that its ineffectiveness correlates positively with therapeutic outcome. For this reason, another paradigmatic approach targets the inhibition of the DDR. Examples are early DDR inhibitors, such as CHK2 inhibitors in pre-clinical and clinical development; or CHK1 inhibitors that sensitize tumorigenic P53-deficient cells more than P53-normal cells[154]. Due to the difference in the DNA repair pathway between cancer and healthy cells, targeting the DDR can have a greater impact on the cancer than on normal tissue. Drugs targeting the enzyme PARP1, which binds the site of damaged DNA helping the repair processes, are non-toxic for normal cells but strikingly cytotoxic towards cancer cells, in particular in BRCA1 and BRCA2 breast cancers[155]. Great progress has been made towards understanding the DDR mechanisms and its functionality in therapeutic development. Not surprisingly, P53 as the main coordinator of the DDR has been studied extensively for its implication in health and disease. I will review its role in neurodegeneration and cancer.

1.3.2 P53 protein: a stern monitor of the cell

The human family of P53-like transcription factors is encoded by three genes: *TP53*, *TP63* and *TP73*. The structure of P53, P63 and P73 proteins is similar. All three have a DNA binding, transcriptional activation domain, with differences in terms of size, structure, and function in the C-terminal region. However all three proteins regulate DNA binding and transcription, and mediate intra- and inter- protein functional interactions[156]. P53 protein was discovered during the peak of tumor virus research as a 53 kDa host protein bound to simian virus 40 large T antigen in viral transformed cells[157]. P53, encoded by the *TP53* gene on chromosome 17, was first described as an oncogene until subsequent works established that P53 protein suppresses growth and oncogenic transformation. In fact, somatic loss-of-function *TP53* mutations are common in human tumors[158]. Mutant *TP53* causes a hereditary dominant autosomal cancer predisposition disorder known as Li-Fraumeni Syndrome. Moreover, P53 knock-out mice develop tumors with a high penetrance[159]. The best understood function of P53 in case of DNA damage consists in promoting cell cycle arrest and inducing the DNA repair machinery. In case of irreversible DNA damage, the cell has to be somehow eliminated and then P53 acts through the activation of the Bcl-2 family members to induce apoptosis, or though P21 to promote senescence, a permanent state of cell cycle arrest. P53 is a 393 amino acid-long DNA binding protein mainly involved in upregulating transcription. Starting from

the N-terminus part of the protein four functional regions are distinguished: the transactivation domain, the proline rich domain, the core domain for DNA binding, and the C-terminal domain encoding its nuclear localization signal and an oligomerization domain needed for transcriptional activity (***Figure 7***).

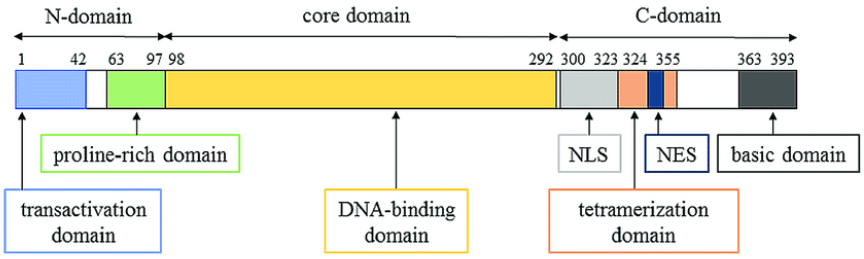

Fig.7 (Tanaka et al. Oncotarget_2018)

Structure of P53 protein

The majority of cancer-associated *TP53* mutations occur in the DNA binding domain. In normal conditions, constitutively transcribed P53 protein is maintained at very low amounts within the cells by several proteins, the main one is the E3 protein ubiquitin ligase MDM2[160]. P53 is stabilized in response to various cellular stress signals, including DNA damage or replication stress induced by deregulated oncogenes. P53 stabilization can result directly by modifying P53 phosphorylation status e.g., in case of DNA damage[161]; or through inhibiting its regulators e.g., with oncogene-mediated induction of the ARF tumor suppressor that inhibits MDM2[162]. Thus, the "guardian of the genome" P53 has the precise role to maintain the genomic integrity of the cell. P53 functions as a tumor suppressor protein in two manners. On one side, P53 can directly eliminate cells carrying DNA damage-mediated oncogenic mutations. In this model, P53 loss promotes cancer by the inability of the cell to reduce the number of mutations[163]. On the other side, P53 can limit the consequences of aberrant oncogene expression that impairs MDM2 activity. In this case, P53 loss enables oncogene-expressing cells to proliferate unabated[164]. An unanticipated way in which P53 maintains this stability is by repressing retrotransposons, which are latent virus-derived genetic elements whose mobility and re-insertion within the genome can lead to mutagenesis [165]. Still, P53 loss with the consequent increase of genome instability leads to the acquisition of additional drivers events, which can accelerate transformation, metastasis and drug resistance[166].

Recently P53 was described to control also "non canonical" programs. As examples, P53 modulates autophagy, alters the cell metabolism, represses pluripotency and cellular plasticity and facilitates an iron-dependent cell death known as ferroptosis[167]. Even at basal level P53 can reinforce multiple other tumor suppressive networks, binding and activating different tumor suppressive genes like *PTEN*, *STK11*, *miR34a*, *FOXO1*, *PHLDA3* and *TNFRSF10B*[168]. An important point to take in consideration is that P53 response is flexible depending on cell type, differentiation state, stress conditions and environmental signals. An example of its flexibility can be done with the non-canonical function of P53 in controlling cellular metabolism. In breast and lung cancer cells,

P53 inhibits glycolysis by attenuating glucose uptake or repressing the glycolytic enzymes. By contrast, in muscle cells P53 induces glycolytic enzymes[169].

While it is often assumed that each P53 effector function is a standalone process, there are now increasing evidences that the existence of a crosstalk between them is fundamental for the success of the process. For example, Young et al[170] highlighted the importance of autophagy activation in the P53-driven cellular senescence. Moreover, some processes can be mutually exclusive to others, as autophagy is described to delay apoptosis by reducing the levels of PUMA[171]. The multifaced functions of P53 are also due to its PTMs; pSer46 or acLys120 stimulate apoptosis[172,173], whereas methylated-P53 by PRMT5 activates P21 and senescence[174]. Collectively, we can conclude that P53 response is not an "on-off" switch; instead, the cellular context is central for both the biochemical activity and the biological outcome of P53.

As mentioned previously, half of human cancers contain mutations in the *TP53* gene, which occur prevalently in the core domain where there is the DNA binding domain. However, the frequency and the distribution of the mutations can vary dramatically between tumor types. As an example, prostate cancer shows few mutations, mainly in the DNA binding domain[175]. On the opposite, head, breast and lung cancer show a high number of mutations distributed along the whole P53 protein, from the transactivation domain to the oligomerization domain[175].

Since P53 plays such a role in tumor suppression and it is highly mutated in carcinogenesis, it appears attractive to search for specific drugs targeting P53 to improve its stability. Nonetheless, this turned out a real challenge and the most advanced efforts have been done by an indirect approach, for example by blocking MDM2. The nutlins are small molecules binding within the MDM2 pocket that recognizes P53, and thus they are able to inhibit its subsequent degradation[176]. Different phase I trials for MDM2 antagonists have been completed for leukemia and liposarcoma, however they give a dose-limiting toxicity. Also, some attempts have been done to increase P53 gene expression[177]. Interesting is the alternative use of MDM2 inhibitors, whereby the term "cyclotherapy" describes their use to stabilize P53 and transiently arrest the cell cycle in normal cells. This allows the use of a higher dose because the normal cells are protected from chemotherapeutics, which in contrast attack dividing cells[176]. Other interesting drugs are designed to cause P53 mutants to regain a sufficient activity for tumor suppression. The most promising ones are the metallochaperones, which allow the reincorporation of the zinc into the unfolded P53, thereby bringing back the normal conformation of the protein and its ability to bind to DNA[178]. Finally, another approach is to target down-stream mechanisms through which P53 promotes invasion, metastasis and tumorigenesis, among which there are HMG CoA reductase, EGFR or PDGFRb inhibitors[179].

Overall, the pharmacological manipulation of the P53 axis carries many risks. Evolution has selected a precise balance of P53 activity, since a too small amount leads to the onset of cancer and excess P53 promotes aging. Increased P53-dependent apoptosis may drive developmental brain disorders or aging-associated neurodegenerative disorders[180]. Mouse models have revealed the centrality and complexity of the P53 network in aging. The aging phenotype can be due to a

dysregulation of MDM2 or chronic stress that causes a stable activation of P53. As example, in naturally aged mouse, longevity is linked with declining P53 functions, whereas increased P53 activity from reduced MDM2 antagonistic function directly accelerates aging[181]. At cellular level, P53 can mediate irreversible growth arrest and senescence causing a "pathological secretion phenotype" contributing to aging[182]. At the same time, the negative regulation of AKT signaling by P53 provides an anti-aging role[183]. Similarly, it has been suggested that P53 is a negative regulator of inflammation, thanks to its antagonism with NF-kB[183]. In an apoE null background, mice without P53 are more prone to accumulate atherosclerotic plaques. In fact, 25% of mice without P53 died before tumor development because of the excessive invasion of active macrophages into plaques, which resulted in an uncontrolled proliferation[184]. In AD, the association between P53 and β-amyloid peptide may explain cell death and high P53 is detected in the frontal cortex[185]. Interestingly, human neurons treated with β-amyloid peptides show decreased Bcl-2, counterbalanced by increased Bax, suggesting a deregulation of P53[186]. This was confirmed by the use of the P53 inhibitor Pifithrin (PFTα), which reduced cell death in cortical neurons by a mechanism of abolished lysosomal branch of apoptotic cascade and the activation of proapoptotic caspases[187]. In the context of PD, P53 KO mice treated with PFTα were protected from cell death of dopaminergic neurons in the substantia nigra pars compacta region of the brain[188]. Finally, a role of P53 was also highlighted in the mitochondrial-associated cellular dysfunction of HD. Mutant huntingtin increases P53 and its transcriptional activity in the nucleus of affected cells, whereas PFTα decreased cytotoxicity in HD cells[189]. Pinpointing which P53 stressors and modifications link P53 to aging will provide further insights in the understanding of human aging processes. Notably the anti-cancer MDM2 inhibitors Nutlin-3 and RITA are associated with an aberrant P53 activation and an alteration of the signaling pathways leading to aging[190]. Also, the ATM blocker KU-60019 preserves low P53 expression and is an inhibitor of ATM-driven senescence[191]. To conclude, P53 activating therapeutics to fight cancer present surprisingly and exciting implications in aging[181].

1.3.3 The P53 influencers: MDM2 and MDM4 lead the way

P53 activity must be strictly regulated to maintain tissue homeostasis. The two main regulators of P53 function are MDM2 and MDM4. The MDM2 (murine double minute 2) protein, also known in human as HDM2, was first identified as a product of a gene amplified over 50-fold on acentromeric extrachromosomal bodies (called in fact "double minutes") found in a 3T3DM spontaneously transformed mouse cell lines[192]. MDM2 protein's structure is composed mainly by three domains. I) The P53-binding domain at the N-terminus of the protein characterized by a deep pocket with high affinity for P53. II) The central domain contains the acidic/zinc domain rich in negative charges and subjected to extensive modifications regulating protein-protein interaction, including with P53 and partners required for poly-ubiquitination of P53. This domain carries also a nuclear translocation signal, which regulates the shuttling between nucleus and cytoplasm and the primary localization in the nucleus. III) The RING domain (Really Interesting New Gene) of the C2H2C4 type. The C2H2C4 designation indicates the order of cysteine and histidine residues

required to form a Zn^{2+} chelating structure, determinant for RING functions. As others RING proteins, MDM2 has an intrinsic E3 protein ubiquitin ligase activity, by which it promotes the transfer of ubiquitin molecules from an E2 conjugating enzyme directly to lysin residue of a target substrate. Moreover, the RING domain of MDM2 is involved in protein-protein interactions and in the formation of the homo- or hetero-dimer with its partner MDM4.

MDM4 (also known in human as HDMX or MDMX), is homolog to MDM2 at 90%, and it was the first one isolated as a P53-binding partner from a mouse cDNA library. The MDM4 structure is like the one of MDM2, except for notable differences. Firstly MDM4 is not sensitive to nutlins; in addition the central domain shows the lowest homology and it lacks the nuclear localization signals causing MDM4 to be primary localized in the cytoplasm if not bound to MDM2; the RING domain stabilizes MDM2 and it is required for its activity but despite the C2H2C4 structure, its ability to ubiquitinate P53 is limited[193] (***Figure 8***).

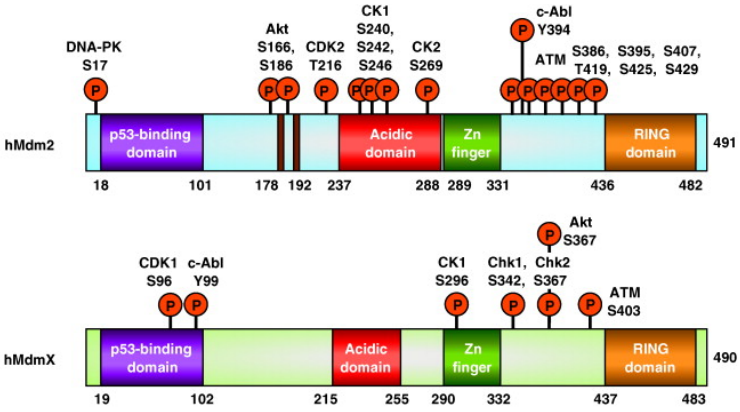

Fig.8 (Wade et al. Trends in Cell Biology _2010)

Structure of MDM2/MDM4 proteins

Transcription of MDM2 is controlled mainly by two promoters: P1 and P2. P1 provides a basal level of transcription, whereas the activity of P2 is induced by several transcription factors, including P53[194]. MDM2 transcription is governed also by a third promoter, P3, which has not been widely studied yet but appears independent to P53. This complex regulation underlines the difficulty in drug-design to target and regulate MDM2. MDM4 transcription is less understood but also based on two promoters P1 and P2, and the control of P53. Human *MDM2* is described to have 72 alternative spliced variants, although not ubiquitous and not all result in protein products. The three main isoforms are: MDM2-A, MDM2-B and MDM2-C[195]. *MDM4* generates the full-length protein and six variants: MDM4-S, MDM4-211, MDM4-G, MDM4-A, MDM4-XAlt1 and MDM4-XAlt2[196]. Due to all these variants and instability of the protein, one interesting target can be the modulation of transcript stability through microRNAs. At least 15 negative microRNA regulators were described for MDM2 and six for MDM4[197].

Given their importance in restraining P53 and possibly other roles in cells, it is not surprising that MDM2/4 are themselves heavily regulated by PTMs and protein-protein interactions. MDM2 ubiquitinates several targets, including itself and its partner MDM4. However, several others E3 ligases were shown to ubiquitinate MDM2/4 like PCAF, APC/C, NEDD4-1, and MARCH7[198]. As in

most cases, ubiquitination may serve to drive proteasomal degradation (polyubiquitination at Lys48[199]) or for other functions (stabilization at Lys63 though monoubiquitylation by NEDD4-1 and MARCH7[198]). MDM2 inhibits P53 activity through three mechanisms: I) polyubiquitination and tethering of P53 to the proteasome, II) competition for the binding of P53 to DNA, rendering P53 inactive as transcription factor, III) translocation of P53 to the cytosol[200]. Interestingly, although MDM2 is the main driver of P53 cytosolic export through a leucin-rich signal, MDM4 may modulate this process[201]. Conversely, P53 activation can be enhanced by increasing the nuclear import[202]. Recently it has been shown that the nuclear import of P53 is mediated by the binding to the importin-3 adapter and this function is negatively regulated by ubiquitination. A nuclear localization signal rich in positively charged Lys residues is located at amino acids 305-322, the same region targeted for MDM2-dependent ubiquitination[203]. Interesting is a role of MDM4 in translocating mono-ubiquitinated P53 into the mitochondria, where it activates transcription-independent apoptosis[204]. MDM2 is also subjected to a positive and negative feedback loop. In fact, MDM2/4 are degraded when complexed with P53 and as a consequence they are absent when it is necessary to activate P53 after stress[205]. Whilst MDM4 exists also as monomer, MDM2 forms homo- or hetero-dimer together with MDM4 via the RING/RING interaction. It is observed, in the absence of stress, that the hetero-dimer is more efficient in targeting P53 for ubiquitination and degradation[206]. However, Cheng et colleagues[207] recently found that the regulation of MDM2 oligomerization is important for P53 stabilization following DNA damage. A mechanism regulated by kinases like ATM that acts by phosphorylating its RING domain at Ser429, thereby enhancing the E3 ligase activity of the MDM2 homodimer without affecting the hetero-dimer[208]. Moreover, the phosphorylation of MDM2 leads to its inability to degrade MDM4, limiting the full activation of P53[209]. Tissue-specific regulation of MDM2/4 activities exist. For example in the nervous system and proliferative intestinal cells both MDM2 and MDM4 are required[210]. In contrast, in smooth muscle cells, MDM2 is able to control P53 without the participation of MDM4[211]. The opposite is not possible, because MDM4 alone cannot compensate the lack of MDM2, which leads inevitably to embryonic lethality[212]. In the absence of exogenous stress there is a balanced turnover of MDM2, MDM4 and P53, where MDM4 seems the more stable of the three[213]. Phosphorylation of MDM2/4 is extensively studied. MDM2 is known to possess more than 20 phospho-sites, whereas MDM4 has six sites[209]. In particular phosphorylation of MDM2/4 is important during the DDR by the action of ATM, Chk1, Chk2, DNA-PK and c-Abl, when it regulates the enzymatic activity, subcellular localization, protein stability and both protein-protein and protein-mRNA interactions. ATM phosphorylates at Ser395 and 407 in MDM2 and at Ser342, -367 and -403 in MDM4. This brings to destabilization of the homo-/hetero-dimer and to the detachment/activation of P53[207]. This effect is reversed after DNA repair by the Wip1 phosphatase that acts on the ATM phospho sites[214]. CK-1 can also phosphorylate MDM2/4 to block P53 degradation and can increase the interaction between MDM4 and P53[215]. In contrast, following growth factor stimulation, Akt phosphorylates MDM2 at Ser166 and -186, to increase MDM2-dependent P53 degradation. Akt is also known to increase MDM2 translocation within the nucleus, with a P53 antagonistic action. Also glycogen

synthase kinase-3 (GSK3) is reported to inhibit P53 by enhancing MDM2-dependent degradation[216]. This evidence highlights that MDM2/4 are critical nodes of multiple cell-signaling pathways. Recent studies have challenged the concept that phosphorylation at the N-terminus of P53 is enough to block the binding to MDM2/4. A crucial determinant seems to be the molecular ratio of MDM2, MDM4 and P53 as shown by *in vivo* and *in vitro* data[217]. Subtle changes in the stoichiometry of P53 and its negative regulators are reported to affect P53 functions in tumor suppression. For example, a polymorphism mediated two-fold increase in MDM2 reduces P53 activation enough to increase the risk of hormone-dependent breast cancer, with also an impressive reduction in the age of onset[218]. Although MDM2/4 acetylation is less well characterized, it has been reported that p300 can acetylate MDM2 at K182 and K185, leading to its stabilization through the inhibition of autoubiquitination[219].

Genetic analysis demonstrates that the major cellular target for MDM2/4 is P53. However, both E3 protein ubiquitin ligases interact with many other targets involved for example in development, morphogenesis and tumorigenesis. Numb was recently described to bind MDM2 and activate P53, which is consistent with reports that shown Numb as a tumor suppressor[220]. In contrast, Notch1, thanks to its ability to enhance MDM2 activity, and its consequent inhibition of P53 function, is considered as an oncogene[221]. Overexpressed MDM2 binds a variety of RNA molecules, including the P53 mRNA, affecting their translation into proteins[209]. MDM2 contributes to stemness and chromatin modifications by acting on H2A K119ub1 and H3K27me3 in cells lacking P53, and again in these cells MDM2 is shown to support the progression of DNA replication forks[222].

The involvement of MDM2 in promoting or preventing tumor development is of big interest. Differently from P53 knock-out mice, mice knocked-out for MDM2 display exaggerated P53 activity and embryonic lethality, which is rescued by P53 depletion, but this may mask any P53-independent contribution of *MDM2*[223]. Whereas P53 loss-of-function contributes to roughly one out of two cancers, a gain in MDM2 activity is less common but it still occurs in 7% of human cancers. Only a subset of sarcomas is caused by *MDM2* amplification on a regular basis. Interestingly, 10% of glioblastoma and invasive breast carcinomas show an amplification of *MDM4* and not *MDM2*, but then again this could lead to increased suppression of P53 because the two proteins are more active as a hetero-dimer. Pathologically, MDM2 overexpression has been correlated with poor clinical prognosis and poor response to cancer therapy[224]. To conclude, it is proved that MDM2/4 play a role in cancer by modulating the oncosuppressor P53, although P53-independent mechanisms are not excluded.

The function of MDM2/4 in regulating P53 may also impact cellular senescence and aging. As mentioned previously evidence for oncogene induced senescence exists[109], whereby oncogenes activate ARF protein, which by binding to MDM2 it interferes with the E3 ligase activity against P53. All these types of senescence have in common the SASP, which create an inflammatory response in the surrounding tissue. The gradual decline in the efficiency to eliminate senescent cells during aging contributes to an increased SASP-mediatory chronic inflammation as a cause of aging. Indeed, in mice the senolytic drugs Dasatinib and Quercetin preferentially target senescence cells, reduce

SASP, slow down physical dysfunction resulting in 36% increased lifespan compared to untreated mice[225]. A conclusion from these observations is that the alteration in MDM2/4 may lead to an overactive P53 pathway, which prevents cancer but accelerates the aging process, which is a main risk factor for neurodegenerative disorders. An elegant study by Lessel et al[226] combined up-to-date genetic approaches including genome-wide linkage mapping and sequencing with in-depth functional characterization of patient-derived primary cell lines with in parallel *in vitro* and *in vivo* analysis. This led to the discovery of a homozygous germline antiterminating *MDM2* mutation linked to aging. Somehow similar, a germline mutation in *MDM4* triggers shortened telomers and increased senescence as well as the bone marrow cancer Dyskeratosis congenita both in human and mice[227].

In terms of therapeutic interventions against MDM2, the hope started to rise with the discovery twenty years ago of the nutlins[228], which were expected to target at least50% of tumors that present wild-type P53. However so far, no FDA approval has been reached for MDM2 antagonists. Liposarcoma was a preferred cancer to test MDM2 inhibitors since 90% of the cases are due to *MDM2* amplifications. However insufficient cancer cell elimination and the occurrence of P53 mutations hampered the clinical success. Oral treatment with RG7112, the first MDM2 inhibitor entering clinical trials, showed the expected activation of P53, increased P21 and apoptosis resulting in anti-tumor activity in 14 out of 20 Liposarcoma patients[229]. However, all treated patients suffered of at least one adverse effect, with 8 patients showing 12 serious adverse events in among which neutropenia and thrombocytopenia, signs of hematological toxicity[229]. More recently proteolysis-targeting chimera (PROTAC) drugs against MDM2 were reported to interfere with MDM2 activities aimed to block cell proliferation[230]. Interestingly, depletion of MDM4 appears to interfere with the proliferation of breast cancer cells carrying mutant P53 through the activation of the CDK inhibitor P27/CDKN1B[231]. This argues in favor of MDM4 presenting a druggable P53-independent cancer promoting activity. As highlighted above targeting the MDM2-P53 axis to block the cell cycle of normal cells, is a strategy aimed at increasing their resistance to chemotoxins. Pharmacological intervention on MDM2/4 may target P53-independent activities mediating cell life and death[232]. Indeed, in P53 negative or mutant mouse lymphoma and sarcoma cells, as well as human breast cancer cells, ablation of MDM2 reduces mitosis by a G2 arrest, although the mechanism of action is still unknown[233]. MDM2 ubiquitination of the HBP1 transcription factor targets it for degradation, thereby delaying DNA damage repair and enhancing tumorigenesis[234]. MDM2-mediated degradation of HER2 induces chemotherapeutic resistance to the HER2 inhibitor Lapatinib, displaying a P53-independent oncogenic property of MDM2[235]. MDM2/4 promote DNA compaction through the stabilization of histone deacetylase and the association with the Polycomb Repressive Complex 2 (PRC2), which results in both histone trimethylation and mono-ubiquitination[236]. MDM2 appears to increase DNA accessibility via degradation of the major methyltransferase suppressor variegation 3-9 homolog 1 (SUV39H1)[237]. Whereas MDM4 is known to have anti-tumor effects in thymus and breast cancer in cells lacking P53[238]. Despite the remarkable opportunity to respond to many medical needs, the development of drugs targeting

MDM2/4 is jeopardized by the broad variety of their biological activities both in terms of P53 modulation and in the context of P53-independent molecular pathways involved in cancer, aging and neurodegenerative disorders.

2. Project Hypothesis & Study Design

The hypothesis of my project is that a loss-of-function of Tau may contribute to human aging-associated diseases.

The experimental approach I selected to assess this hypothesis is to prove the existence of a Tau dependent response in cells exposed to a toxic insult and, in case of a positive outcome, to elucidate the molecular mechanisms regulating this cellular response. For this purpose, I analyzed the phenotype of Tau-deprived cells when exposed to a genotoxic pharmacological treatment.

The accumulation of DNA damage contributes to aging and represents a risk factor for aging-associated diseases like cancer and neurodegeneration. With the development of different cellular models and technologies, I first demonstrated a role of Tau in regulating the fate of cells subjected to a chemotoxic insult and I reported that this new function of Tau occurred through the modulation of P53. In its function as the guardian of the genome, P53 is deeply involved in the pathogenesis of cancer, whereas its role in aging and neurodegeneration is just emerging. Following this discovery, I then obtained evidence of a direct interaction between Tau and MDM2, the E3 protein ubiquitin ligase that predominantly regulates the stabilization and activity of P53. Notably, I also showed abnormal amounts of MDM2 co-localizing with Tau lesions in the Alzheimer's disease brain. In the last part of my project, I started to define the molecular determinants for the interaction between Tau and MDM2, which may facilitate the discovery of experimental drugs targeting this protein-protein interaction that has obvious implications in major aging-associated human disorders and unmet medical needs.

I divided the description of my data in two sections reflecting the main project outcomes:

A. Tau-dependent, P53-driven regulation of fate decisions in cells exposed to a DNA damage
B. Description of a Tau-MDM2 complex as a molecular mechanism of this new Tau function

3. Results

3.1 Tau affects P53 function and cell fate during the DNA damage response

It is widely accepted that the pathogenic role of Tau in neurodegeneration is associated to a gain-of-toxic-function, characterized by self-oligomerization, brain deposition in fibrils composing NFTs and neuropil threads, and destabilization of microtubules. However, the physiological function of Tau may go beyond that linked to its ability to bind microtubules as suggested e.g., by its nuclear localization where it binds to DNA[239]. Indeed, nuclear Tau has a role in DNA protection[68], and its nuclear translocation increases in the presence of a DNA damage[61]. The emerging function of Tau in genomic stability suggests an additional loss-of-function contribution of Tau to disease. As a follow up study to a project that investigated in our laboratory nuclear translocation of Tau[61], in this part of my thesis I investigated the possible contribution of Tau in modulating the DDR.

To perform my studies, I generated neuroblastoma cell lines knocked-out or knocked-down for Tau that I subjected to an acute DNA damage. Then I carefully analyzed the cell phenotype of Tau-depleted cells when compared to cells expressing Tau, as well as key proteins involved in the DDR. These studies have revealed an altered Tau-dependent P53 response to stress, which resulted in reduced cell death and increased cellular senescence in absence of Tau.

This study allowed to demonstrate a new function of Tau as a modulator of the "guardian of the genome" P53, which may have implication for P53 as a modifier of neurodegenerative tauopathies as well as a role of Tau in cancer.

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Tau affects P53 function and cell fate during the DNA damage response

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Cells are constantly exposed to DNA damaging insults. To protect the organism, cells developed a complex molecular response coordinated by P53, the master regulator of DNA repair, cell division and cell fate. DNA damage accumulation and abnormal cell fate decision may represent a pathomechanism shared by aging-associated disorders such as cancer and neurodegeneration. Here, we examined this hypothesis in the context of tauopathies, a neurodegenerative disorder group characterized by Tau protein deposition. For this, the response to an acute DNA damage was studied in neuroblastoma cells with depleted Tau, as a model of loss-of-function. Under these conditions, altered P53 stability and activity result in reduced cell death and increased cell senescence. This newly discovered function of Tau involves abnormal modification of P53 and its E3 ubiquitin ligase MDM2. Considering the medical need with vast social implications caused by neurodegeneration and cancer, our study may reform our approach to disease-modifying therapies.

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Tauopathies are disorders of Tau protein deposition best represented by Alzheimer's disease (AD), where Tau accumulation in neurofibrillary tangles of the brain correlates with the clinical course in terms of number and distribution[1,2]. Also, mutations in the *MAPT* gene encoding for Tau lead to frontotemporal dementia with Parkinsonism 17[1,2]. Since Tau is a microtubule-associated protein, an accepted concept explaining the pathogenesis of tauopathies is that abnormal phosphorylation and folding cause Tau detachment from microtubules, Tau accumulation, and neuronal dysfunction[3,4]. In addition to microtubule association, Tau localizes in the cell nucleus and binds DNA[5–8] and also forms a complex together with P53, Pin1, and PARN regulating mRNA stability through polyadenylation[9]. Nuclear Tau was shown to have a role in DNA protection, whereby heat or oxidative stress cause nuclear Tau translocation[10]. Enhanced DNA damage was observed in Tau-KO neurons when compared to normal neurons[11]. We reported that drug-induced DNA damage also causes Tau nuclear translocation and affects Tau phosphorylation[12]. Notably, checkpoint kinases controlling DNA replication and cell cycle following a DNA damage phosphorylate Tau[13]. Together with chromosomal abnormalities found in AD-derived fibroblasts[14] and increased DNA damage in AD brains[15,16], the emerging function of Tau in DNA stability offers an alternative role of Tau in neurodegeneration and, importantly and insufficiently investigated, also in the DNA damage response (DDR). DNA is continuously damaged by genotoxic agents originating from the environment or generated intracellularly. The integrity of the genome is ensured by an efficient DDR signaling network regulating cell cycle and the DNA repair machinery, but also the activation of cell death or senescence when DNA damage persists. DDR deregulation causes accumulation of DNA errors and genomic instability, both implicated in age-related pathologies as cancer and neurodegenerative disorders[17].

In order to evaluate a role of Tau in this process, we depleted Tau in human cells and then carefully analyzed the DDR. We demonstrate that Tau deficiency renders cells less sensitive to DNA damage-induced apoptosis, which is counterbalanced by increased senescence. We show that this activity of Tau is mediated through a P53 modulation. Overall, our findings propose a role of P53 in tauopathies and a role of Tau in P53 dysregulation, a key event in oncogenesis.

Results

Generation and characterization of Tau-KO and Tau-KD cells. We opted the use of human SH-SY5Y neuroblastoma cells for generating Tau knock-out (Tau-KO) cells by the CRISPR-Cas9 technology and Tau knock-down (Tau-KD) cells by shRNA interference (Fig.1). For disruption of the *MAPT* gene, we designed gRNAs targeting Cas9 endonuclease to two sequences in the first coding *MAPT* exon. CRISPR-Cas9 cell lines were screened for Tau expression by fluorescent confocal microscopy and immune protein blotting with the human-specific N-terminal Tau13 antibody. So, we identified cell lines devoid of Tau (Fig.1a and Supplementary Fig. 7a). Since the Tau13 epitope is within the Cas9-targeted exon, false negatives may perhaps result from in-frame indels or abnormal mRNA processing. With the HT7 antibody against amino acid 159–164 of Tau_{441}, we confirmed the isolation of Tau-KO lines lacking full-length or truncated Tau expression (Fig. 1a and Supplementary Fig. 7a). We finally selected the cell lines 232P and 231K presenting alleles modified at the expected gRNA-sites by indels causing frame-shifts into stop codons within the same exon (Fig.1a). The 231A cell line underwent an unsuccessful CRISPR-Cas9 procedure and had normal Tau expression (Fig. 1a).

To obtain Tau-KD cells, we screened shRNAs targeting the coding sequence or the 3′ untranslated region of the Tau mRNA. Culturing shRNA transduced cells in the presence of puromycin resulted in the isolation of cell populations with constitutive down-regulation of Tau for three shRNAs as shown by immune staining and western blot (Fig.1b and Supplementary Fig. 7b).

Tau deficiency protects against DNA damage-induced apoptosis. Persistent DNA damage induces cell death or senescence. Thus, as a functional readout for the DDR, we assessed the cytotoxicity following a mild exposure to etoposide[18], a DNA topoisomerase II inhibitor causing double-stranded DNA breaks (DSBs). Cell viability was first tested with the well-established LDH and the MTS assays. Tau-KO cells exposed to a short (30 min) 60 μM etoposide treatment did not release LDH in the culture medium and more efficiently converted MTS when compared to Tau-expressing cells, which exhibited substantial etoposide-dependent cytotoxicity in both assays (Fig. 2). To test the involvement of apoptosis, we immune-stained cells for cleaved active caspase-3 (clCasp3), an initiator of apoptosis. Whilst <1% of the untreated Tau-expressing cells were positive for clCasp3, etoposide exposure increased the apoptotic population to 13–15%, apoptosis was induced in only 4–5% of Tau-KO cells (Fig.2). The presence of activated clCasp3 in Tau-expressing cells exposed to etoposide and its almost complete absence in Tau-KO cells was confirmed by western blot analysis with the same antibody for the cleaved enzyme form (Fig. 2 and Supplementary Fig. 8). As a whole, we found a positive association between Tau expression and DSB-induced apoptosis in SH-SY5Y cells.

Tau depletion induces cellular senescence. In alternative to cell death, unresolved DNA damage may provoke cellular senescence[17]. Inhibition of cyclin-dependent kinase by p21 causes cell cycle arrest and induction of senescence[19,20]. When compared to untreated conditions, at three recovery days after etoposide exposure (Fig. 3a), higher amounts of p21 were detected by western blot in both Tau-expressing and Tau-KO cells (Fig. 3b). When comparing the extent of this effect in the absence or the presence of Tau, we found that Tau depletion increased p21 both at basal conditions as well as after etoposide treatment when compared to wt cells (Fig. 3b). The increased amount of p21 present in Tau-KO cells suggests that Tau-depletion may prone cells to enter a senescence state further accelerated in the presence of DSBs. We determined the number of cells entering in a senescent state based on their mean cell size and by the senescence-associated β-galactosidase (SA-βGal) staining procedure at mild acidic conditions. When compared to untreated conditions, significantly increased cell size and SA-βGal-positive cells were found at three recovery days after etoposide exposure both for wt and Tau-KO cells (Fig. 3b). Again, Tau-depleted cells at basal conditions displayed a larger proportion of senescent cells in terms of cell size, SA-βGal staining and p21 expression (Fig. 3b). A consistent observation was made also for Tau-KD cells when compared to control shRNA cells (Fig. 3c, d). We concluded that reduced expression of endogenous Tau changed the fate of SH-SY5Y cells as a consequence of DSBs, favoring cellular senescence induction at the expense of activation of programmed cell death.

DNA damage and DDR activation are not reduced. DSBs lead to rapid recruitment and phosphorylation of the H2A histone family member at the site of DNA damage, and so the presence of γH2A-X is utilized as a surrogate marker of DNA damage. We first performed an accurate etoposide dose-response by in-cell

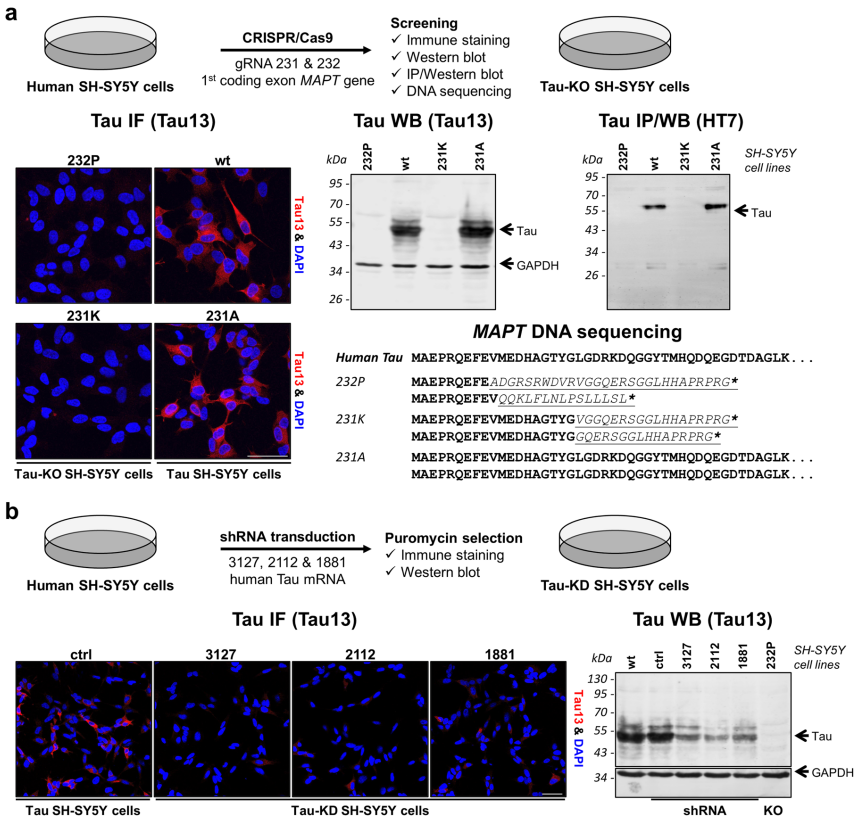

Fig. 1 Generation of Tau-KO and Tau-KD SH-SY5Y cells. a Scheme of the procedure used to generate CRISPR-Cas9-targeted cells and their characterization. Immune staining was performed with Tau13 antibody and nuclear staining with DAPI, western blot with Tau13 (loading control GAPDH) and immune precipitation and western blot with HT7 antibody, parental cells (wt) served as control. Amino acid sequences of the first *MAPT* coding exon in all lines demonstrate successful CRISPR-Cas9-editing causing frameshift (underlined in italics) into early stop codons (asterisks) for both alleles of 232P and 231K cells. **b** Scheme of procedure used to generate Tau-KD cell lines and their characterization by immune staining and western blot for Tau expression when compared to parental cells (wt) or cells transfected with the parental shRNA plasmid (ctrl). Scale bar 50 µm.

western for γH2A-X staining normalized by nuclear DAPI staining and observed an increase detection of γH2A-X total staining in Tau-KO cells when compared to control cells (Fig. 4a). The Comet assays is a direct measure of the magnitude of DSBs in single cultured cells. No difference between control and Tau-KO cells was found at the end of the etoposide treatment or during the recovery, which was rapid and complete before the 6 h washout time point independently on the presence or absence of Tau. However, Tau deletion led to more DSBs at basal conditions (Fig. 4b). Our data were thus consistent with a role of Tau in DNA-protection[10,11]. In contrast, the relatively small increase in etoposide-mediated DNA damage in Tau-KO cells inadequately explained reduced DNA damage-induced cell death in Tau-depleted cells. To corroborate this observation, we performed an etoposide dose-response and determined by confocal microscopy the presence of immune-stained nuclear γH2A-X and of the DSB-activated forms of ATM and Chk2[21,22]. This demonstrated a robust and dose-dependent induction of all three markers at 30 min after etoposide treatment (Fig. 4c). The difference between wt and Tau-KO cells was relatively minor and suggested a slightly stronger activation of the early DDR in Tau-KO cells, although the results obtained at 0 and 6 h recovery were less conclusive (Fig. 4d). Overall, the modest and somehow opposite effect of Tau depletion on the early DDR when compared to cell death induction, suggested a downstream contribution of Tau in modulating cell death.

Tau modulates DDR-dependent stabilization of P53 protein. A key DDR regulator is the tumor suppressor protein P53, which first halts cell division and then dictates cell fate when DNA damage persists[23,24]. To check the requirement of P53 for apoptosis induction in our cell model, we transduced cells with viral pseudoparticles and isolated stable P53 shRNA expressing cells (Supplementary Fig. 1a). The effect of the shRNA was negligible at basal conditions, i.e. when the cells maintain a minimal amount of P53 due to its efficient degradation. In

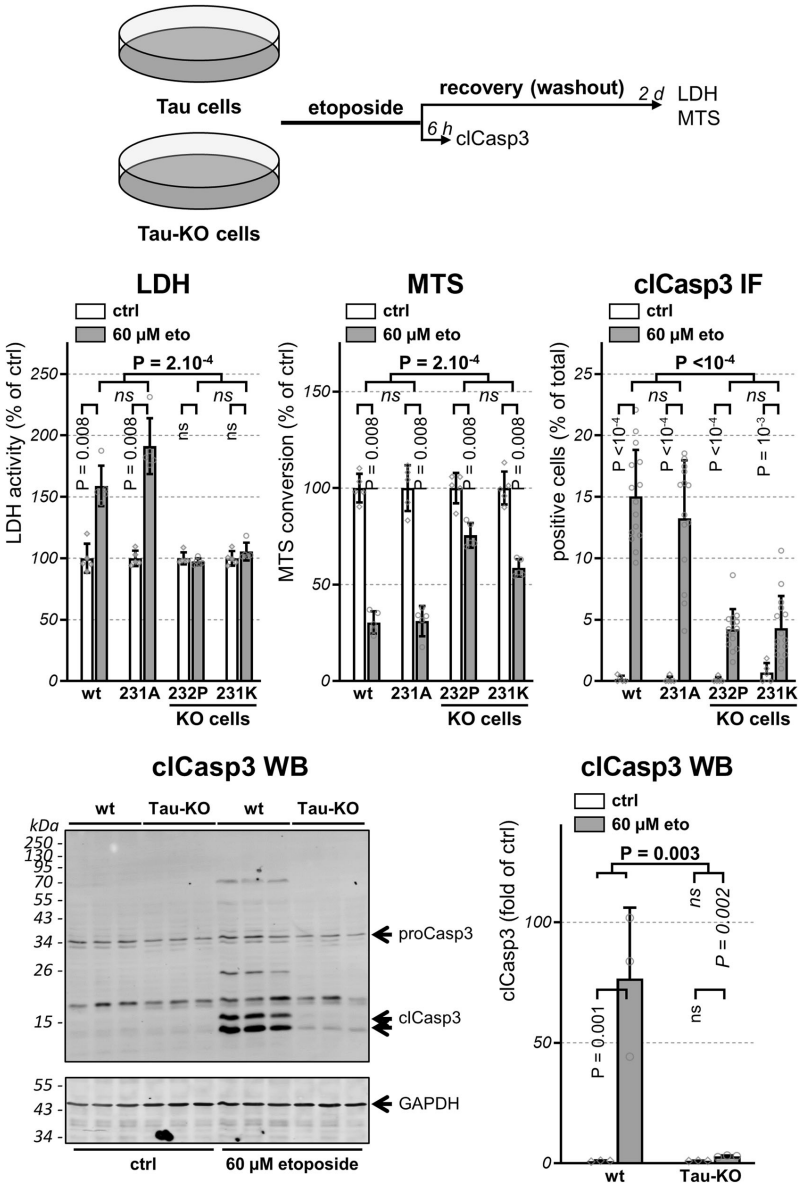

Fig. 2 Tau deficiency confers resistance to etoposide-induced apoptosis. Scheme representing the design of the experiment with parental and 231A (Tau) cells compared to 232P and 231K (Tau-KO) cells treated 30 min with 60 µM etoposide and recovered as indicated before analysis. LDH and MTS values are shown as percentage of parental cells (wt), mean ± SD of five biological replicates. To measure activation of apoptosis, percent positive cells for cleaved-caspase-3 (clCasp3) is determined on confocal microscope images and normalized for total DAPI-positive cells, mean ± SD of five images for the untreated cells (ctrl) and of 15 images for etoposide-treated cells (60 µM eto), $n > 500$ cells/condition, representative experiment of $n > 3$ biological replicates. Activated clCasp3 was also analyzed by western blot with GAPDH as loading control and 15 and 17 kDa clCasp3 quantified by normalization with GAPDH, mean ± SD ($n = 3$ biological triplicates). Statistical analysis by independent measures ordinary two-way ANOVA, source of variation for cell lines (in bold), multiple Bonferroni pairwise comparisons for treatment between lines (in italics) or for each line (in vertical).

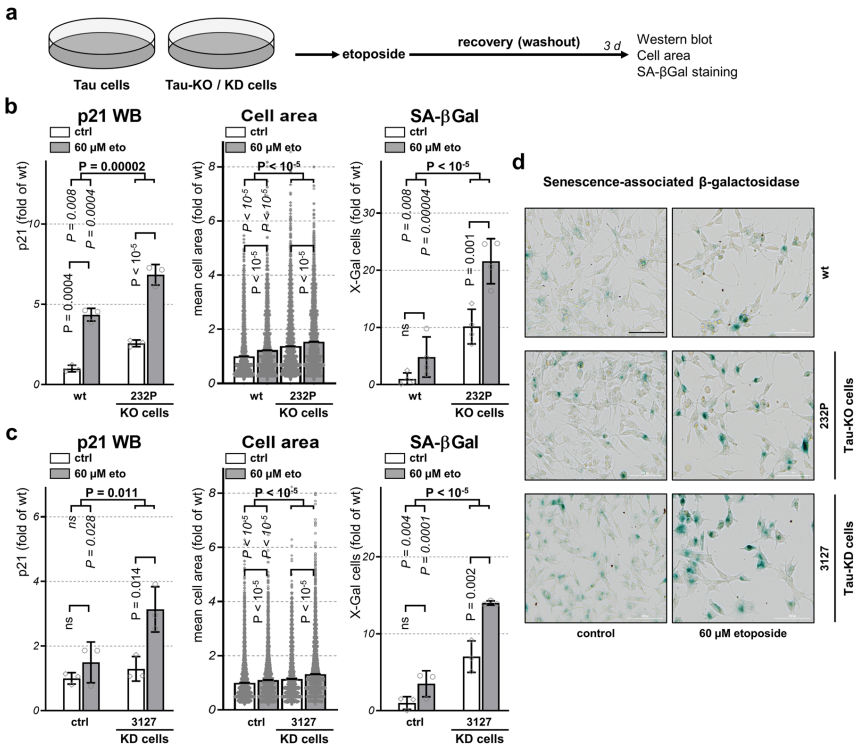

Fig. 3 Tau depletion increases cellular senescence. a Scheme of the procedure followed to assess cellular senescence upon 30 min treatment with 60 µM etoposide followed by 3 days of recovery. **b** Quantification of p21 amount in cell lysates by western blot in parental (wt) or 232P (Tau-KO) cells under control conditions (ctrl) or following etoposide treatment (60 µM eto) normalized for GAPDH, mean ± SD of three biological replicates. Quantification of mean cell area and percent positive cells for senescence-associated β-galactosidase (SA- βGal) determined with a high-content microscope scanner, mean ± sem of four (Tau-KO cells) or three (Tau-KD) independent experiments, $n > 8000$ cells. Data are shown as fold of wt cells at basal conditions. **c** Same as in **b** for mock shRNA (ctrl) or Tau 3127 shRNA (Tau-KD) cells. **d** Representative images of SA-βGal staining (in blue), bright-field, scale bar = 100 µm. Statistical analysis by independent measures ordinary two-way ANOVA, source of variation for cell lines (in bold), multiple Bonferroni pairwise comparisons for treatment between lines (in italics) or for each line (in vertical).

contrast, P53-KD cells displayed reduced etoposide-dependent P53 stabilization when compared to control cells as demonstrated by western blot analysis with the monoclonal antibodies DO-1 and Pab 1801 and confirmed by immune staining with DO-1 (Supplementary Fig. 1b, c). Cell lysates obtained from the neuroblastoma cell line SK-N-AS carrying a homozygous deletion in the *TP53* gene and therefore not expressing P53[25], were used as a negative control for P53 immune detection. Etoposide treatment induced apoptosis in ~2% P53-KD cells compared to ~14% of the control cells (Supplementary Fig. 1d). These data confirmed the involvement of P53 in DSB-induced apoptosis also in SH-SY5Y cells.

Having exposed the contribution of P53 and Tau in modulating DNA damage-dependent apoptosis in SH-SY5Y cells, we next asked whether Tau may modulate P53 activation. Tau-KO cells presented reduced DNA damage-induced nuclear P53 when compared to Tau-expressing cells as shown by immune staining and western blot (Fig. 5a and Supplementary Fig. 2a, b). Reduced P53 was observed when Tau-KO cells were exposed to 30, 60 or 90 µM etoposide and let recover for 30 min or 6 h (Fig. 5a). Reduced etoposide-induced apoptosis in Tau-KO cells displayed a similar dose-dependent effect (Fig. 5b).

Further documenting the role of Tau in etoposide-induced cytotoxicity, re-expressing high levels of human Tau_{441} in Tau-KO cells (Supplementary Figs. 3a and 11) increased P53 stabilization in etoposide-treated cells (Supplementary Fig. 3b) and restored sensitivity in the LDH and clCasp3 assays (Supplementary Fig. 3c). In order to obtain reconstituted Tau expression at a level similar to that of endogenous Tau, in a second set of experiments Tau-KO cells were transiently transfected with a 1:10 mixture of Tau_{410} and GFP plasmids or of empty and GFP plasmids. Tau expression was then analyzed in GFP-positive cells co-transfected either with the Tau_{410} or the empty plasmid by immune staining. This led to determine a level of ectopic expression corresponding to ~2-fold that of endogenous Tau determined in parental SH-SY5Y cells (Supplementary Fig. 3d). Under these conditions, 6 h after etoposide exposure Tau_{410}-transfected Tau-KO cells displayed increased P53 stabilization

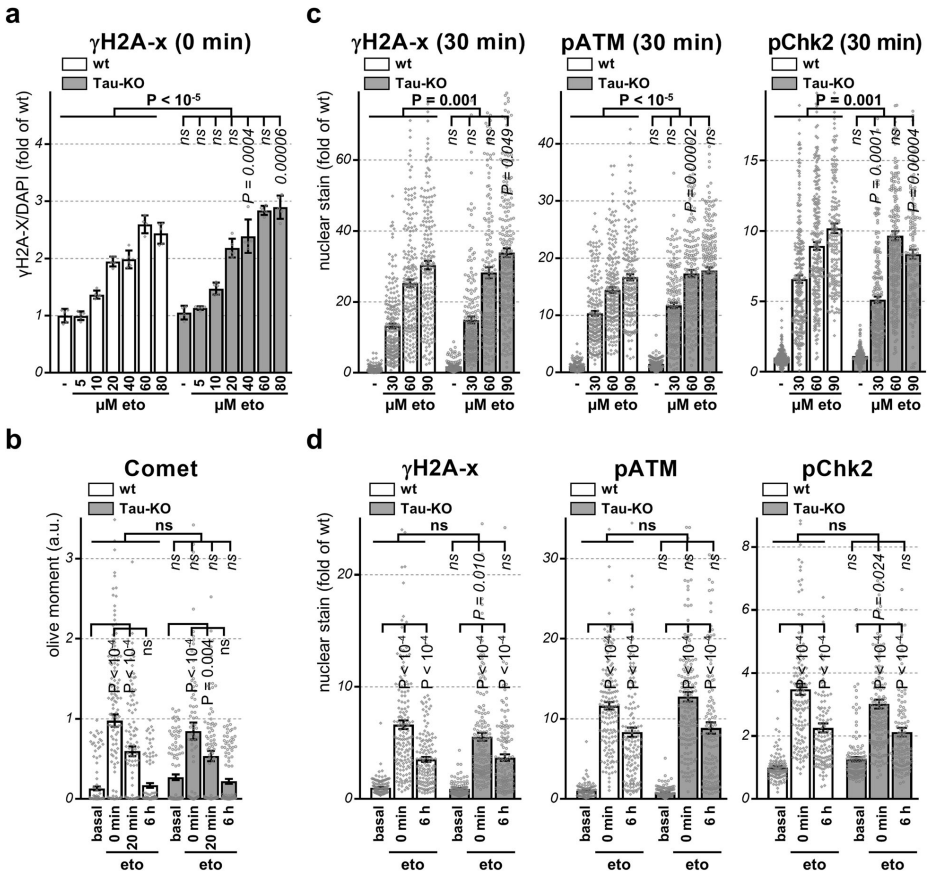

Fig. 4 Reduced P53 in Tau-KO cells is not caused by DDR activation. For all panels, parental (wt) or 232P (Tau-KO) cells were treated 30 min with the indicated etoposide concentrations and recovery times. **a** Mean intensity ± SD of γH2A-X staining normalized for DAPI staining by in-cell western is shown as fold of wt cells at basal conditions, $n = 5$ of biological replicates in a 96-well plate. Non-parametric independent Mann–Whitney U test between lines (in bold), or for each dose between lines (in italics). **b** Olive moment in the Comet assay is shown as mean ± sem, $n = 84$–146 cells/condition. **c**, **d** Mean intensity ± sem of single-cell nuclear γH2A-X, pATM or pChk2 staining (DAPI mask, ImageJ) is shown as fold of wt cells at basal conditions, $n > 100$ cells/condition distributed over five images. Statistical analysis by independent measures ordinary two-way ANOVA, source of variation for cell lines (in bold), multiple Bonferroni pairwise comparisons of each condition between lines (in italics) and of time points for each line (**b**, **d**, in vertical).

when compared to that detected in empty plasmid-transfected Tau-KO cells (Supplementary Fig. 3e).

Tau-KD-cells with reduced Tau-expression corresponding to 71 ± 1% for the 3127 shRNA and 64 ± 2% for the 2112 shRNA (Fig. 5c) when exposed to etoposide also showed reduced P53 activation (Fig. 5d) and apoptosis (Fig. 5e) in a Tau-dose-dependent manner. On the other hand, 60 ± 1% reduced Tau in 1881 shRNA cells did not affect P53 protein level and apoptosis (Fig. 5c–e). Single-cell analysis of the whole Tau-KO or Tau-KD cell population revealed that when we applied a threshold just above background to count P53-positive cells, etoposide-dependent P53 stabilization was better described by a change in the relative number of P53-positive cells rather than by a gradual correlation between Tau and P53 expression (Fig. 5f).

Reduced P53 and apoptosis occurs in other neuroblastomas. In order to validate the observation made in SH-SY5Y cells, we tested the effect of Tau down-regulation in IMR5 and IMR32 human neuroblastoma cell lines. Similar to SH-SY5Y cells, these two cell lines express a wild-type functional P53[26,27]. Several other neuroblastoma cell lines were disregarded because P53 mutations were causing either constitutive activation or expression loss of P53[27]. Tau expression in IMR5 cells was down-regulated ~4-fold in the presence of the 2112 shRNA (Supplementary Fig. 4a). Under these conditions, we observed lower etoposide-induced P53 stabilization in Tau-KD when compared to mock-transduced IMR5 cells as determined by western blot and immune staining analysis (Supplementary Fig. 4b). Similar to what observed in SH-SY5Y cells, etoposide-induced clCasp3 was

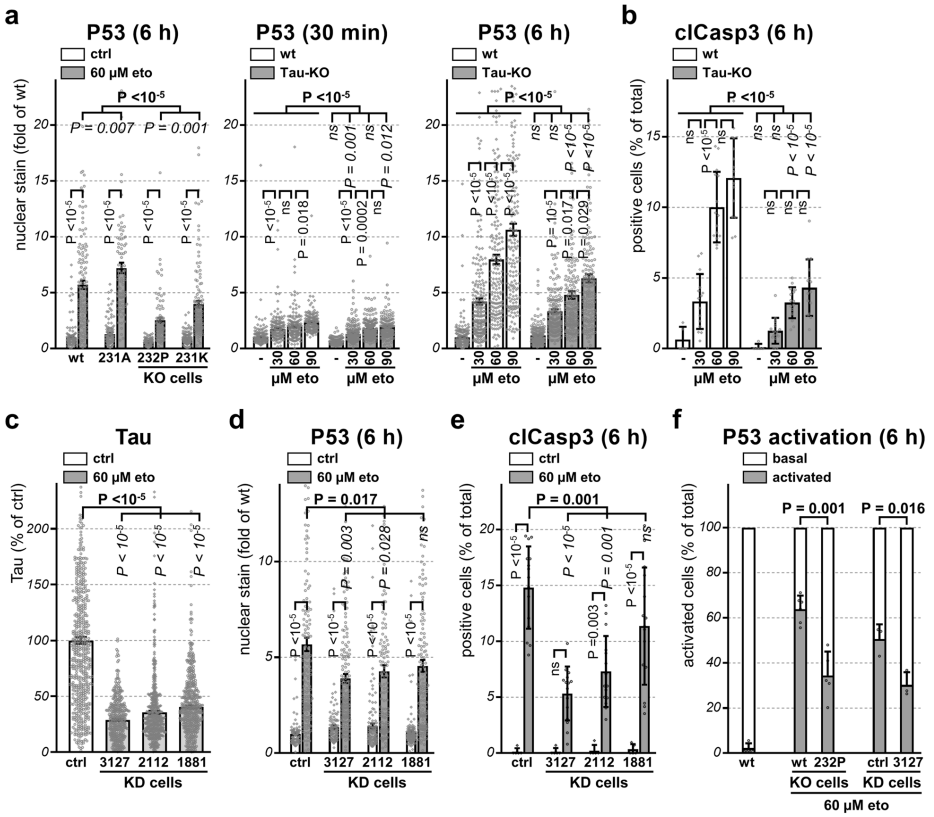

Fig. 5 Tau depletion decreases P53 level and apoptosis. For all panels the indicated cell lines (Tau-KO are 232P cells) were treated 30 min with the indicated etoposide concentrations and recovery times. **a** Mean intensity ± sem of single-cell nuclear P53 staining (DAPI mask, ImageJ) is shown as fold of wt cells at basal conditions, $n > 100$ cells/condition distributed over five images. **b** Percent clCasp3-positive cells are shown as mean ± SD of five images for the untreated cells and of 15 images for etoposide-treated cells, $n > 500$ cells/condition. **c** Mean intensity ± sem of single-cell Tau staining (tubulin mask, ImageJ) for the indicated cell lines is shown as percent of mock shRNA cells (ctrl), $n > 160$ cells/condition distributed over five images. **d** Mean intensity of single-cell nuclear P53 staining quantified as in **a**, $n > 380$ cell/condition distributed over 15 images from $n = 3$ biological replicates. **e** Percent clCasp3-positive cells quantified as in **b**, $n > 500$ cell/condition. **f** An arbitrary threshold was applied in order to count P53-positive cells as percentage ± SD of total DAPI-positive cell number, $n > 100$ cells/conditions. For the comparison between the four cell lines (**a**), non-parametric independent Mann–Whitney U test for genotype (in bold) and Kruskal–Wallis pairwise comparison of treatment for cell lines with same genotype (in italics) or for each line (in vertical). For the dose-dependency (**a**, **b**), non-parametric independent Mann–Whitney U test between cell lines (in bold), Kruskal–Wallis pairwise comparison for each dose (in italics) and between doses (in vertical). Non-parametric independent Mann–Whitney U test (**c**–**e**) between control and the three Tau-KD lines (in bold) and Kruskal–Wallis pairwise comparison for each Tau-KD line (in italic) and for each treatment (in vertical). Unpaired two-tailed t test with Welch's correction (**f**).

also reduced in Tau-depleted IMR5 cells (Supplementary Fig. 4c). The presence of the 3127 shRNA in IMR32 cells lowered Tau expression by ~40%, which resulted in reduced P53 stabilization and caspase-3 activation in cells exposed to the etoposide treatment (Supplementary Fig. 4d–f).

Tau regulates P53 expression post-translationally. To assess whether lower P53 protein level observed in Tau-KO cells was occurring by transcriptional or post-translational mechanism, we first determined by quantitative RT-PCR the amount of the P53 mRNA in wt and Tau-KO cells before or 6 h after the acute etoposide treatment. At basal conditions Tau-KO and Tau-KD cells showed a significant but modest increase in *TP53* transcription when compared to Tau-expressing cells. Etoposide exposure slightly increased the P53 transcript in all cell lines, but there was no difference when comparing treated Tau-expressing and treated Tau-KO cells (Fig. 6a, b). Overall, these data essentially dismissed the premise that the effect of Tau depletion on P53 stabilization occurred at the transcriptional level, rather suggesting a translational or post-translational control. On the other hand, etoposide treatment resulted in the expected

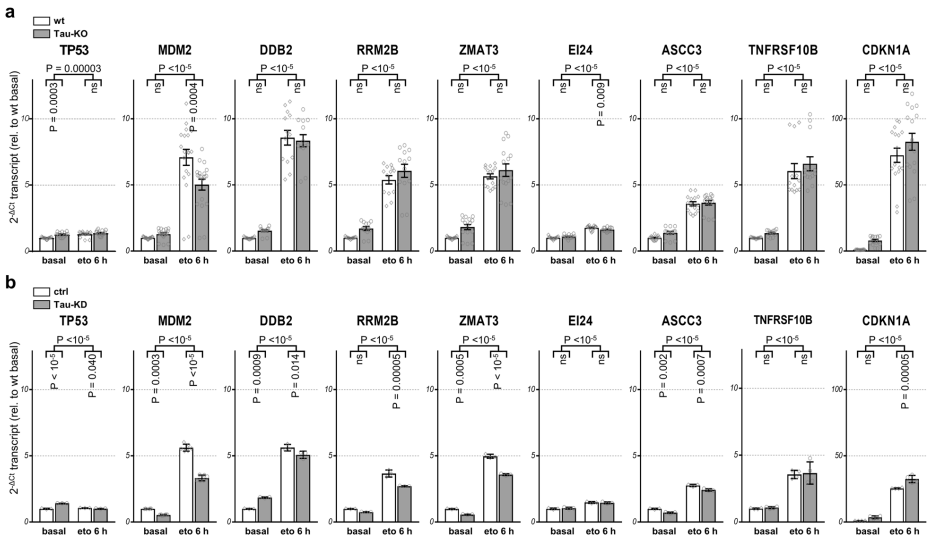

Fig. 6 Differential regulation of P53 transcription targets. Extracted RNA from parental (wt) and 232P (Tau-KO) cells in **a** or from control shRNA plasmid (ctrl) and 3127 (Tau-KD) cells in **b** at basal conditions or after 30 min 60 μM etoposide and 6 h recovery, was subjected to reverse-transcription and qPCR with primers specific for the indicated transcripts. Mean ± SD of relative mRNA levels ($n = 3$) shown as fold of the respective basal conditions for parental or control cells. Statistical analysis by independent measures ordinary two-way ANOVA, source of variation for cell lines (horizontal), multiple Sidak pairwise comparisons for treatment for each line (in vertical).

P53-dependent upregulation of *HDM2* transcription, but this was markedly reduced in Tau-KO and in Tau-KD cells (Fig. 6a, b), a result that was confirmed also at the MDM2 protein level (Supplementary Fig. 5). Analysis of additional direct targets of P53[28,29] showed a differential transcriptional response to etoposide in Tau-depleted cells. Whilst, transcription of the *EI24* gene was reduced in etoposide-treated Tau-KO cells, that of *RRM2B*, *TNFRSF10B*, *DDB2*, *ZMAT3, ASCC3* and *CDKN1A* was not affected in Tau-KO cells (Fig. 6a). Dysregulation of transcription of the P53 targets was more evident in Tau-KD cells, which showed reduced etoposide-induction for the *MDM2*, *DDB2*, *RRM2B*, *ZMAT3*, *ASCC3* transcripts, and no effect on the *EI24* and *TNFRSF10B* transcripts (Fig. 6b). Interestingly, as observed for the *CDKN1A* protein product p21 (Fig. 3c), also the *CDKN1A* transcript was increased in Tau-KD cells after etoposide treatment (Fig. 6b), whereas this did not reach significance in Tau-KO cells (Fig. 6a). A different regulation of direct P53-dependent genes after etoposide exposure, conveyed by a positive or negative difference in the degree of transcription activation between cells with normal or reduced Tau expression, substantiated a Tau-dependent modulation of P53 function at a post-translational level. Also, the heterogeneous response observed among the different P53 targets cannot be explained solely by a change in P53 protein stability but implied a more complex modulation of P53 activity.

Tau affects P53 and MDM2 modification. A post-translational clearance mechanism keeps P53 protein at low levels in the absence of a cellular stress[30]. This occurs mainly, but not exclusively, by the activity of the E3 ubiquitin ligase MDM2 (also known as HDM2) that associates with P53 to favor its degradation and interfere with its function[30,31]. We determined the amount of nuclear MDM2 and found that induction of MDM2 was lower in Tau-KO cells when compared to Tau-expressing cells, possibly explained by reduced gene transcription (see above, Fig. 6), whereas MDM2 expression in untreated cells was not modulated by Tau (Supplementary Fig. 5). Post-translational modification of P53 through the action of DDR transducing kinases causes the dissociation of the P53-MDM2 complex and induces stress-dependent P53 stabilization. In the presence of DSBs, this occurs mainly by N-terminal phosphorylation of the P53 transcription-activation domain by the ATM-Chk2 axis[22]. We tested two small molecules interfering with this process. KU-55933 is an ATM inhibitor blocking ATM-dependent P53 phosphorylation thus preserving the P53-MDM2 complex and its degradation. Nutlin-3 binds to the P53-binding pocket of MDM2 thus inhibiting their association and degradation. KU-55933 had no effect on P53 and MDM2 expression when tested alone (Fig. 7a). As expected, adding the drug after etoposide treatment severely impaired DSB-induced P53 and MDM2 stabilization and also blocked apoptosis activation (Fig. 7a, b). Consistent with its mode of action, the presence of nutlin-3 led to a strong increase in P53 and MDM2, which was higher to that caused by etoposide but, notably, did not induce apoptosis (Fig. 7a, b). Nutlin-3 potentiated the effect of etoposide in terms of P53-MDM2 stabilization in wt cells. In Tau-KO cells exposed to etoposide, nutlin-3 eliminated the drop in MDM2 and partly also that in P53 (Fig. 7a, b). Determination of P53 phosphorylation at S_{15} when normalized for total P53 protein showed a similar etoposide-dependent relative occupancy in Tau-KO cells when compared to Tau-expressing cells (Supplementary Fig. 6a). This was an unexpected result because amino-terminal P53 phosphorylation should stabilize P53 by interfering with the binding to MDM2, and thus increased P53 destabilization in Tau-KO cells should be reflected by a reduction in P53 phosphorylation. A

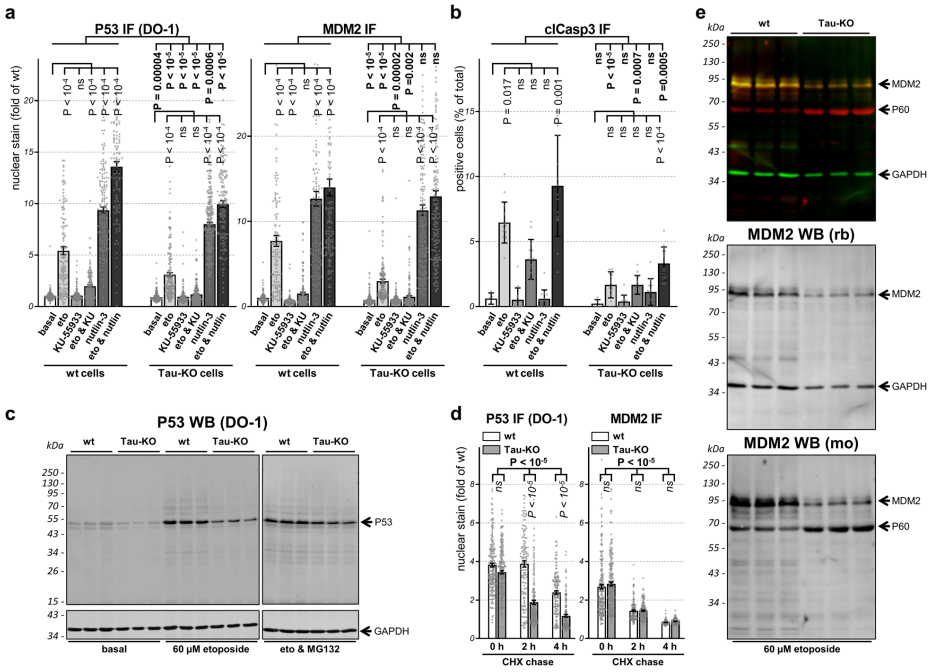

Fig. 7 Role of P53 and MDM2 modifications for P53 function and stability. a, **b** Parental (wt) or 232P (Tau-KO) cells treated 30 min without (basal) or with 60 µM etoposide and recovered for 6 h in the absence (eto) or presence of 10 µg/mL KU-55933 and/or 5 µg/mL nutlin-3. **a** Mean intensity ± sem of single-cell nuclear P53 or MDM2 (DAPI mask, ImageJ) shown as fold of basal conditions, $n > 100$ cells/condition distributed over five images. **b** Percent clCasp3-positive cells shown as mean ± SD of five images (basal) or 15 images (treatments), $n > 500$ cells/condition. Non-parametric independent samples test and Kruskal–Wallis pairwise comparison between cell lines (in bold) or for treatment for each cell line (in vertical). **c** Western bot analysis of P53 in parental (wt) or 232 P (Tau-KO) cells at basal conditions, after 30 min 60 µM etoposide and 4 h recovery without or with 10 µM MG132. GAPDH served as loading control. **d** Parental (wt) or 232P (Tau-KO) cells pre-treated for 30 min with 60 µM etoposide followed by 4 h with 10 µM MG132, were incubated with 25 µM of cycloheximide (CHX) for the indicated chase times. Single-cell nuclear P53 or nuclear MDM2 (DAPI mask, ImageJ) shown as fold of wt cells at basal conditions. Mean intensity ± sem of $n > 100$ cells/condition distributed over five images. Independent measures ordinary two-way ANOVA, source of variation for cell lines (bold), multiple Bonferroni pairwise comparisons of treatment for each line (in italic). **e** Parental (wt) or (Tau-KO) 232P cells treated for 30 min with 60 µM etoposide and 6 h recovery analyzed with a 90 kDa MDM2 rabbit antibody (green and middle panel with GAPDH as loading control) or a 60 and 90 kDa MDM2 mouse antibody (red and bottom panel).

possible explanation is that reduced DSB-dependent stabilization of P53 caused by the absence of Tau might be compensated by a change in P53 phosphorylation. Etoposide-induced P53 phosphorylation at S_{15} was severely impaired by the ATM inhibitor KU-55933 (Supplementary Fig. 6b), but this only partially blocked apoptosis induction in wt cells and had no effect in Tau-KO cells when compared to etoposide-exposure alone (Fig. 7b). In addition, P53 stabilization by nutlin-3 did not involve S_{15} phosphorylation (Supplementary Fig. 6b) and poorly induced apoptosis (Fig. 7b). Our attempts to analyze pS_{46}-P53 phosphorylation was unsuccessful as no signal was detected also under conditions of prolonged etoposide treatment both in Tau-expressing and Tau-KO cells. We concluded that in SH-SY5Y cells, DSB-induced apoptosis was at least in part dependent on P53 modification.

Tau-depletion increases P53 degradation rate. To address if Tau-KO cells displayed faster P53 degradation possibly accounting for the lower detection of P53 protein, we first inhibited the ubiquitin-proteasome system by treating the cells with MG132. The presence of MG132 during the recovery phase from etoposide exposure restored P53 stabilization in Tau-KO cells but had no effect in wt cells, suggesting that the absence of Tau favored P53 degradation (Fig. 7c and Supplementary Fig. 9c).

Taking advantage of the fact that MG132 was able to restore similar P53 protein levels in wt and Tau-KO cells exposed to etoposide, we then analyzed the rate of degradation of P53 and MDM2 by removing MG132 and adding the translation inhibitor cycloheximide. Under these conditions we observed a faster P53 degradation rate at 2 and 4 h wash-out in Tau-KO cells when compared to wt cells (Fig. 7d). In contrast, no difference was observed in terms of MDM2 degradation (Fig. 7d).

Western blot analysis of MDM2 with the same rabbit antibody used for immune staining of the cells confirmed reduced MDM2 expression in Tau-KO cells exposed to etoposide when compared to wt cells (Fig.7e and Supplementary Fig. 9e). Interestingly, when using a mouse antibody for MDM2, we also detected a 60 kDa MDM2 form (Fig.7e and Supplementary Fig. 9e), likely representing the amino-terminal caspase-2 cleavage product of

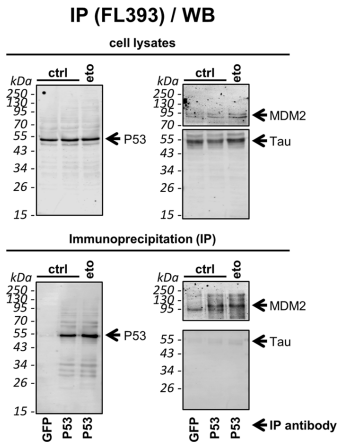

Fig. 8 Tau does not directly interact with P53. Cell lysates of SH-SY5Y treated with 10 μM of MG132 to stabilize P53 expression, without (ctrl) or with (eto) a 30 min pre-treatment with 60 μM etoposide, were subjected to immune precipitation of endogenous P53 with a rabbit antibody (P53) or with a rabbit GFP antibody as negative IP control (GFP). Western blot analysis for co-precipitation of MDM2 or Tau with the respective mouse antibodies as indicated. The blots on the top show the analysis of the starting material (cell lysates), those on the bottom the immunoprecipitation (IP). The P53 blots are entirely shown, whereas for MDM2 and Tau, the blots were cut between the 55 kDa and the 95 kDa protein size markers and analyzed separately.

full-length 90 kDa MDM2[32,33]. An opposite effect of etoposide-exposure was obtained for these two forms of MDM2 in Tau-KO cells when compared to wt cells. Although reduced 90 kDa MDM2 detection was confirmed in Tau-KO cells, in the same cells we found that etoposide markedly increased 60 kDa MDM2 (Fig.7e and Supplementary Fig. 9e).

Tau does not interact with P53. In the presence of the proteasome inhibitor, the interaction between P53 and MDM2 was confirmed by a co-precipitation experiment both in the presence and in the absence of etoposide treatment (Fig. 8 and Supplementary Fig. 10). However, although etoposide increased the amount of MDM2 detected in cell lysates when compared to the control, for both conditions a similar amount of MDM2 was co-precipitated by P53; a result consistent with decreased P53-MDM2 interaction as a consequence of DNA damage. In contrast, we did not detect any interaction between P53 and Tau in the presence or absence of DNA damage (Fig. 8 and Supplementary Fig. 10), suggesting that the modulatory function of Tau on P53 stabilization may not occur by a direct interaction between the two proteins in SH-SY5Y cells.

The data obtained implied that in neuroblastoma cells Tau modulated P53 in a manner that went beyond a simple regulation of its stabilization, but accounted also for a deregulation of P53 and MDM2 post-translational modification ultimately affecting the activity and function of P53 in cell fate decisions dependent on DNA damage.

Discussion

We report a new function of Tau as a regulator of DSB-induced cell fate and describe that this occurred by a deregulation of P53 activity. Our data support a role for Tau as a P53 modifier in neurodegeneration. Uncontrolled P53 activity in the presence of P53 or MDM2 mutations is one of the main pathomechanism of cancer[34]. Not surprisingly, the P53-MDM2 axis includes a variety of factors regulating their modification and localization[35]. Based on our data, Tau should now be listed as a modifier of wild-type P53 function, with possible implications in cancer biology.

The involvement of apoptosis in neurodegeneration is documented[36], but whether this is modulated by Tau remains questionable[37,38]. We show now that a brief DNA damaging insult positively associates Tau to programmed cell death and negatively to cellular senescence. Positive association to cell loss was already shown in Bloom's syndrome cells under continuous DNA damage for three days, although a distinction between cell death and senescence was not clear[39]. Age-dependent increase in senescent cells promotes tissue deterioration and neuronal dysfunction[40,41]. Moreover, increased senescent glia cells were found in a tauopathy model characterized by reduced soluble Tau and, remarkably, senescent cell removal prevented functional neuronal decline[42]. Nevertheless, direct evidence that Tau loss-of-function may promote senescence was not yet reported.

In the adult human brain, post-mitotic neurons express Tau in multiple alternative spliced isoforms that differ depending on the presence of up to two N-terminal inserts (0N, 1N, 2N) and on the presence of three or four microtubule-binding repeats (3R, 4R). In early development, 3R-Tau isoforms are predominant whereas in the adult brain the 3R and 4R isoforms are detected at a similar level, although they ratio is substantially altered in a peculiar manner for distinct tauopathies[43]. The cell lines used in our study (SH-SY5Y, IMR-32 and its subclone IMR-5) express predominantly the embryonic isoform 3R-Tau when actively dividing[44–46]. Our results showing that the effect of endogenous Tau deletion was reversed by ectopic expression of either 4R-Tau_{441} or 3R-Tau_{410} suggest that the modulatory role of Tau on P53 is possibly shared by all Tau isoforms. Tau phosphorylation in undifferentiated SH-SY5Y is increased[46], so at this time we cannot exclude that the modulatory effect of Tau on P53 may require modification of Tau by phosphorylation.

The balance between apoptosis and senescence is regulated by an intricate mechanism, which varies in response to distinct stressors[47]. In terms of DNA damage, crucial determinants are the nature and intensity of the stress. Since P53 drives both the induction of apoptosis and senescence, cell fate is finely tuned by changes in P53 kinetics and transcriptional activity under post-translational modification control[48]. The inference that Tau modifies P53 protein expression and the balance between cell death and senescence, suggests Tau as a modifier of P53 post-translation modification by acting on P53 modulators. P53 is modified by most types of substituents, which dictate the complex response to a wide range of cellular conditions[49]. Ubiquitination and phosphorylation control P53 stability, subcellular localization and transcriptional versus non-transcriptional activity, as well as cellular senescence[50] and apoptosis[51]. P53 acetylation may govern transcriptional-regulation of gene targets involved in growth arrest, and the choice to enter senescence or apoptosis[52]. In our cellular model, treatment with nutlin-3 restored P53 stabilization without involving P53 phosphorylation in Tau-depleted cells exposed to etoposide, but poorly reversed reduced activation of the apoptotic pathway. Nutlin-3 restored also the expression of the P53 negative regulator MDM2, which targets P53 for degradation, regulates its nuclear localization, and the interaction of P53 with transcriptional co-factors[35]. Therefore, considering only the protein amount of P53 or MDM2 inadequately explains the role of Tau as an effector balancing apoptosis and senescence. Additional P53-interacting protein such as WW domain-containing oxidoreductase (WWOX), which modulate Tau phosphorylation, may be involved in Tau-dependent regulation of P53[53,54].

Table 1 Argeted gRNA sequences for human *MAPT* gene.

Oligo	Sequence
231	CACGCTGGGACGTACGGGTTGGG
232	CCGCCAGGAGTTCGAAGTGATGGG

Tau has been described recently to be part of a complex containing P53, PARN and Pin1 involved in the regulation of mRNA stability through regulation of polyadenylation[9]. In this report, in HCT-116 colon cells Tau expression modulated the level of the P53 transcript and P53 that of Tau. In our cellular model, we also observed that Tau was able to modulate P53 expression, but we excluded that this occurred at the level of gene transcription and instead showed a post-translational mechanism. Nevertheless, the action of Tau on P53 degradation, on P53 activity as transcription factor and on P53 function as cell fate mediator reported herein for SH-SY5Y cells may well be regulated by a Tau complex similar to that described for HCT-116 cells. However, HCT-116 and other colon cancer cell lines modify Tau to an hyperphosphorylated form resembling the one deposited in tauopathy brains[55], suggesting a difference in function between pathogenic and physiological Tau.

Increased DNA damage has been reported as a consequence of Tau deletion and heat stress, indicating a protective role of Tau against DNA damage, which may require nuclear Tau translocation[10,11,35]. We reported that also etoposide exposure increased nuclear Tau and reduced its phosphorylation[12]. In the present study, determination of DNA integrity assessed by the Comet and γH2A-X assays confirmed the protective role of Tau, but rebuffed the possibility that decreased DNA damage in the absence of Tau may be the reason for decreased DSB-induced cell death. Moreover, we did not observe an overt effect on the ATM-Chk2 axis, implying that a downstream pathway was affected in Tau-KO cells.

P53 stabilization and apoptosis induction depended on the activity of the ATM-Chk2 axis, because they were stopped by the ATM inhibitor KU-55933. An alternative reading is that Tau may intervene on P53 modification or on its functional modulators. Whether this occurs in the cytosol, possibly based on its activity on microtubules, or following its nuclear translocation is yet to be examined. P53 has been shown to associate to and be regulated by microtubules[56,57]. Therefore, Tau may affect P53 interaction with the cytoskeleton by modulating microtubule dynamics. However, Tau is also present in the nucleus in normal and stress conditions and some functions associated to nuclear Tau were suggested[10,11,58–60].

The implication of the neurodegeneration-associated Tau protein in the biology of P53, the "guardian of the genome", is a thrilling finding that may explain the role of P53 and DDR dysfunction in neurodegeneration and the link between Tau and cancer. Abnormal P53 species are potential biomarkers of AD[61–63], the most common tauopathy with an high incidence of P53 mutations[64] and P53 deregulation[65]. Genetic manipulation of P53 family members in mice affects aging, cognitive decline, and Tau phosphorylation[66,67]. Cell cycle activators are upregulated in post-mitotic neurons by stress conditions and tauopathies, possibly representing a cause for neurodegeneration[68,69]. Increased DNA damage is found in AD[15,16] and persistent DDR causes neuronal senescence and upregulation of pro-inflammatory factors[70]. Our finding that cellular senescence is increased by DSBs in Tau-KO cells is consistent with these observations.

Intriguingly, hyperphosphorylated and insoluble Tau is detected in some prostate cancer[71], FTDP-17 *MAPT* mutations increase the incidence of cancer[72], and higher levels of phosphoSer199/202-Tau have value as predictors of non-metastatic colon cancer[73]. More recently several reports described that high Tau expression improves survival in several types of cancers[74–77]. Intriguingly, Tau deficiency resulting in reduced P53 stabilization reported herein provides a mechanism to explain why reduced Tau represents a negative prognostic marker. Moreover, since our data show that Tau expression also modulates etoposide cytotoxicity, we would like to propose that Tau protein level may acquire value as a response marker of genotoxic therapy.

Cancer and neurodegenerative diseases may involve common signaling pathways balancing cell survival and death[78–80] and may be defined as diseases of inappropriate cell-cycle control as a consequence of accumulating DNA damage. Epidemiological studies show an inverse correlation between cancer and neurodegeneration[79], although not consistently[81,82], and chemotherapy is associated to a lower predisposition for AD[83,84]. The study of Tau as a modifier of P53 and, importantly, P53 control of cell death and senescence is crucial because of the implication that Tau may modulate cell death and senescence in neurodegenerative tauopathies and in cancer. Considering the unmet medical need with vast social implications caused by these—unfortunately frequent —disorders, our finding holds sizeable scientific importance and may lead to innovative approaches for disease-modifying therapeutic interventions.

Methods

Cell culture and DNA transfections. Human neuroblastoma IMR5, IMR32, and SK-N-AS were kindly provided by Dr. Chiara Brignole and Dr. Mirco Ponzoni from the IRCCS Istituto Giannina Gaslini in Genova. These cells and the human neuroblastoma SH-SY5Y cells (94030304, Sigma-Aldrich) were cultured in complete DMEM: Dulbecco's Modified Eagle Medium (61965–059, Gibco) supplemented with 1% non-essential amino acids (11140035, Gibco), 1% penicillin-streptomycin (15140122, Gibco) and 10% fetal bovine serum (10270106, Gibco). Cells were grown at 37 °C with saturated humidity and 5% CO_2, and maintained in culture for <1 month. Cells grown on poly-D-lysine (P6407, Sigma-Aldrich) were transfected with jetPRIME (114–15, Polyplus) or Lipofectamine 3000 (L3000008, Invitrogen) according to the manufacturer's instructions or with the calcium phosphate method[85].

Targeted disruption of Tau expression. For disruption of the *MAPT* gene encoding for Tau by the CRISPR-Cas9 method, the two gRNAs 231 and 232 (Table 1) targeted exon 2 containing the initiating ATG (ENST00000344290.9). Cells in six-well plates were transfected with the plasmid kindly provided by Dr. Zhang[86] (52961, Addgene) driving expression of one of the two gRNA, Cas9 nuclease and puromycin resistance. One day post-transfection, cells were transferred to 10 cm plates and incubated for 2 days with 20 μg/mL puromycin (P8833, Sigma-Aldrich). Single colonies were isolated, amplified, and stored in liquid nitrogen. The cDNA encoding for human Tau isoform of 441 amino acids (Tau441) in the expression plasmid pcDNA3 and selection in 0.5 mg/mL Geneticin (11811031, Gibco) served to generate reconstituted Tau expression in the Tau-KO 232P cell line.

Sequencing of the targeted *MAPT* gene. Cell pellets were resuspended in 400 μL TNES buffer (0.6% SDS, 400 mM NaCl, 100 mM EDTA, 10 mM Tris pH 7.5) and 0.2 mg/mL proteinase-K (Abcam, ab64220) under continuous shaking 3–4 h at 50 °C, and then supplemented with 105 μL of 6 M NaCl. Genomic DNA was precipitated with one volume of ice-cold 100% ethanol, washed with 100% ethanol and with 70% ethanol, and air-dried. The genomic fragment containing the CRISPR-Cas9-targeted regions was amplified by PCR with primers containing BamHI or XhoI restriction sites (Table 2) with the AccuPrime™ Pfx SuperMix (12344–040, Invitrogen). PCR reactions were purified with the GeneJET PCR purification kit (K0701, ThermoFisher Scientific) and subcloned in pcDNA3. DNA from single bacterial colonies were analyzed by restriction mapping with NdeI and XhoI or Van91I, in order to verify the presence and the *MAPT* origin of the inserts. Inserts with different size were selected in order to increase the chance of sequencing both alleles (Microsynth).

Down-regulation of Tau or P53 expression. Short hairpin RNAs (shRNAs, Table 3) were inserted in the pGreenPuro vector (SI505A-1, System Biosciences). The design of shRNAs targeting Tau or P53 was done following the manufacturer's instructions or with the tool provided by Dr. Hannon at http://katahdin.cshl.org//siRNA/RNAi.cgi?type=shRNA. Pseudo-lentiviral particles were produced in

Table 2 PCR Primers (all specific for homo sapiens mRNAs).

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
MAPT	GATCAGGATCCGTGAACTTTGAACCAGGATGGC	GATCAGGATCCGTGAACTTTGAACCAGGATGGC
MDM2	TGTTTGGCGTGCCAAGCTTCTC	CACAGATGTACCTGAGTCCGATG
TP53	CCTCAGCATCTTATCCGAGTGG	TGGATGGTGGTACAGTCAGAGC
CDKN1A	AGGTGGACCTGGAGACTCTCAG	TCCTCTTGGAGAAGATCAGCCG
DDB2	CCAGTTTTACGCCTCCTCAATGG	GGCTACTAGCAGACACATCCAG
ZMAT3	GCTCTGTGATGCCTCCTTCAGT	TTGACCCAGCTCTGAGGATTCC
RRM2B	ACTTCATCTCTCACATCTTAGCCT	AAACAGCGAGCCTCTGGAACCT
ASCC3	GATGGAAGCATCCATTCAGCCTA	CCACCAAGGTTCTCCTACTGTC
EI24	GCAAGTAGTGTCTTGGCACAGAG	CAGAACACTCCACCATTCCAAGC
GAPDH	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG
HPRT1	TGACACTGGCAAAACAATGCA	GGTCCTTTTCACCAGCAAGCT

Table 3 Oligonucleotide annealed for shRNA sequences.

P53	Sense	5′gatccgactccagtggtaatctaccttcctgtcagagtagattaccactggagtcttttg 3′
	Antisense	3′cgactccagtggtaatctaccttcctgtcagagtagattaccactggagtcttttgaatt 5′
Tau 1881	Sense	5′gatcctggtgaacctccaaaatcacttcctgtcagatgattttggaggttcaccatttttg 3′
	Antisense	3′ctggtgaacctccaaaatcacttcctgtcagatgattttggaggttcaccatttttgaatt 5′
Tau 2112	Sense	5′gatccaactgagaacctgaagcaccagcttcctgtcagactggtgcttcaggttctcagtgtttttg 3′
	Antisense	3′caactgagaacctgaagcaccagcttcctgtcagactggtgcttcaggttctcagtgtttttgaatt 5′
Tau 3127	Sense	5′gatccagcagacgatgtcaaccttgtgcttcctgtcagacacaaggttgacatcgtctgccttttg 3′
	Antisense	3′cagcagacgatgtcaaccttgtgcttcctgtcagacacaaggttgacatcgtctgccttttgaatt 5′

HEK-293 cells (HEK 293TN, System Biosciences) with the pPACKH1 kit (LV500A-1, System Biosciences) by the calcium phosphate transfection method[85]. Particles were harvested 48–72 h later, concentrated on a 30K MWCO Macrosep Advance Spin Filter (MAP030C37, Pall Corporation), aliquoted and stored at −80 °C until use. Particle titers were determined at 72 h post-transduction by calculating the percent of GFP-positive cells and the mean GFP intensity. Tau-KD and P53-KD cells were obtained upon transduction and selection in 5 µg/mL puromycin for one to 2 weeks.

Drug treatments. Etoposide (100 mM stock in DMSO; ab120227, Abcam) treatment was followed by three washes with complete DMEM and cells were allowed to recover for the indicated times. The concentration of etoposide was adapted depending on the cell line, most treatments of SH-SY5Y cells were performed at 60 or 100 µM etoposide, whereas 15 µM etoposide was used for IMR5 cells and 30 µM etoposide for IMR32 cells. When specified, recovery was done in the presence of 5 µg/mL nutlin-3 (5 mg/mL stock in DMSO; SC-45061, Santa Cruz), 10 µg/mL of KU-55933 (10 mg/mL stock in DMSO; SML1109, Sigma-Aldrich), 10 µM MG132 (10 mM stock in DMSO, M7449, Sigma-Aldrich) or 25 µM cycloheximide (10 mg/ml stock in H_2O, 01810, Sigma-Aldrich). Vehicle DMSO was added in the controls.

Immune assays. For immune staining, cells were grown on poly-D-lysine coated eight-well microscope slides (80826-IBI, Ibidi). Cells were fixed in paraformaldehyde and stained[12] with primary antibodies 1 µg/mL Tau13 (sc-21796, Santa Cruz), 0.5 µg/mL pS_{129}-H2A-X (sc-517348, Santa Cruz), 0.13 µg/mL pS_{1981}-ATM (#13050, Cell Signaling), 0.2 µg/mL pT_{68}-Chk2 (#2197, Cell Signaling), 0.4 µg/mL P53 DO-1 (sc-126, Santa Cruz,), 1 µg/mL pS_{15}-P53 (ab223868, Abcam), 0.2 µg/mL MDM2 (#86934, Cell Signaling), 0.05 µg/mL $clAsp_{175}$-Caspase3 (#9661, Cell Signaling), 0.5 µg/mL alpha-tubulin (ab18251, Abcam). Detection of endogenous Tau entailed the testing of a number of commercial antibodies so to find the human-specific Tau13 monoclonal antibody against an amino-terminal epitope as reagent providing the most reliable detection of endogenous Tau. Although determination of DSBs by γH2A-X is mostly performed by counting positive nuclear foci, we noticed that at the concentration of etoposide used here, single foci were poorly discernible, we nevertheless confirmed that mean intensity of nuclear γH2A-X staining correlated with foci counting and decided to apply the first method for our quantifications of DNA damage. Detection by fluorescent laser confocal microscopy (Nikon C2 microscope) was done with 2 µg/mL secondary antibodies anti-mouse IgG-Alexa594 or -Alexa 488 (A-11032, A-11001, ThermoFisher Scientific) or anti-rabbit IgG-Alexa594 or -Alexa488 (A-11037, A-11034, ThermoFisher Scientific). Nuclei were counterstained with 0.5 µg/mL DAPI (D9542, Sigma-Aldrich). Images were usually taken with a line by line scan with a sequence of excitations, i.e. 405 nm laser with 464/40–700/100 nm emission filter, 488 nm laser with 525/50 nm emission filter, and 561 nm laser with 561/LP nm emission filter. ImageJ was used for all image quantifications.

For biochemical analysis by western blot, cells plated in 6 well plates were directly lysed in 40 µL SDS PAGE sample buffer (1.5% SDS, 8.3% glycerol, 0.005% bromophenol blue, 1.6% β-mercaptoethanol and 62.5 mM Tris pH 6.8) and incubated 10 min at 100 °C.For immune precipitation, cells from 10 cm plates were rinsed with PBS and collected by scraping and low speed centrifugation. Cell lysates were prepared in 400 µL ice-cold RIPA buffer (R0278, Sigma-Aldrich), supplemented with protease and phosphatase inhibitor cocktails (S8820, 04906845001, Sigma-Aldrich) and treated with benzonase (707463, Novagen) 15 min at 37 °C.

Protein immune precipitation was performed by a batch procedure using Protein G-Sepharose® beads (101241, Invitrogen) overnight at 4 °C with 40 µL 30% slurry beads and 1 µg of HT7 antibody (MN1000, Invitrogen) or P53 antibody (FL-393, bs-8687R, Bioss Antibodies) antibody. The cell lysates for P53 immune precipitation were cleared by centrifugation at 20,000 g per 10 min. For immune blots[12], we used 0.2 µg/mL Tau13 (sc-21796, Santa Cruz), 0.18 µg/mL GAPDH (ab181602, Abcam), 0.4 µg/mL P53 DO-1 (sc-126, Santa Cruz), 4 µg/mL P53 Pab 1801 (sc-98, Santa Cruz), 0.5 µg/mL pS_{15}-P53 (ab223868, Abcam), 0.1 µg/mL rabbit D1V2Z MDM2 (#86934, Cell Signaling), 0.2 µg/mL mouse SMP14 MDM2 (sc965, Santa Cruz), 0.05 µg/mL $clAsp_{175}$-Caspase3 (#9661, Cell Signaling), or 0.2 µg/mL p21 (sc53870, Santa Cruz). Immune precipitated Tau protein was detected with 0.1 µg/mL biotinylated HT7 antibody (MN1000B, ThermoFisher Scientific) and 0.2 µg/mL streptavidin-IRDye (926–32230, Licor Biosciences). In the co-IP experiments, the antibodies used were 0.4 µg/mL P53 DO-1, 1 µg/mL Tau13 and 0.1 µg/mL D1V2Z MDM2.

For in cell western, cells plated on poly-D-lysine-coated 96-well microtiter plates were fixed with cold 4% paraformaldehyde in PBS 15 min at 4 °C, stained with 0.5 µg/mL pS_{129}-H2A.X (sc-517348, Santa Cruz), anti-mouse IgG-IRDye680 (926–68070, Licor), and analyzed on a dual fluorescent scanner (Odyssey CLx, LICOR). Determination of 0.5 µg/mL DAPI staining for normalization was performed with an absorbance reader (Infinite M Plex, Tecan).

Cell death and senescence assays. The LDH (Pierce LDH Cytotoxicity Assay Kit; 88954, ThermoFisher Scientific) and MTS assay (CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay; G5421 Promega) were done following the manufacturer's instructions. For LDH, conditioned medium from cells plated in a 96-well microtiter plate was analyzed by measuring absorbance at 490 and 680 nm (Infinite M Plex, Tecan). Colorimetric measurement for MTS was performed at 490 nm (Infinite M Plex, Tecan).

Senescence-associated β-galactosidase staining was determined on cells plated in six-well plates, fixed with 2% paraformaldehyde 10 min at RT and washed twice with gentle shaking 5 min at RT. Then, cells were incubated with 1 mg/mL X-gal (20 mg/mL stock in DMF; B4252, Sigma-Aldrich,) diluted in pre-warmed 5 mM potassium ferricyanide crystalline (P-8131, Sigma-Aldrich), 5 mM potassium ferricyanide trihydrate (P-3289, Sigma-Aldrich), and 2 mM magnesium chloride (M-8266, Sigma-Aldrich) in PBS at pH 6.0. Acquisition and quantification of the

images for SA-βGal and cell area was done with an automated live cell imager (Lionheart FX, BioTek).

Comet assay. The assay was performed according to the manufacturer's instructions (STA-351, Cell Biolabs INC.). In short, cells were plated in 6 well plates for drug treatments, collected, counted, resuspended at 100,000 cells/mL and washed with PBS at 4 °C. Cells were mixed at 1:10 with low melting agarose at 37 °C, and poured on a glass microscope slide. After cell lysis, the slides were maintained at 4 °C in alkaline buffer (pH > 13) for 20 min. After electrophoresis, the slides were washed three times with a neutralizing buffer and stained with the Vista Green DNA Dye (#235003, Cell Biolabs). All manipulations were performed protected from direct light. Analysis was performed by capturing Z-stack images with a laser confocal microscopy and measurement of Olive tail moment with the CaspLab software.

RNA extraction and RT-qPCR. Total RNA extraction using the TRIzol™ Reagent (15596026, Invitrogen) and cDNA synthesis using the GoScript™ Reverse Transcription Mix, Random Primers (A2800, Promega) were done according to the manufacturer's instructions. Amplification was performed with SsoAdvanced™ Universal SYBR® Green Supermix (1725271, BioRad) with 43 cycles at 95 °C for 5 s, 60 °C for 30 s and 60 °C for 1 min (for the primers see Table 2). Relative RNA expression was calculated using the comparative Ct method and normalized to the geometric mean of the GAPDH and HPRT1 mRNAs.

Statistics and reproducibility. Statistical analysis was performed with the aid of GraphPad Prism version 8.4 using the method specified in the legend of each figure. Exact *p*-values are specified in the figures. All quantifications were performed based on at least three independent biological replicates, sample size, number of replicates and how they are defined is specified in the figure legends. When indicated, western blots and microscopic images are shown for representative data.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The raw data for all the figures (Supplementary Data 1) and Supplementary Figures (Supplementary Material) are included as a Supplementary Data Files. All genomic sequencing data generated by the external provider (Microsynth AG, Balgach, Switzerland) for the CRISPR/Cas9 edited exon of *MAPT*, and all maps and sequences of the gene-editing plasmids and shRNA plasmids are included as supplementary material (Supplementary Data 2). All the data generated and/or analyzed, all plasmids and cell lines included in the current study are available from the corresponding authors on reasonable request.

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Author contributions

M.S. and C.M. designed, performed, analyzed and described most of the experiments; G.P. designed the strategy and established the knock-out cell lines; S.Pi. helped in implementing several analytical procedures; A.S. helped in creating, isolating and characterizing cell lines; S.Pa. supervised the whole study and wrote the first draft, P.P. finalized the paper; all authors revised the paper; S.Pa. and P.P. conceived and designed the study and provided the financial support for this study.

Competing interests

The authors declare no competing financial and non-financial interests. P.P. and S.Pa. owns stocks of AC Immune SA. No funding organizations was involved in the conceptualization, design, data collection, analysis, decision to publish, preparation of the paper, or may gain or lose financially through this publication. There are no patents, products in development, or marketed products to declare.

Additional information

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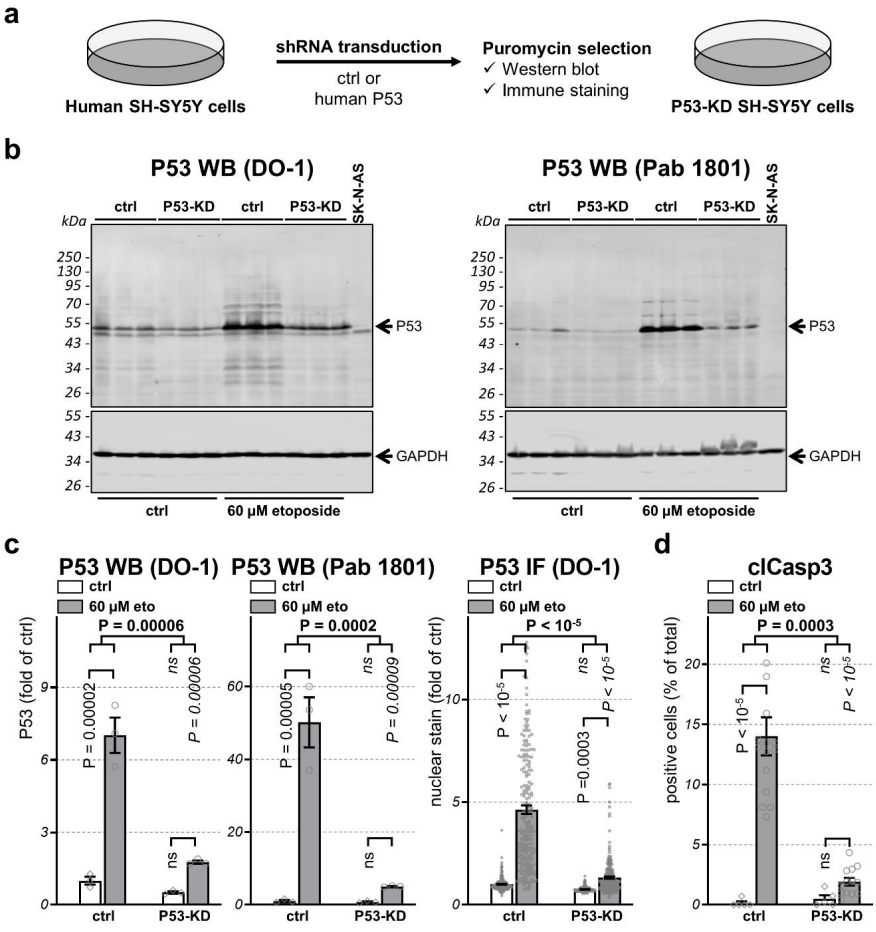
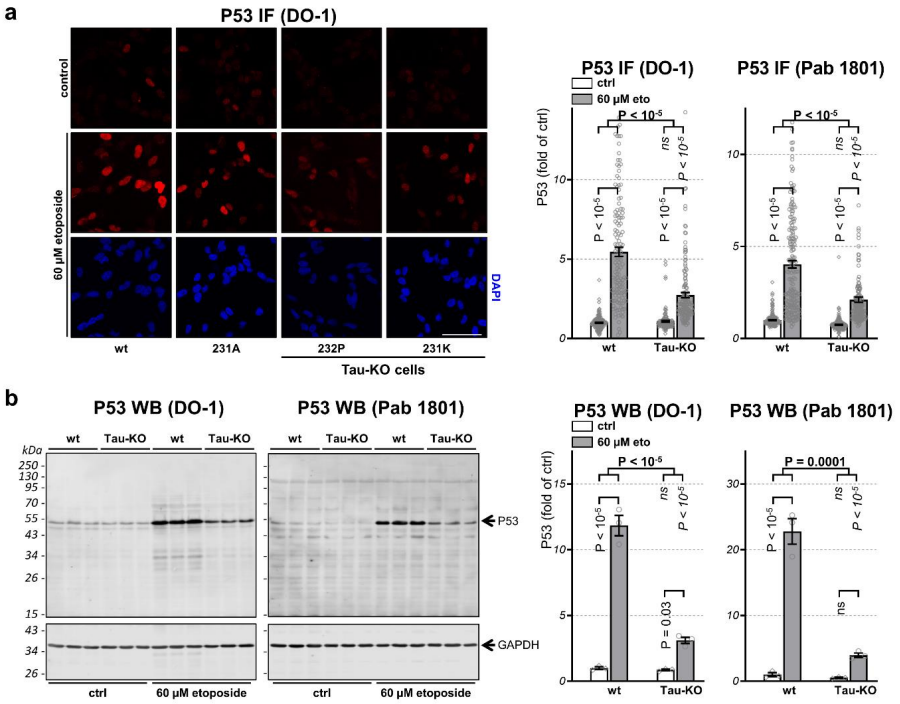


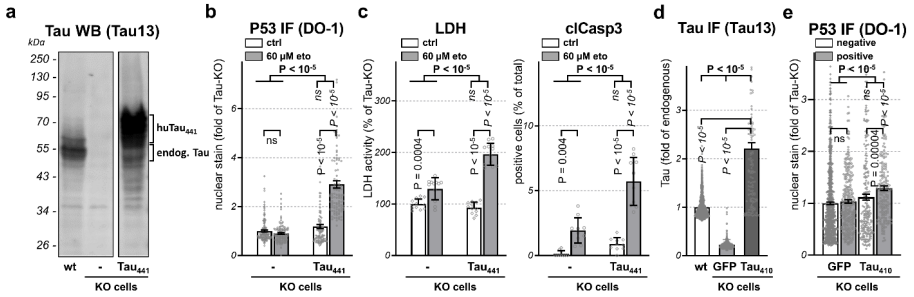
Supplementary Figure 1. DSBs-induced apoptosis is P53-dependent in SH-SY5Y cells. a. Scheme of procedure used to generate P53-KD cells and for their characterization by western blot and P53 immune staining when compared to cells transduced with the parental shRNA plasmid (ctrl). **b.** Cells at basal conditions (ctrl) or treated 30 min with 60 µM etoposide and recovered for 6 h before western blot analysis with the indicated P53 antibodies. GAPDH served as loading control. **c.** Western blot quantification of P53 signal intensity normalized for GAPDH shown as fold of control cells at basal conditions, mean +/- SD of n = 3 biological replicates. On the left is show the quantification of P53 immune staining as fold of control cells at basal conditions, mean

intensity +/- sem of single cell nuclear P53 staining (DAPI mask, ImageJ), n >219 cells/condition distributed over 5 images. **d**. Quantification of clCasp3 immune staining shown as percent of total DAPI-positive cells, mean ± SD of 5 images for the untreated cells (ctrl) and of 15 images for etoposide-treated cells (60 µM eto), n >500 cells/condition. Statistical analysis by independent measures ordinary 2way ANOVA, source of variation for cell lines (in bold), multiple Bonferroni pairwise comparisons among lines for each treatment (in italics), or among treatment for each line (in vertical).



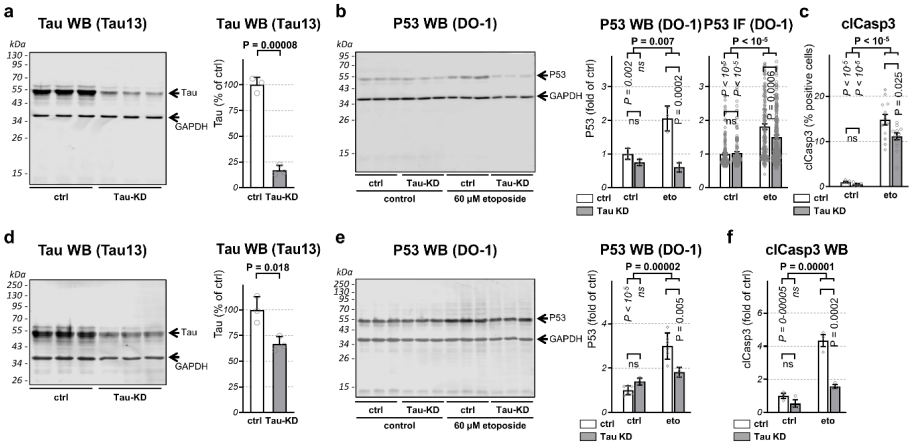
Supplementary Figure 2. Tau deficiency reduces cellular P53. a. Representative laser confocal microscopy images of P53 DO-1 antibody-immune stained parental cells (wt) and the indicated CRISPR-Cas9 cell lines at basal conditions (control) or treated for 30 min with 60 µM etoposide and recovered for 6 h. Shown are also the nuclear staining with DAPI. Scale bar 50 µm. Quantification of mean intensity +/- sem of single cell nuclear P53 staining (DAPI mask, ImageJ) shown as fold of wt cells at basal conditions using the indicated P53 antibodies, n >90 cells/condition distributed over 5 images. **b.** Parental (wt)

or 232P (Tau-KO) cells at basal conditions (ctrl) or treated for 30 min with 60 μM etoposide followed by 6 h recovery were analyzed for P53 by western blot with the indicated P53 antibodies, n = 3 biological replicates. GAPDH served as loading control. Quantification of P53 signal intensity normalized for GAPDH, mean +/- SD shown as fold of control (wt or ctrl cells at basal conditions). Statistical analysis by independent measures ordinary 2way ANOVA, source of variation for cell lines (in bold), multiple Bonferroni pairwise comparisons for each treatment among lines (in italics), or among treatment for each line (in vertical).



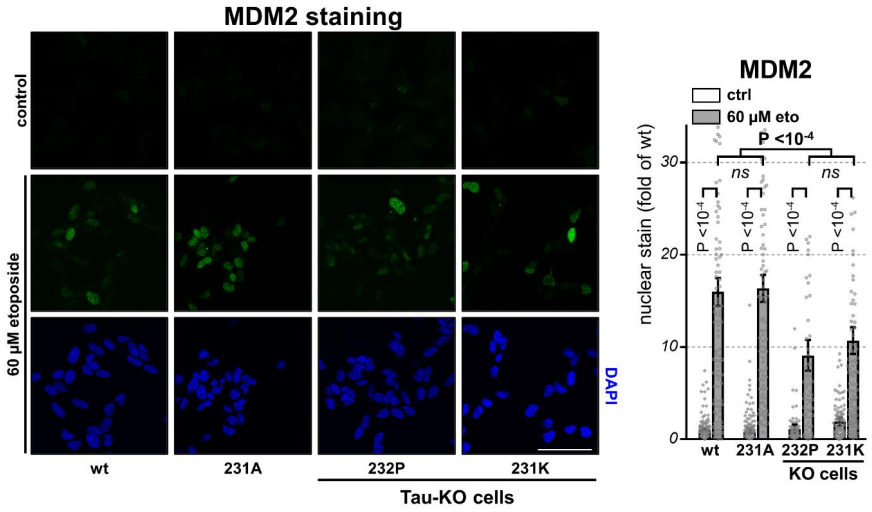
Supplementary Figure 3. Restoring Tau expression in Tau-KO cells rescues P53 and the apoptotic phenotype. a. Parental (wt), 232P (KO cells) or 232P cells stably re-expressing 4R-Tau (Tau_{441}) analyzed by western blot with the Tau13 antibody. **b.** Quantification of single cell nuclear P53 immune staining (DAPI mask, Image J) of 232P Tau-KO cells or 232P-Tau_{441} cells at basal conditions (ctrl) or treated 30 min with 60 μM etoposide and allowed to recover 6 h (60 μM eto), mean ± sem of n >100 cells/condition distributed over 5 images shown as fold of 232P cells at basal conditions. **c.** LDH release from the same cells treated as in **b.** (2 d recovery). Values are shown as percentage of Tau-KO cells at basal conditions, mean ± SD of 12 wells from three independent experiments. Cells were also analyzed by immune staining for clCasp3 at 6 h recovery. Percent clCasp3-positive cells, mean ± SD of 10 images, n >500 cells/condition. **d.** Quantification of Tau13 immune stained Tau in parental cells (endogenous Tau, wt), or in GFP-positive 232P cells (KO cells) transiently transfected with a 1:10 ratio of empty:GFP plasmids (GFP) or of 3R-Tau:GFP plasmids (Tau_{410}). Cells were stained 3 days after transfection (GFP mask, Image J). **e.** Immune staining quantification of nuclear P53 (DAPI mask, Image J) in GFP-positive 232 cells (KO cells) transiently transfected as in **d.** and treated for 30 min with 60 μM etoposide and recovered for 6 h, mean intensity ± sem

shown as fold of control-transfected 232P cells, n >100 cells/condition distributed over 5 images. Statistical analysis by independent measures ordinary 2way ANOVA, source of variation between non-transfected and Tau-transfected conditions (in bold), multiple Bonferroni pairwise comparisons among lines for each treatment (in italics), among treatment for each line (**b** and **c**, in vertical).

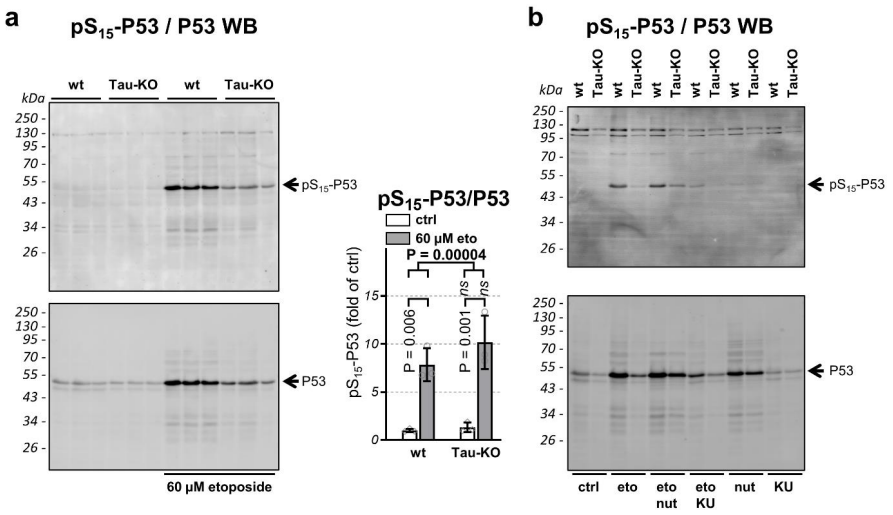


Supplementary Figure 4. Reduced etoposide-induced P53 protein in Tau-KD IMR5 and IMR32 cells. a. Cell lysates obtained from IMR5 cells transduced with the parental shRNA plasmid (ctrl) or with the Tau shRNA plasmid 2112 (Tau-KD) were analyzed by western blot for Tau expression. The Tau signal was normalized for GAPDH and reported as percent of the control, mean +/- SD, n = 3 biological replicates. Unpaired student t-test. **b.** The two IMR5 cell lines at basal conditions or treated 30 min with 15 μM etoposide and recovered for 6 h were analyzed by western blot with the P53 DO-1 antibody. The Tau signal was normalized for GAPDH and reported as fold of the control, mean +/- SD, n = 3 biological replicates. Same conditions were applied to quantify immune stained P53, mean intensity +/- sem of single cell nuclear P53 staining (DAPI mask, ImageJ) shown as fold of untreated control cells (ctrl), n >100 cells/condition distributed over 5 images. **c**. Apoptotic cells were determined by clCasp3 immune staining and reported as percent clCasp3-positive cells of total DAPI-positive cells, mean ± SD of 5 images for the untreated cells (ctrl) and of 15 images for etoposide-treated cells (15 μM eto), n >500 cells/condition. **d**. Same as in **a.** for IMR32 cells transduced with the parental shRNA plasmid (ctrl) or with Tau shRNA plasmid 3127 (Tau-KD). **e.** Same as in **b.** for the western blot analysis. **f**.

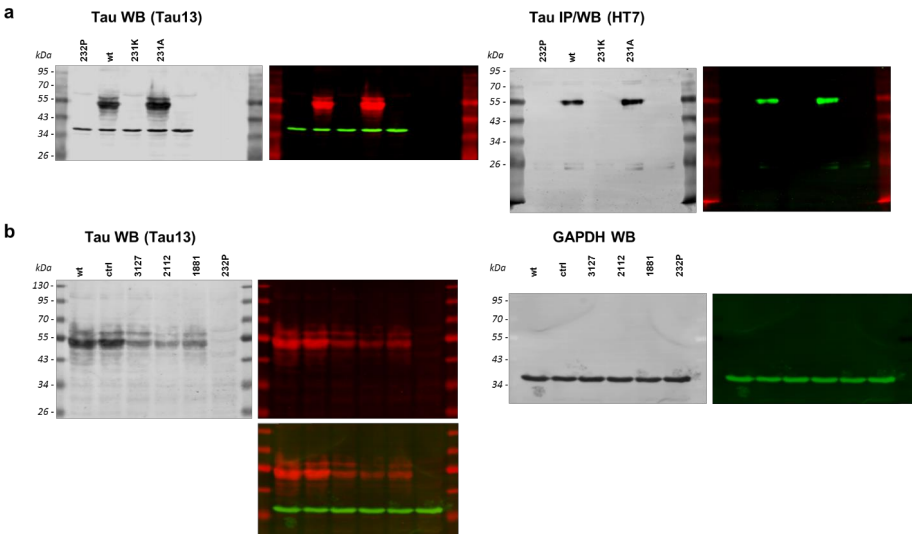
Same as in **c.** for 15 and 17 kDa clCasp3 fragments determined by western blot, mean +/- SD (n = 3 biological triplicates). Statistical analysis by independent measures ordinary 2way ANOVA, source of variation for cell lines (in bold), multiple Bonferroni pairwise comparisons for treatment between lines (in italics) or for each line (in vertical).



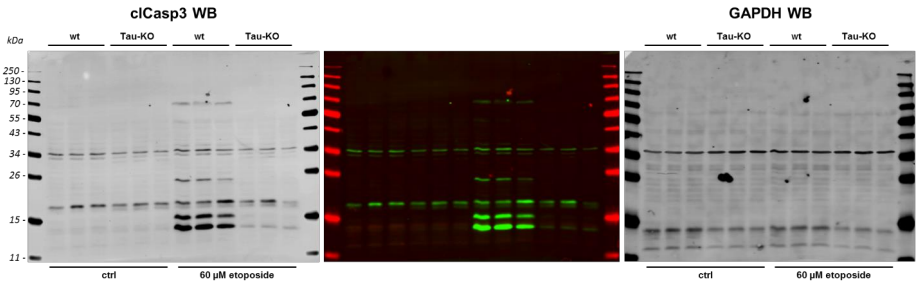
Supplementary Figure 5. Tau deficiency affects MDM2. a. Representative images by laser confocal microscopy of parental (wt) or CRISPR-Cas9 cell lines immune stained with the rabbit MDM2 antibody and counter-stained with DAPI. Cells at basal conditions (control) or after 30 min 60 µM etoposide and 6 h recovery, scale bar 50 µm. Mean intensity +/- sem of single cell nuclear MDM2 staining (DAPI mask, ImageJ) shown as fold of wt cells at basal conditions, n >90 cells/condition distributed over 5 images. Statistical analysis by independent measures ordinary 2way ANOVA, source of variation for genotype (in bold), multiple Bonferroni pairwise comparisons for etoposide treatment between lines with same genotype (in italics) or for the etoposide treatment for each line (in vertical).



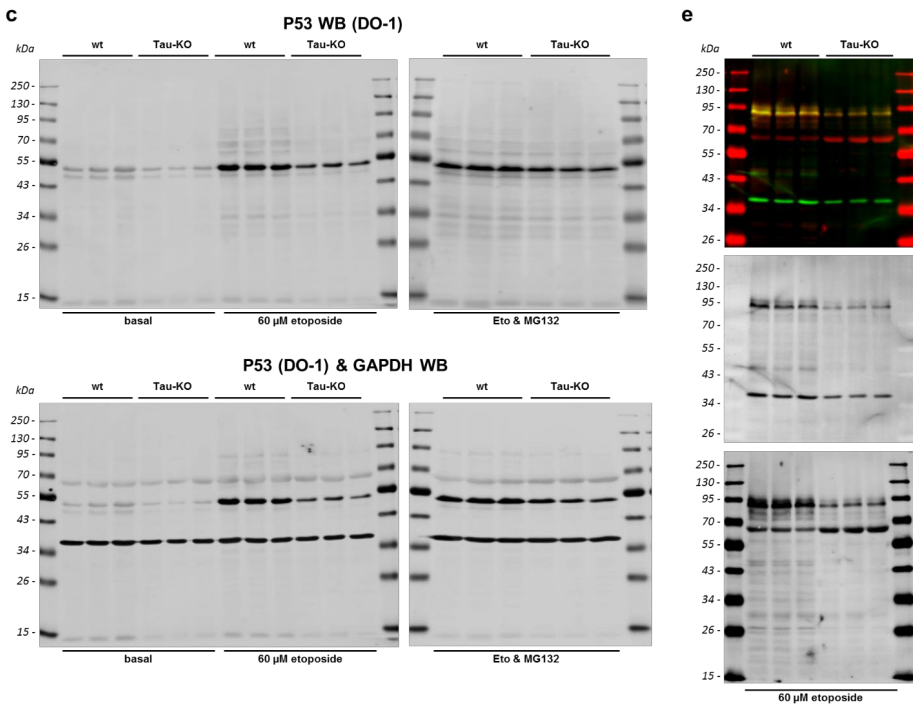
Supplementary Figure 6. Analysis of pS$_{15}$-P53 phosphorylation. a. Parental (wt) or 232P (Tau-KO) cells at basal conditions or after 30 min 60 μM etoposide and 6 h recovery were analyzed for pS$_{15}$-P53 or P53 by western blot, n = 3 biological triplicates. Quantification was performed by measuring the signal intensity of pS$_{15}$-P53 normalized for the signal of total P53, mean +/- SD shown as percent of control (parental cells at basal conditions). Statistical analysis by independent measures ordinary 2way ANOVA, source of variation for cell lines (in bold), multiple Bonferroni pairwise comparisons among lines for each treatment (in italics), or of treatment for each line (in vertical). **b.** Parental (wt) or 232P (Tau-KO) cells at basal conditions (ctrl) or analyzed after 6 h recovery from a 30 min treatment in the absence or presence of 60 μM etoposide in the absence (eto), the recovery was performed in the absence or presence of 5 μg/mL nutlin-3 (eto nut) or 10 μg/mL KU-55933 (eto KU) as indicated. Analysis by western blot for pS$_{15}$-P53 or P53, a representative experiment out of three experiments is shown.



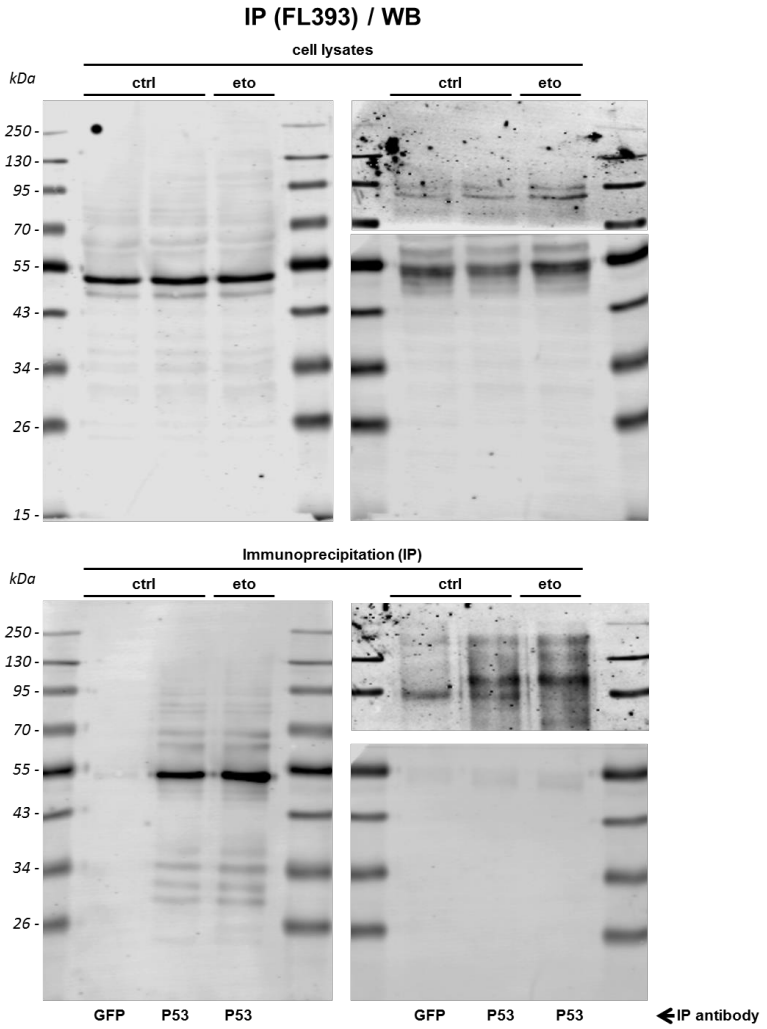
Supplementary Figure 7. Unprocessed western blot images (in grey tones) used for creating the corresponding panels in Fig.1a and 1b. Shown are also the original dual fluorescence images.



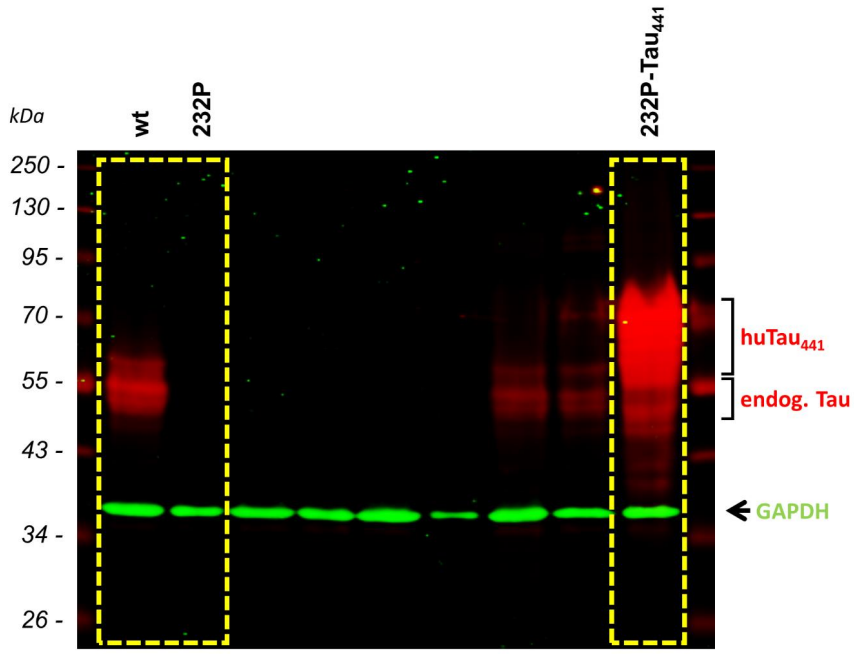
Supplementary Figure 8. Unprocessed western blot images (in grey tones) used for creating the corresponding panels in Fig.2. Shown are also the original dual fluorescence image.



Supplementary Figure 9. Unprocessed western blot images used for creating the corresponding panels in Fig.7c and 7e.



Supplementary Figure 10. Unprocessed western blot images used for creating the corresponding panels in Fig.8. The MDM2 and Tau blots shown on the left were cut between the 55 kDa and the 95 kDa protein size markers and analyzed separately.



Supplementary Figure 11. Unprocessed gel image used for creating Supplementary Fig.3a.

3.2 Tau binds to MDM2 and modulates P53 activity

Based on the data showing a Tau-dependent increasing activity of P53, the aim of the second part of my project was to elucidate the molecular mechanism responsible for this effect.

P53 stability and activity is tightly suppressed by MDM2 by at least three distinct mechanisms: P53 polyubiquitination and proteasomal degradation, inhibition of P53 binding to DNA, cytosolic translocation of nuclear P53[161]. Multiple structural domains of MDM2 contributes to its activity towards P53. The N-terminal domain forms a deep pocket binding to an alpha-helix of P53. CK1-phosphorylation of the central domain of MDM2 forms a second binding domain for P53[241]. The RING domain at the C-terminus of MDM2 represent the E3 catalytic domain for P53 ubiquitination.

In this part of my project, I implemented different assays to demonstrate the formation of a complex between Tau and MDM2, and to characterize the protein domains driving the interaction. Furthermore, I provide evidence that the binding of Tau to MDM2 has a functional impact on its E3 ubiquitin ligase activity towards P53 and that this is impaired when Tau carries a point mutation causing an autosomal variant of FTD. I also reported an abnormal accumulation of MDM2 colocalizing with Tau tangles in the AD and FTD brain.

This study offers insight on the molecular mechanisms utilized by Tau to regulate P53 activity and possibly additional function of its E3 ligase MDM2. The data may facilitate the discovery of experimental drugs targeting this protein-protein interaction that has obvious implication in major aging-associated human disorders and unmet medical needs.

The data obtained are described in a manuscript currently drafted for submission for peer-reviewed publication.

Tau Binds to MDM2 and Modulates P53 Activity

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INTRODUCTION

Fibrillar Tau protein deposition in the brain is the hallmark of tauopathies, a group of disorders best represented by Alzheimer's disease (AD)[1]. Tau accumulation in brain neurofibrillary tangles correlates with the clinical course[2,3]. Mutations in the *MAPT* gene encoding for Tau lead to fronto-temporal dementia with Parkinsonism 17 (FTDP-17)[2,3]. Since Tau is a microtubule-associated protein, an accepted concept explaining the pathogenesis of tauopathies is that abnormal phosphorylation and folding cause Tau detachment from microtubules followed by oligomerization, fibril formation, acquisition of toxic properties, deposition in neurofibrillary tangles and neuropil threads, a cascade of events tightly associated with neuronal dysfunction and death[4,5].

In addition to its well-characterized role in neurodegeneration, an implication of Tau in cancer is emerging with several studies describing positive or negative correlations between Tau expression and survival in various types of cancers[6–8]. Microtubule-related and -unrelated functions of Tau may underly these correlations. Indeed, new roles of Tau in addition to the one well characterized on microtubules have been shown. Tau localizes in the cell nucleus where it binds to the DNA[9–12]. Nuclear Tau protects the DNA, whereby heat or oxidative stress favor nuclear Tau translocation[13]. Enhanced DNA damage was observed in Tau-KO neurons when compared to normal neurons[14]. In addition, chromosomal abnormalities and increased DNA damage are found in AD-derived fibroblasts[15] and in AD brains[16,17]. We reported that also an acute DNA damage increases nuclear translocation of Tau and that nuclear Tau present a distinct phosphorylation patter when compared to cytosolic Tau[18]. More recently, we described that Tau modulates P53 stability and activity leading to a change in cell fate response in cells with an unresolved DNA damage[19].

Somatic mutations in the guardian of the genome P53 or its gene *TP53* account for >50% of human cancers[20–22]. Under basal conditions, cells maintain a low amount of P53 by post-translational mechanisms tightly regulated by a complex protein network with the E3 ubiquitin ligase MDM2 as a main actor[23]. All three structural domains of MDM2 participate in steering P53 degradation by the ubiquitin proteasome system or the subcellular distribution of P53[9–12]. The N-terminus of MDM2 forms a deep hydrophobic pocket binding to an α-helix in the transactivation domain of P53 thus also antagonizing the transcription of P53 target genes[24]. The C-terminal RING domain of

MDM2 executes the E3 ligase activity that modifies lysine residues at the C-terminus of P53[25,26], but also contributes to tethering the MDM2-P53 complex to the 19S subunit of the proteasome[27]. The phosphorylation of the central acidic region, mainly by CKIδ, enhances the association of MDM2 to P53 and, notably, the same region also binds intramolecularly the RING domain thus impairing interaction with the proteasome[28]. The P53-MDM2 axis is a fundamental pathway to overcome cellular stress due to its role as central coordinator of cellular stress response. As an example, P53-MDM2 drive the recovery process during the DNA damage response, but also balance survival, senescence and apoptosis of irreversibly lesioned cells[21].

Here we characterized the molecular mechanism underlying Tau-dependent P53 modulation. The starting point of our study was the observation that Tau-depletion also modified MDM2 and that the effect of Tau on P53 was reversed by nutlin-3, a specific inhibitor of the P53-MDM2 interaction[19]. We report now that Tau binds to MDM2 and that this inhibits P53 ubiquitination in a manner sensitive to the presence of the P301L mutation linked to FTDP-17. Notably, we also observed an abnormal accumulation of MDM2 in Tau tangles of AD and FTD. The minimal domains interacting with each other in the Tau-MDM2 complex correspond to the microtubule-binding domain (MBD) of Tau and the central acidic domain of MDM2. An interaction that is reminiscent to that of the MBD with the negatively charged microtubule surface.

RESULTS

Tau binds to MDM2 in HeLA and SH-SY5Y cells. Our first attempt to assess the existence of a Tau-MDM2 complex was performed starting from full-length human proteins ectopically expressed in human HeLa cells and analysis by immune precipitation / western blot (IP/WB). Analysis of cell extracts revealed MDM2 (95-100 kDa) and Tau (70-80 kDa) at the expected apparent molecular weight range (**Fig 1A**). We observed co-isolation of Tau following immune precipitation of MDM2 with a specific anti-tag antibody but not with a control antibody (**Fig 1A**). Reversed immune isolation with three different anti-Tau antibodies revealed co-isolation of MDM2 when the amino-terminal and central Tau antibodies were used by not when with the antibody directed against the MBD of Tau, possibly indicating epitope masking when Tau is bound to MDM2 (**Fig 1B**). As the co-immune isolated proteins gave a relatively weak

signal, we switched to a homogenous immune assay based on the highly sensitive AlphaLisa® technology performed in small sample volumes. AlphaLisa® requires two antibodies coupled to donor and acceptor beads with bead proximity measured by an oxygen radical-induced fluorescent signal. Total Tau was detected with a commercial kit using the antibody pair Tau12 (epitope corresponding to amino acids 6-18 of Tau2N4R) and BT2 (epitope 194-198). The MDM2 assay was developed in house with the antibody pair D1V2Z (MDM2 epitope surrounding Val280) and SMP14 (154-167). Both assays detected the corresponding proteins ectopically expressed in HeLa cells with high sensitivity since the cell extracts were diluted 1:10'000 for Tau and 1:50 for MDM2 (**Fig 1C**). Specificity of the signals was confirmed by omitting one of the two antibodies forming the detection pair. The Tau-MDM2 interaction was assessed with the antibody pair HT7 (Tau epitope 159-163) and D1V2Z. The Tau-MDM2 complex was detected in cell extracts diluted 1:250(**Fig 1C**). This highlights the exquisite sensitivity of the AlphaLISA® that is performed with roughly 20'000-time less cell extract than the 240 µg total protein used for the IP/WB protocol. We therefore opted for the use of the Tau-MDM2 AlphaLisa® to detect the endogenous interaction in human neuroblastoma SH-SY5Y cells or, as a negative control, in Tau-knock-out SH-SY5Y cells[19]. We obtained a signal for endogenous Tau corresponding to ~19-fold the background signal measured in Tau-KO cells, whereas the MDM2 signal was ~2.3-fold that of the no-D1V2Z control in wild-type and Tau-KO cells (**Fig 1D**). Importantly. the Tau-MDM2 assay confirmed the presence of the endogenous Tau-MDM2 complex with a signal ~2.7-fold that measured in Tau-KO cells SH-SY5Y cells (**Fig 1D**).

The microtubule-binding domain of Tau binds to the acidic region of MDM2. To define the interacting domains in the Tau-MDM2 complex, we generated plasmids encoding for truncated polypeptides of Tau and of MDM2 (**Fig 2A**) and assessed the co-isolation of the Tau fragments by western blot after immune isolation of MDM2. Firstly, we selected three complementary MDM2 fragments of corresponding to the P53 binding domain (amino acids 1-101), the central region (102-361), and the C-terminal portion (361-491) the caspase cleavage site and including the RING domain domains. When co-expressed in HeLa cells, full-length Tau2N4R was co-immune precipitated with the central region of MDM2 whereas no interaction was observed with the other two fragments of MDM2 (**Fig 2B**). Then, the central region of MDM2 was co-expressed with two complementary halves of Tau truncated between the proline-reach

region and the MBD (**Fig 2A**), to show specific immune isolation of the C-terminal half (**Fig 2C**). In fact, the MBD of Tau (K18, 244-372) was found to interact with the central region of MDM2 (**Fig 2C**). In an additional experiment to assess whether the tagging of Tau may interfere with the co-isolation, full-length T_{11}β1-MDM2 was co-expressed with a set of Tau fragments carrying the T_{10}-tag either at their N- or at their C-terminus. Again, immune isolated MDM2 was found to interact with the MBD of Tau both when fused down-stream or up-stream of the tag (**Suppl Fig 1**).

Subcellular localization of the Tau-MDM2 complex in living cells. Tagging of two proteins each with one β-strand of GFP (T_{10} or T_{11}) and their co-expression with the complementary GFP sequence (GFP_{1-9}) allow confirming the interaction between the two tagged proteins because of biofluorescent trimolecular complementation (TriFC) of the 11-stranded GFP (**Fig 3A**). First, we expressed the T_{11}β1-MDM2 fragments with the GFP_{1-10} sensor in order to determine the amount of each MDM2 fragment present in cells by bimolecular complementation (biFC, **Fig 3B, left panel**). Then the three T_{11}β1-MDM2 fragments were co-expressed with Tau MBD-HAT_{10} and the GFP_{1-9} sensor. Single-cell analysis by cytofluorimetry was used to determine the relative number of intact cells containing a Tau-MDM2 complex (**Fig 3B, central panel**). Since the variable between the three conditions is represented by the different MDM2 fragments, we normalized the triFC signal (Tau-MDM2 complex) with the biFC signal (MDM2)[29]. This demonstrated a higher percentage of cells positive for TriFC when expressing the central region of MDM2 together with the MDB of Tau thereby validating the IP/Western-blot results (Fig 2). Analysis of the TriFC signal by confocal fluorescent microscopy, showed again complex formation when HeLa cells expressed MBD of Tau and the central region of MDM2 and allowed demonstrating that the complex was preferentially located in the nucleus (**Fig 3C**). We also evaluated a possible interaction of Tau with MDM4, the MDM2 homolog that forms heterodimer with MDM2. We detected a strong interaction of Tau with MDM4 by TriFC, both by confocal microscopy and cytofluorimetry analysis **(Fig 3D)**. Based on biFC using either MDM2 or MDM4, we detected a nuclear localization of MDM2 whereas MDM4 is mainly cytoplasmic. The triFC signal between Tau and the two homologs was nuclear when Tau was co-expressed with MDM2 and cytoplasmic when Tau was co-expressed with MDM4. This interaction between Tau and MDM4 is not surprising due the high level of homology between MDM4 and MDM2.

TriFC is associated to irreversible GFP reconstitution thus impeding dissociation of the protein complex. In contrast reconstitution of luciferase enzymatic activity is reversible process, allowing flexible analysis of kinetic aspects of the protein-protein interaction. ReBiL is a luciferase reconstitution assay obtained by comparable and simultaneous transcription of the mRNAs of two complementing fragments by an inducible bidirectional promoter[30]. For the ReBiL technology, we generated constructs encoding for the different fragments of interest, all tagged with either at their C-terminus or N-terminus one of the two luciferase fragments (N-Luc, C-Luc). We studied the reconstitution of the luciferase signal in transiently transfected human U2OS cells, which were used to study the P53-MDM2 complex previously[30]. The test was performed after transfection of the plasmids and the luminescence was detected in cells lysates (**Fig 3D**). We observed that the cells overexpressing the MBD of Tau and the central region of MDM2 showed a stronger luminescence signal compared to the one expressing MBD with the C-terminal region of MDM2 (**Fig 3D**).

Tau inhibits P53 ubiquitination by MDM2. We tested the impact of Tau on the activity of MDM2 using a commercial kit containing all required reagents necessary for ubiquitination of P53. The reaction was performed in triplicates according to the protocol specified by the manufacturer and ubiquitinated P53 was detected a multiples bands migrating slower than unmodified P53 (**Fig 4A**). Western blot analysis was performed to show that all conditions had similar amounts of MDM2 and P53. Strickling, supplementing the reaction with recombinant Tau expressed in bacteria MDM2 activity showed a ~80% reduction. A result in good agreement with our observation that Tau depletion increased MDM2-dependent degradation of P53 by the proteasome[19]. Notably, the autosomal dominant FTDP-17 point mutation P301L of Tau reduced by ~50% the inhibitory effect of Tau on MDM2-dependent P53-ubiquitination (**Fig 4A**). The P301L mutation of Tau is located in a highly conserved stretch of the MBD, which we showed to bind to the central region of MDM2.

Abnormal MDM2 accumulation of MDM2 in brain Tau tangles. Prompted by the data obtained so far, we investigated whether fibrillar Tau deposition in the brain may alter MDM2 expression. Twenty µm frozen sections obtained from an AD patient were stained with antibodies against Tau or MDM2. As expected, the presence of hallmark neurofibrillary tangles and neuropil threads was revealed by the TauAS polyclonal antibody as well as the AT8 antibody against a pathological phosphorylation of

Tau(Malia, Teplyakov et al. 2016). Consecutive sections derived from the same donors were then immune stained with the MDM-323 antibody. A strong immune reactivity for MDM2 was observed in the tauopathy brains. MDM2 staining appeared to colocalize with Tau in a subset of neurofibrillary tangles (**Fig 5)**.

DISCUSSION

We demonstrated a yet unreported interaction between the proteins Tau and MDM2 in multiple human cell lines that we observed by orthogonal technologies. With about 200 proteins the MDM2 interactome encompasses ribosomal proteins, transcription factors, tumor suppressors, DNA repair mediators, cell fate modulators, E2 ubiquitin-protein ligases and other regulators of cell function[31]. The large number of binding partners is likely to result from the scaffold nature of MDM2 organized in distinct structural domains linked by disordered amino acid sequences. This apparent promiscuity contrasts with the selective and tightly regulated functions attributed to MDM2. Perhaps, it could be rationalized by a model where different PTMs and conformations of MDM2 restrict the set of interacting partners[32]. Tissue-specific absence of binding partners may further limit the spectrum of MDM2 activity in differentiated cells. Then again, preferential expression of Tau in specific cell populations may explain why the Tau-MDM2 complex eluded previous studies.

We showed that the minimal interacting domains are the MBD of Tau and the central domain of MDM2, composed of an acidic domain (residues 243–301) and a zinc-finger domain (residues 290–335). This region of MDM2 also interacts with the DNA binding region of P53 and favors a conformational switch in P53[33], possibly regulated by CKIδ phosphorylation[28]. Moreover, the central region of MDM2 forms an intramolecular interaction with the RING domain, that impairs the binding of this latter to the proteasome. It is therefore predictable that the binding of Tau to the central domain of MDM2 may interfere with these functions regulating P53 stability and activity. Consist with this hypothesis, we report that the presence of Tau interferes with MDM2-dependent ubiquitination of P53 in an *in vitro* assay based on recombinant proteins. However, numerous proteins bind to the central region of MDM2 and regulate its E3 ubiquitin-protein ligase activity. A prominent ligand is the tumor suppressor ARF, which inhibits the E3 ligase activity of MDM2 and targets MDM2 to the nucleolus thereby

reducing its nuclear availability[34,35]. The zing finger domain of MDM2 interacts with factors regulating cell growth and proliferation, as well as RNA proteins when released following a ribosomal stress[36]. Whether Tau may interfere with these additional functions of MDM2 is a matter of future studies.

Tau is described as an unfolded protein[4], which acquires a limited defined structure e.g. when the MBD binds to microtubules[37]. So, it has been described that the net positive charge of the MBD and the acidic character of the microtubule surface represent contributing determinants of their interaction, which then is inhibited when the MBD is phosphorylated. The MBD is also subjected to splicing generating the 4R and 3R isoforms of Tau, which show differential function and implication in disease[38,39]. Indeed, the MBD of Tau is prone to multimerize into β-sheet structured fibrils in disease[40]. Moreover, most autosomal dominant FTDP-17 mutations target the MBD[41], possibly affecting its splicing, its modification by PTMs and its ability to acquire defined structures when interacting with other macromolecules or when multimerizing. In this contest, it is intriguing that the FTDP-17 mutation P301L interferes with the inhibition of MDM2 by Tau. Notably, FTDP-17 MAPT mutations increase the incidence of cancer[42], hyperphosphorylated and insoluble Tau is detected in some prostate cancers[43], and Tau expression affect survival in various cancers[6–8].

The central domain of MDM2 also contains the nuclear import/export signals important for the subcellular distribution of MDM2. Interestingly, we observed that the Tau-MDM2 interaction occurs mainly in the cell nucleus, whereas the MDM4-Tau complex is predominantly cytoplasmic. We can maybe speculate that MDM2 may contribute to the nuclear localization of Tau in the nucleus in presence of a DNA damage[18]. Also, the binding of Tau to MDM2 and MDM4 suggests that Tau may modulate P53 by acting on both proteins. In this contest, it is interesting that in the central nervous system MDM2 needs MDM4 to regulate P53[44], whereas in other tissues MDM2 alone is sufficient to prevent accumulation of P53[45]. Since MDM4 lacks the E3 ligase activity, binding of Tau to MDM4 may not affect P53 stability. Instead, Tau may modulate MDM4 inhibition of P53 transcriptional[46]. MDM2 and MDM4 play a key role in cancer by controlling P53 activity but they are also associated with aging and senescence in a P53-independent manner. As an example, a germline mutation in MDM4 is associated to shortened telomers and increase of senescence linked to dyskeratosis congenital[47].

The presence of MDM2 in human brain or in neurodegenerative disorders has not been investigated so far. Our observation that MDM2 accumulation is associated to neurofibrillary tangles in the AD brain calls for filling this gap in knowledge. However, a possible role of P53 in CNS disorders has been proposed[48]. Whereas cancers are linked to P53 inactivation, in neurodegeneration the level and activity of P53 appear increased[49]. A relatively high incidence of P53 mutations is reported in AD[50]. In addition, some abnormal P53 species are considered as potential biomarkers of AD [51–54]. Genetic manipulation of P53 family members in mice affects aging and cognitive decline[55,56]. Increased DNA damage is found in AD[17,57] and persistent DDR causes neuronal senescence and upregulation of pro-inflammatory factors[58]. The link between Tau and the P53-MDM2/MDM4 axis may likely explain the de-regulation of the P53 pathway and other P53 and/or MDM2/MDM4-dependent signaling in neurodegeneration as well as the observed correlation between Tau expression and cancer survival.

MATERIALS AND METHODS

DNA Plasmids

For most experiments, the plasmid pcDNA3 drove ectopic expression in human cell lines of Tau, MDM2 and P53. We took advantage of parental plasmids for N- or C-terminal protein tagging already available in the laboratory[29]. Ectopic proteins carry thus the tenth (T10 with the HA epitope) or the eleventh (T11 with the epitope recognized by the β1 antibody. Biofluorescence reconstitution was obtained with the first nine ($GFP_{1–9}$) or ten ($GFP_{1–10}$) β-strands of an optimized form of superfolder GFP[29,59]. The cDNA encoding protein fragments (**Supplementary Table 1**) were obtained by the polymerase chain reaction (PCR) using human full-length templates and specific oligonucleotide primers (**Supplementary Table 2**).

For generating ReBiL cell lines we started from parental targeting plasmids generously provided by Drs. Leo Li and Geoffrey Wahl (Salk Institute, La Jolla, California). The plasmids carry a bidirectional tetracycline-inducible promotor (TREbi) for expression of N-terminal (nLuc) and C-terminal (cLuc) luciferase fragments and HA-tags[30]. Plasmid pSME-501 was obtained starting from pLI635 by in frame cloning of the cDNAs encoding for the MBD of Tau and the central domain of MDM2 amplified

by PCR (**Supplementary Table 2**). This resulted in inducible expression of nLuc-HA-MBD and cLuc-HA-101-365 (**Supplementary Table 1**). A similar strategy was followed to obtain pSME-502 that drives the inducible expression of the MBD of Tau (nLuc-HA-MBD) and the RING domain of MDM2 (cLuc-HA-362-491). Finally, plasmid pSME-506 was built starting from pLI772 for the inducible expression of the central domain of MDM2 (nLuc-HA-101-365) and the MBD of Tau (MBD-HA-cLuc).

Cell Culture and DNA Transfections

Human neuroblastoma SH-SY5Y cells (94030304, Sigma-Aldrich) and human cervical cancer HeLa cells (kindly provided by Prof. Magdalini Polymenidou, University of Zurich, Switzerland) were cultured in complete DMEM containing Dulbecco's Modified Eagle Medium (61965–059, Gibco) supplemented with 1% non-essential amino acids (NEAA, 11140035, Gibco), 1% penicillin-streptomycin (PS, 15140122, Gibco) and 10% fetal bovine serum (FBS, 10270106, Gibco). Human osteosarcoma U2OS cell lines 134-8 HyTK8 (parental), 134-385 (inducible expression of P53 and MDM fragments) and 134-354 (P53 and MDM4 fragments) were kindly provided by Drs. Leo Li and Geoffrey Wahl (Salk Institute, La Jolla, California)[30]. Parental U2OS cells were maintained in DMEM with 10% FBS, 400 µg/ml G418 (11811-031, Gibco), 10 µg/ml ciprofloxacin (S2027, Lubio) and 200 µg/ml hygromycin B (10843555001, Roche). U2OS ReBiL cells were maintained in the same medium with 4 µg/ml blasticidin (A11139-03, Gibco) instead of hygromycin B. When indicated cells were treated with the proteasome inhibitor MG132 at 10 µM (10 mM stock in DMSO, M7449, Sigma-Aldrich) or with 0.1% DMSO for 4 h before analysis.

All cells were grown at 37 °C in saturated humidity and 5% CO_2 and maintained in culture for less than one month. For transient plasmid transfections with jetPRIME (114–15, Polyplus) according to the manufacturer's instructions, cells were grown overnight on plates coated with poly-D-lysine (P6407, Sigma-Aldrich). For U2OS ReBiL lines, parental 134-8 HyTK8 cells at 80% confluency in a 6-well plate were transfected with ReBiL targeting plasmids and a cre-recombinase plasmid (pOG231) (Cre-recombinase) at a 2:1 ratio using jetPRIME. Transfected cells were then replated at clonogenic density in a 10 cm plate and selected with a transient treatment with 2 µM ganciclovir (G2536, Sigma-Aldrich)[30]. Mixed populations were selected and maintained in 4 µg/ml blasticidin and 1 ng/ml doxyciclin (D9891, Sigma-Aldrich).

Immune Precipitation and Western blot

For direct analysis by western blot, total cell lysates from cells cultured in 6-well plates were lysed in 50 µL of SDS-PAGE sample buffer (1.5% SDS, 8.3% glycerol, 0.005% bromophenol blue, 1.6% β-mercaptoethanol and 62.5 mM Tris pH 6.8) and incubated for 10 min at 100 °C. 15 µL/lane of the sample (40 µg protein) was loaded.

For immune precipitation, the cells were rinsed with PBS and lysed on ice in 100 µL AlphaLisa® Lysis Buffer (AL003, PerkinElmer) supplemented with protease and phosphatase inhibitor cocktails (S8820 & 04906845001, Sigma-Aldrich). Cell lysates were treated with benzonase (707463, Novagen) for 15 min at 37 °C, centrifuged at 20'000 g for 10 min at 4°C and the supernatants collected as cell extracts. These latter were diluted in HiBlock® (10205589, PerkinElmer) and incubated overnight at 4 °C with 0.5 µg of the following primary antibodies: Tau13 (sc-21796, Santa Cruz), HT7 (MN1000, Invitrogen), MDM2-D12 (sc-5304, Santa Cruz), FL-393 rabbit antiserum (bs-8687R, Bioss Antibodies), or β1 antibody[60]. Protein G-Sepharose® beads (101241, Invitrogen) were added for 1 h at room temperature and the beads were washed three times in PBS with 0.1%Tween-20. Bead-bound proteins were eluted in SDS-PAGE sample buffer by boiling for 10 min at 100 °C. After SDS PAGE, transferred protein were incubated with primary antibodies as indicated in the figure legends: 0.2 µg/mL Tau13, 0.2 µg/mL Tau13-AlexaFluor (sc-21796 AF680, Santa Cruz), 0.2 µg/mL HT7, 0.2 µg/mL TauAS rabbit antiserum (ab64193, Abcam), 0.1 µg/mL rabbit monoclonal D1V2Z (86934, Cell Signaling), 0.2 µg/mL MDM2-323 (sc56154, Santa Cruz), 0.4 µg/mL DO-1 (sc-126, Santa Cruz), 0.1 µg/mL FL-393 rabbit antiserum, 2.3 µg/mL β1, or 0.18 µg/mL GAPDH (ab181602, Abcam). Primary antibodies were revealed with anti-mouse IgG coupled to IRDye RD 680 or anti-rabbit IgG coupled to IRDye 800CW (Licor Biosciences, 926–68070 & 926–32211) on a dual infrared imaging scanner (Licor Biosciences, Odyssey CLx 9140) and quantified with the provided software (Licor Biosciences, Image Studio V5.0.21, 9140–500).

AlphaLISA®

Cell extracts were obtained as described for immune precipitation. The amount of Tau and P53 was determined with AlphaLISA® kits (Tau-AL271C and ALSU-TP53-A-HV, PerkinElmer) following the manufacturers' instructions. A home-made assay was developed for MDM2, using D1V2Z coupled to CaptSure (D1V2Z-CaptS) and

CaptSure-conjugated acceptor beads (ALSU-ACAB, PerkinElmer), paired to biotinylated SMP14 (sc-965B, SantaCruz) and streptavidin-conjugated donor beads. Similarly, the Tau-MDM2 complex was detected with D1V2Z-CaptS and biotinylated HT7 (MN1000B, ThermoFisher). Determinations were performed using a 384-well plate (6005350, PerkinElmer) by 1 h incubation at room temperature in the dark with 10 µL cell extract and 5 µL of a mix containing biotinylated antibody (1 nM final concentration), antibody-CaptS bound to acceptor beads (10 µg/mL final concentration) in HiBlock®; followed by the addition of 5 µL of donor beads (20 µg/mL final concentration) in HiBlock® for another 1 h incubation. Acquisition was done with a multi-plate reader (Victor Nivo, PerkinElmer) and data were analyzed using the provided software (Victor Nivo, PerkinElmer) using the emission range of 575/110 nm with excitation time of 50 ms and emission time of 700 ms.

Immune staining of human brain tissue

The brain samples were obtained from The Netherlands Brain Bank, Netherlands Institute for Neuroscience, Amsterdam (www.brainbank.nl). All material derived from donors from whom a written informed consent for a brain autopsy and the use of the material and clinical information for research purposes had been obtained. Brain tissue from age-matched healthy controls, AD or FTD patients were cut in 20 µm frozen sections using a cryostat. Sections were fixed in 100% cold methanol for 15 min at -20 °C. After 1 h in blocking solution, the sections were stained overnight at 4°C with antibodies 0.2 µg/mL TauAS rabbit antiserum, 0.4 µg/mL AT8 (MN1020, ThermoFisher) or 0.4 µg/mL MDM2-323; followed by 1 h at room temperature with 2 µg/mL secondary antibodies anti-mouse IgG-Alexa594 (A-11032, ThermoFisher Scientific) or anti-rabbit IgG-Alexa488 (A-11034, ThermoFisher Scientific). Nuclei were counterstained with 0.5 µg/mL DAPI (D9542, Sigma-Aldrich). Images were acquired on a fluorescent laser confocal microscope (Nikon C2 microscope).

BiFC and TriFC Analysis

GFP biofluorescence reconstitution was analyzed on fixed cells co-transfected with the plasmids encoding for proteins tagged with the T_{11} tag and GFP_{1-10} (biFC), of with plasmids for proteins tagged with T_{10}, T_{11} and GFP_{1-9} (triFC). Fixation for 15 min at room temperature was done by adding one volume of 8% PFA in PBS. After three washes with 100 mM glycin in PBS and one wash in PBS, fixed cells were blocked for

30 min in 300 µL/well of blocking buffer (5% normal goat serum, 0.3% Triton X-100 in PBS) followed by three PBS washes. Immune staining was done as described[19]. Nuclei were counterstained with 0.5 µg/mL DAPI (D9542, Sigma-Aldrich). Images were acquired on a fluorescent laser confocal microscope (Nikon C2 microscope). For cytofluorimetric determinations, transfected cells were collected by a trypsin treatment and cautiously resuspended in culture medium in order to obtain a single-cell suspension. Cells were then washed with ice-cold PBS, resuspended in 0.5 mL ice-cold PBS and kept on ice until analysis. 10'000 cells were analysed on an analytical device (Beckman, cytoFLEX) using the 488 nm excitation laser and the FL1 emission channel (525/40 nm). Values collected included total cell number, gated cell number and geometric mean fluorescence.

Recombinant Bimolecular Luciferase Complementation (ReBiL)

For the ReBiL assay in living cells, cells were plated at 8'500 cells/well in a 96-well plate in DMEM, 10% FBS, 10 µg/mL ciprofloxacin, 500 ng/mL doxycycline and 100 µM D-luciferin (L-8220, Biosynth). The plate was sealed with MicroAmp Optical adhesive film (4311971, Life Technologies) and cell luminescence determined in a luminometer (M200, Tecan) with 2 sec integration time every 10 min for up to 24 h at 37 °C. To detect a ReBiL signal in cell lysates, cells were induced with 500 ng/mL doxycycline for 48 h, washed with PBS and lysed in 100 mM Tris-HCL pH 7.5, 0.5 mM EDTA, 150 mM NaCl, 0.1% Triton X-100, 1 mM sodium orthovanadate, 50 mM sodium fluoride, and protease inhibitor cocktail. Cell extracts were obtained by centrifugation at 13'000 g for 5 min at 4 °C and diluted with DMEM. Fifty 50 µL/well of cell extract diluted in DMEM was pipetted into a 96-well plate containing 1 volume of D-luciferin and immediately analyzed in the luminometer with 0.5 sec integration time every 3 min for up to 30 min at 26 °C.

P53 Ubiquitination Assay

MDM2-dependent ubiquitination of P53 was determined with a commercial kit (K-200B, BostonChem, BioTechne) according to the manufacturer's instruction. When indicated the reaction was performed in the presence of 100 ng of recombinant human Tau2N4R protein produced in *E.coli* (wild-type or Pro301Leu, kindly provided by Prof. Luca Colnaghi, Università Vita-Salute San Raffaele, Milan). The reaction was initiated by the addition of the ubiquitin-containing solution, run for 30 min at 37 °C, and

terminated with the addition of 5X SDS-PAGE sample buffer with 5 min at 90 °C. Protein was run on a low percentage acrylamide SDS-PAGE and transferred protein incubated with 0.25 µg/mL MAB1355 antibody provided in the kit. In addition, MDM2 and Tau were detected with 0.1 µg/mL rabbit antiserum D1V2Z and 0.2 µg/mL TauAS rabbit antiserum. Immune-positive proteins detected with IRDye-coupled secondary antibodies on a dual infrared imaging scanner.

Statistics and reproducibility.

Statistical analysis was performed with GraphPad Prism version 8.4 using the method specified in the legend of each figure. Exact p-values are specified in the figures. All quantifications were performed based on at least three independent biological replicates. Sample size, number of replicates and how they are defined is specified in the figure legends. When indicated, western blots and microscopic images are shown as representative data.

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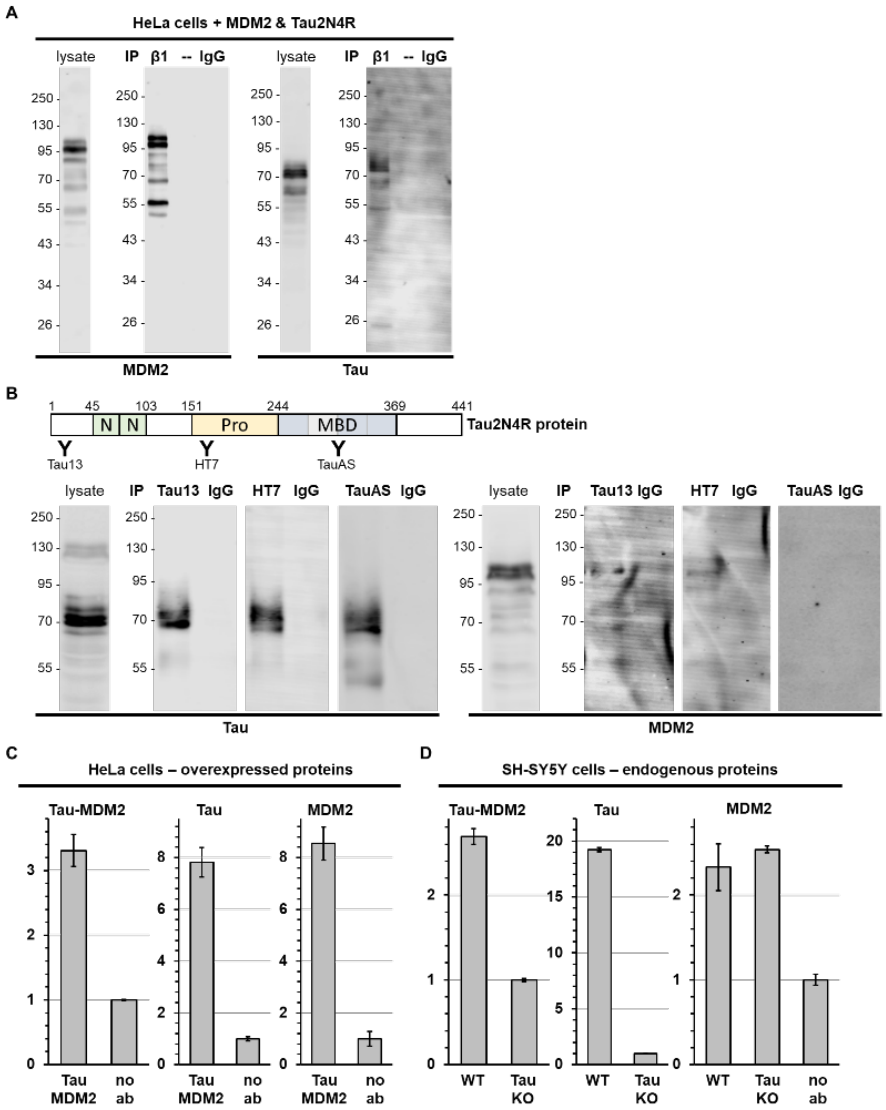


Figure 1. Tau-MDM2 interaction. A. Hela cells transfected with plasmids encoding for T_{10}HA-Tau2N4R and $T_{11}\beta$1-MDM2 were treated with MG132 for 4 h before lysis. Cell extracts (lysate) were subjected to immune precipitation (IP) with the monoclonal β1 or control (IgG) mouse antibodies. Immune isolated proteins were analyzed by

western blot with the rabbit D1V2Z antibody and anti-rabbit IgG IRDye 800CW (MDM2) or the mouse Tau13-AF680 antibody (Tau). Molecular weight markers in kDa are given on the left of the blots. **B.** Cell extracts obtained from a second transfection with the same plasmids were subjected to a reversed immune precipitation with the indicated antibodies recognizing the three Tau epitopes shown in the scheme. Samples were then analyzed as described for panel A. **C.** Hela cells transfected with plasmids encoding for full-length $T_{11}\beta$1-MDM2 and T_{10}HA-Tau2N4R and treated with MG132 were lysed and subjected to AlphaLisa® to detect the Tau-MDM2 complex as well as the total amount of Tau or MDM2. Shown is one representative experiment of at least three replicates. Values are given as mean fold over negative control omitting one of the two primary antibodies (no ab) ± SD of technical triplicates. **D.** SH-SY5Y treated with MG132 were lysed and subjected to AlphaLisa® to detect the total amount of endogenous Tau-MDM2 complex as well as endogenous Tau or MDM2. Shown is one representative experiment of at least three replicates. Values are given as mean fold over negative control obtained from Tau-KO cells for Tau-MDM2 and Tau, or omitting one of the two primary antibodies (no ab) for MDM2 ± SD of technical triplicates.

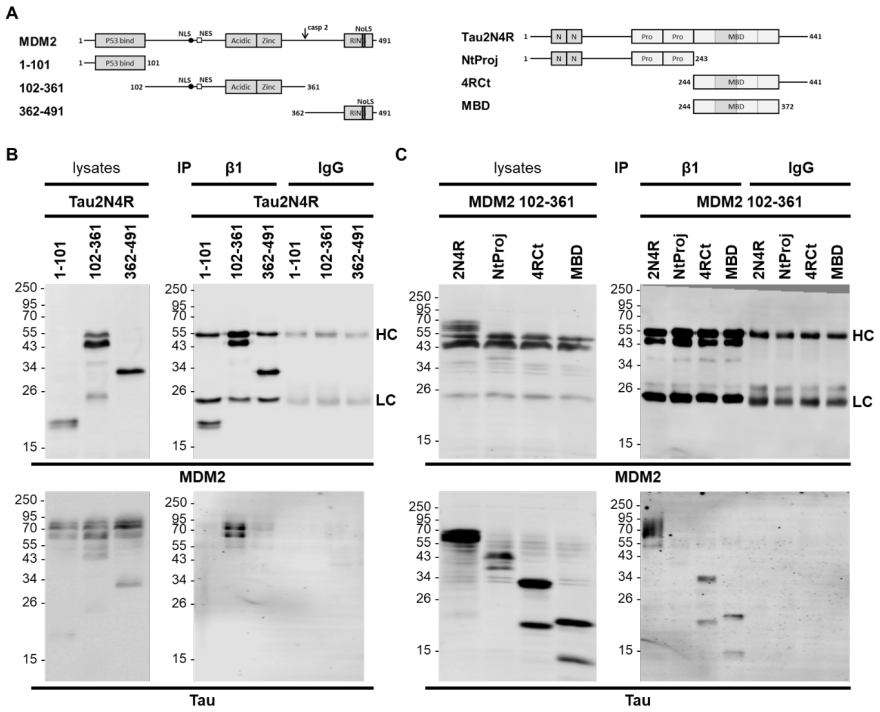


Figure 2. TauMBD binds to MDM2 central domain. A. Schematic representation of the MDM2 and Tau truncated variants. MDM2 fragments carry a N-terminal $T_{11}\beta1$ tag whereas Tau fragments carry a N-terminal T_{10}HA tag. **B.** Hela cells co-transfected with the indicated plasmids were treated with MG132 for 4 h before lysis. Cell extracts (lysates) were subjected to immune precipitation (IP) with β1 or control (IgG) mouse antibodies. Immune isolated proteins were analyzed by western blot with the mouse β1 antibody (MDM2) followed by the mouse Tau13-AF680 antibody (Tau). Heavy chains (HC) and light chains (LC) of β1 are indicated. **C.** Hela cells were co-transfected with $T_{11}\beta1$-MDM2(102-361) and the indicated Tau fragments and processed as described for B. Tau was detected with a mix of two Tau antibodies, Tau13 against the N-terminus and the TauAS against the MBD. Molecular weight markers in kDa are given on the left of the blots.

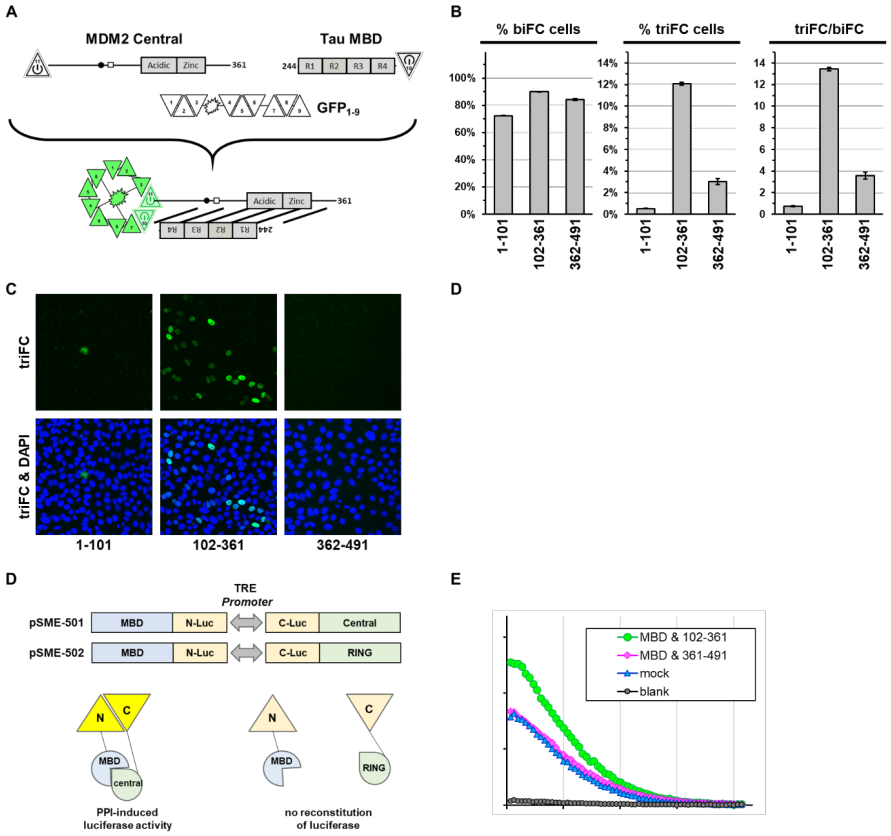


Figure 3. Validation by orthogonal techniques and nuclear localization of the Tau-MDM2 complex. A. Scheme of the TriFC principle using $T_{11}\beta$1-MDM2 fragments and TauMBD-HA-T_{10}. **B.** Hela cells co-transfected with the indicated plasmid encoding for a $T_{11}\beta$1-MDM2 fragment and $GFP_{1\text{-}10}$ were treated with MG132 and analyzed by cytofluorimetry for the biFC signal corresponding to the percent cells (mean ± SD) expressing the MDM2 fragments. In parallel, Hela cells co-transfected with the same plasmids together with TauMBD-HA-T_{10} and $GFP_{1\text{-}9}$ were treated with MG132 and analyzed by cytofluorimetry for the triFC signal corresponding to the percent cells (mean ± SD) positive for the Tau-MDM2 complex. In the third graph the percentage of cells positive for triFC was normalized for the percentage of cells positive for biFC (mean ± SD). **C.** Hela cells co-transfected with the indicated $T_{11}\beta$1-MDM2 fragments, TauMBD-HA-T_{10} and $GFP_{1\text{-}9}$ were treated with MG132 and analyzed by confocal

microscopy for the triFC signal. Cells were fixed and counterstained with DAPI. **D.** Scheme of the ReBiL cassettes for birectional inducible expression of the indicated cDNAs and principle of the luciferase reconstitution assay. U2OS cells transfected with plasmids encoding for C-Luc-MDM2(102-361)/N-Luc-TauMBD or C-Luc-MDM2(361-491)/N-Luc-TauMBD were lysed and incubated in presence of luciferin. The signal then recorded up to 30 min.

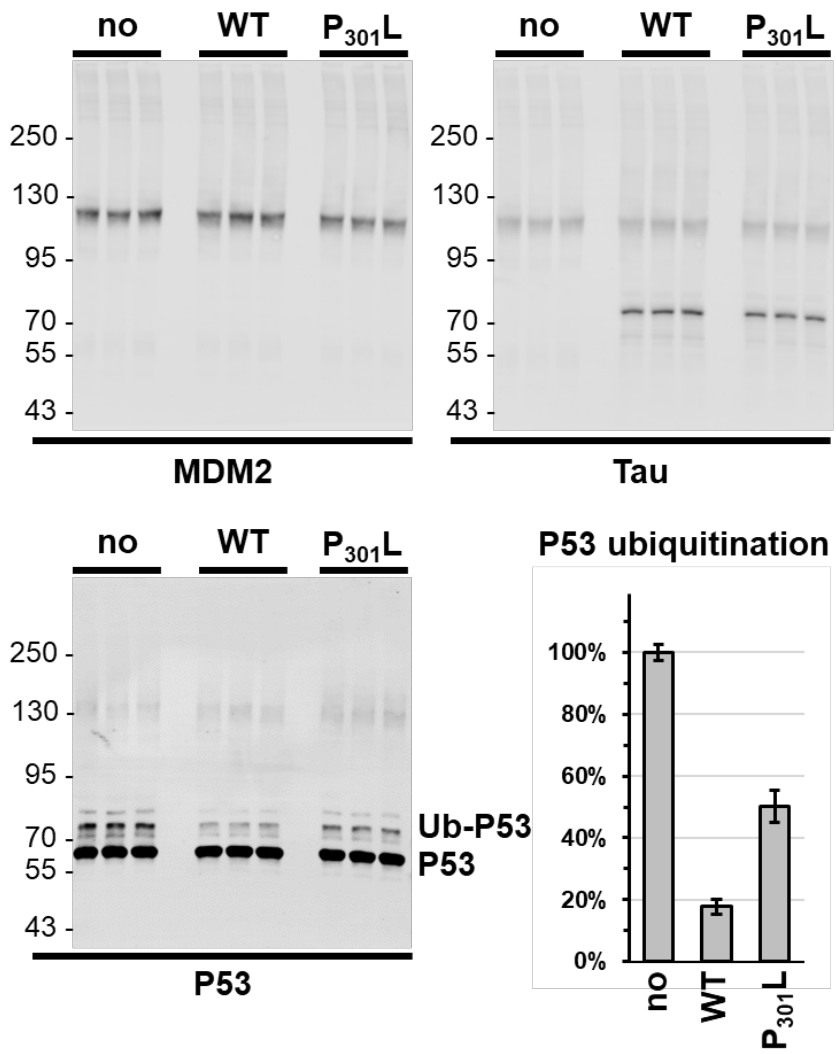


Figure 4. Tau inhibits MDM2-dependent ubiquitination of P53. An *in vitro* P53 ubiquitination assay was performed in the absence (no) or the presence of wild-type Tau2N4R (WT) or mutant Tau2N4R ($P_{301}L$). Triplicate conditions of the reaction was analyzed by western-blot using antibodies against MDM2, Tau and P53. The graph shows the quantification of ubiquitinated P53 (Ub-P53) normalized for P53. Shown is a representative experiment of at least three independent experiments. The mean ± SD of the value obtained in the absence of Tau was set as 100%.

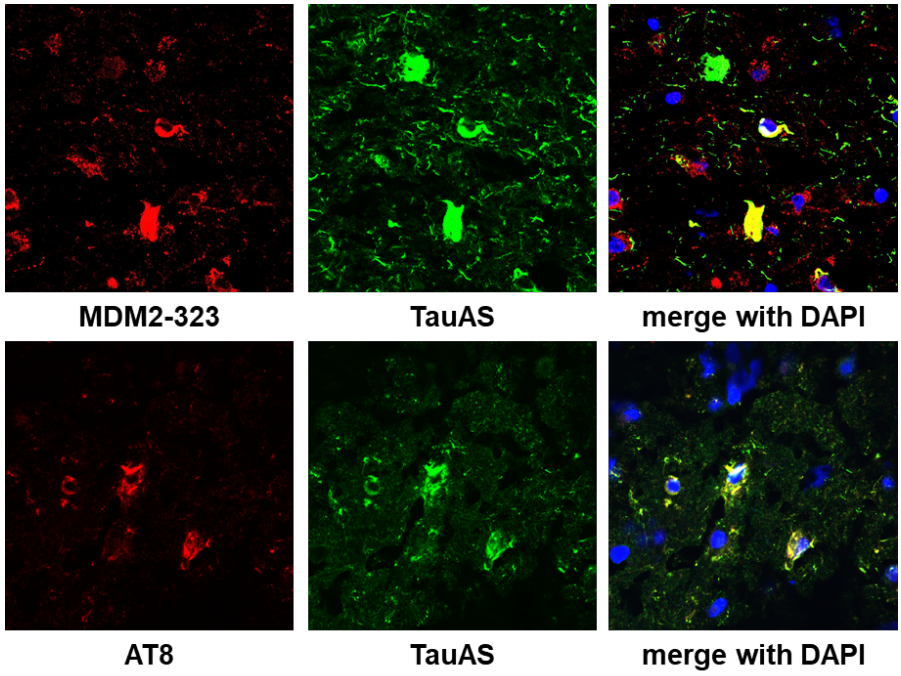
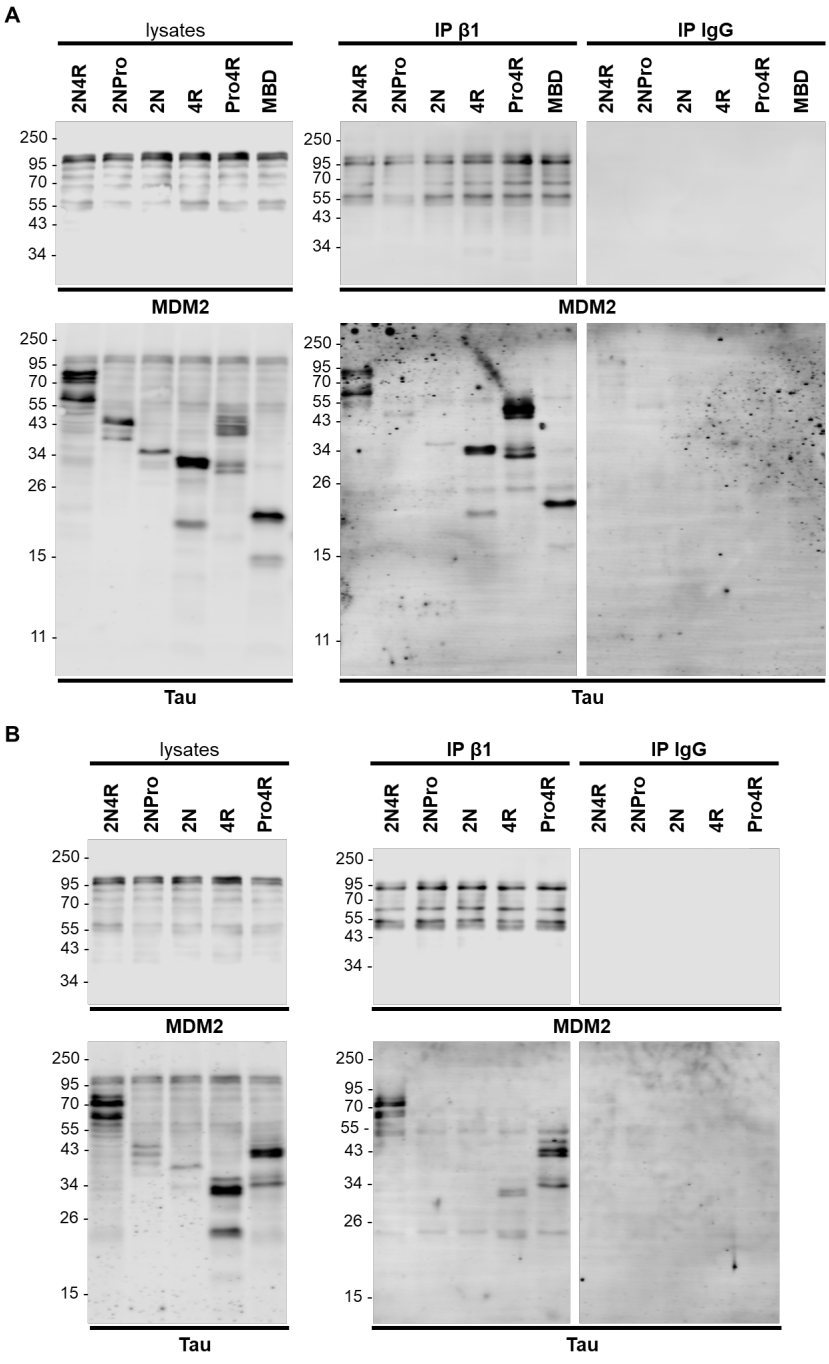


Figure 5. Abnormal MDM2 accumulation in Tau neurofibrillary tangles in the AD brain. Frozen brain sections from a patients affected by Alzheimer's disease were stained using antibodies against MDM2 and Tau (top row) or phosphorylated Tau and Tau (bottom row). The slides were counterstained with DAPI. Calibration bar = xx µm.


A
lysates
IP β1
IP IgG
2N4R
2NPro
2N
4R
Pro4R
MBD
250
95
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34
26
15
11
MDM2
Tau
B
lysates
IP β1
IP IgG
2N4R
2NPro
2N
4R
Pro4R
250
95
70
55
43
34
26
15
MDM2
Tau

Supplementary Figure 1. C-terminal or N-terminal tags do not alter the Tau-MDM2 interaction. A. Hela cells transfected with plasmids encoding for $T_{11}\beta1$-MDM2 and T_{10}HA-Tau fragments and treated with MG132 were lysed and subjected to immunoprecipitation with an anti-β1 antibody. Lysates and proteins immunoprecipitated were analyzed by western blot with the Tau13-Alexa, TauAS and β1 antibody. **C.** Hela cells transfected with plasmids encoding for $T_{11}\beta1$-MDM2 and Tau-T_{10}HA fragments and treated with MG132 were lysed and subjected to immunoprecipitation with an anti-β1 antibody. Lysates and proteins immunoprecipitated were analyzed by western blot with the Tau13-Alexa, TauAS and β1 antibody.

4. Debate and future perspectives

Until recently, Tau was mainly known for its role in microtubule stabilization and the acquisition of toxic properties causing neurodegenerative tauopathies. Starting from the hypothesis that a loss-of-function of Tau may contribute to human aging-associated diseases, in my thesis project I demonstrated that in neuroblastoma cells exposed to an acute DNA damage, Tau depletion is associated with a decrease in apoptotic cell death balanced by an increase in cell senescence. By dissecting carefully the DDR cascade, I showed that this phenotype is caused by the ability of Tau to increase the stability and transcriptional activity of P53. In order to study the molecular mechanism underlying this observation, I focused on MDM2, the main E3 ubiquitin ligase driving P53 degradation. With the implementation of new orthogonal technologies, I discovered that Tau binds to MDM2 and MDM4. Then, through the generation of expression plasmids encoding for truncated proteins, I defined that the microtubule binding domain of Tau and the central region of MDM2 are critical for the Tau-MDM2 interaction. Finally, I reported that the binding of Tau to MDM2 has a functional impact by reducing P53 ubiquitination. Validating my data in a more general context, I observed that a disease-associated Tau mutation perturbs the inhibitory function of Tau on the MDM2 ligase activity. Notably, I also found an abnormal MDM2 accumulation associated to Tau NFTs in the AD brain. My findings suggest that Tau may not only modulate P53 activities, but also other functions associated to MDM2 and MDM4, and position the Tau-MDM2-P53 axis at the crosstalk between neurodegeneration and cancer. Also, aging-associated cellular stress may contribute to neurodegenerative disorders by a loss-of function of Tau when stabilizing P53. In view of the importance of the P53-MDM2 axis in cancer, my data offers a new approach for the study of the emerging implication of Tau in cancer.

MDM2 forms homodimers or heterodimers with its close homolog MDM4 to bind, ubiquitinate and target P53 for degradation[242]. MDM2 and MDM4 contribute to P53 regulation depending on the tissue origin: in the CNS both MDM2 and MDM4 are required[210], whereas in other tissue such as smooth muscle cells, MDM2 alone is sufficient to prevent accumulation of P53[211]. Other studies showed that MDM2 and MDM4 are essential in a non-redundant manner for preventing p53 activity in the same cell type, irrespective of the proliferation/differentiation status of the cells. In addition to contribute with MDM2 to prevent accumulation of the p53 protein, MDM4 contributes to the overall inhibition of p53 transcriptional activity independently of MDM2[242]. The newly discovered interaction between Tau and both MDM2 and MDM4 indicates that Tau may modulate P53 activity by interfering with both proteins, independently or, perhaps, by a synergistic effect.

In addition to their function to control P53, MDM2 and MDM4 exert several P53-independent functions. MDM2 is implicated in regulating a number of cellular processes, including cell-cycle control, apoptosis, differentiation, genome stability, and transcription, among others[243]. MDM2 exerts these functions through a wide variety of mechanisms: in addition to ubiquitination of alternative targets[244], MDM2 stimulates the activity of transcription factors[245], binds to mRNAs to regulate their stability[246], and acts as chromatin modifier[236]. MDM4 also carries out p53-independent functions. The lack of MDM4 promotes genome instability[247]. When expressed

ectopically, MDM4 inhibits DNA break repair by associating with Nbs1, a member of the MRN complex that also consists of Mre11 and Rad50[248]. Moreover, MDM4 negatively regulates E2F1[249] but it can also mediate the degradation of the E2F1 antagonist RB[250]. Similarly to MDM2, MDM4 is also able to increase the proteasomal turnover of p21[251]. Through its interaction with MDM2 and MDM4, Tau may also influence the P53-independent activities of these proteins.

MDM2 and MDM4 play a key role in cancer by inhibiting P53 activity but they are also associated with aging and senescence in a P53-independent manner. The link between Tau and the P53-MDM2/MDM4 axis may likely explain the de-regulation of the P53 pathway and other P53 and/or MDM2/MDM4-dependent signaling in neurodegeneration as well as the observed correlation between Tau expression and cancer survival.

The interaction between Tau and MDM2 occurs through the MBD of Tau and the central region containing an acidic domain and a zinc finger domain. Also the binding of Tau to tubulin requires the MBD, which is also known to have pro-fibrillogenic properties[252] and to be affected by most of the mutations associated to FTDP-17 pathology[253]. The MBD is also subjected to splicing generating the 4R and 3R isoforms of Tau which show differential function and implication in disease[254,255]. Our observation that one FTDP-17 mutation is less potent to inhibit MDM2-dependent ubiquitination of P53 suggests that the binding of Tau to MDM2 may have a protective role against neurodegenerative tauopathies.

The data I generated in the context of this thesis projects support the need for follow-up studies to better explore the physiological and pathological relevance of the Tau-MDM2-P53 axis, also considering the potential, in term of therapeutic development, to identify innovative disease-modifying approaches for major unmet medical needs.

To go further in the comprehension of the role of the Tau-MDM2 complex, following aspects are worth investigating:

- Validate *in vivo* whether a loss of function of Tau impacts P53 stability and transcriptional activity. As a first step, a genotoxic treatment inducing P53 activity in tissues expressing Tau could be applied to Tau-KO mice and wild-type counterparts. Phenotypes such as the activation of P53, cell death, senescence as well as the propensity to develop tumors could then be compared between the two mouse genotypes.
- Analyze the correlation between Tau, P53 and MDM2 protein expression in tumor biopsies.
- Investigate whether MDM2/4 have a functional impact on Tau protein stability and microtubule-dependent and -independent activities
- Identify the exact molecular determinants of the Tau-MDM2 interaction, also supported by computational methods to predict the protein structures. Particularly important is to also investigate the contribution of PTMs for complex formation and identify other associated proteins e.g., by quantitative analysis like mass spectrometry. These data could be capitalized for the generation of new Tau mouse models expressing Tau forms with point mutations abrogating only the interaction with MDM2, and so avoid interfering with important microtubule-associated functions of Tau that may confound the results obtained in Tau-KO mice.

5. Abbreviations

- AD = Alzheimer's disease
- MCI = mild cognitive impairment
- TDP-43 = TAR DNA-binding protein 43
- PiD = Pick's disease
- CBD = corticobasal degeneration
- PSP = progressive supranuclear palsy
- GGT = globular glial tauopathy
- AGD = argyrophilic grain disease
- FTD = frontotemporal dementia
- GSS = Gerstmann–Sträussler–Scheinker syndrome
- AChEIs = acetylcholinesterase inhibitors
- PTMs = post-translational modifications
- PET = positron emission tomography
- MAPs = microtubule associated proteins
- *MAPT* = microtubule associated protein Tau gene
- KDa = kilo Dalton
- 4R / 3R = microtubules-binding repeats of Tau proteins
- MAPK = mitogen-activated protein kinase
- GSK-3 = glycogen synthase kinase-3
- Cdk5 = cyclin-dependent kinase-5
- NFTs = neurofibrillary tangles
- PHFs = B-sheets-rich paired helical filaments
- CNS = central nervous system
- Cys = cysteines
- Lys = lysines
- Phospho-Tau = phosphorylated Tau
- HAT p300 = histone acetyltransferase p300
- CREB = cAMP response element binding protein
- UPS = ubiquitin proteasome system
- EVs = extracellular vesicles
- K18 = truncated form of Tau containing only the 4 microtubule binding repeats
- NDMA = N-metyl-D-aspartate receptor
- AMPA = alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
- DNA = deoxyribonucleic acid
- 0N / 1N = amino-terminal inserts of Tau protein
- HSP70 = heat shock protein 70
- P301L = from proline 301 to leucine mutation
- SIRT6 = sirtuin 6

- ApoBDs = apoptotic bodies
- TNF = tumor necrosis factor receptor
- FAS = Fas cell surface death receptor
- ER = endoplasmic reticulum
- Bax = Bcl-2 associated X
- BH3 = Bcl-2 associated protein
- Bcl-2 = B cell lymphoma 2
- MOMP = mitochondrial outer membrane permeabilization
- HIV = human immunodeficiency virus
- Z-VAD-FMK = carbobenzoxy-valyl-alanyl-aspartyl-O-methyl-fluoromethylketone
- PAI-1 = plasminogen activator type 1
- SA-β-gal = senescence-associated β-galactosidase
- DEC1 = embryonic chondrocyte-expressed 1
- DCR2 = decoy death receptor 2
- miRNAs = micro ribonucleic acid
- SASPs = senescence-associated secretory pathway
- OIS = oncogene-induced senescence
- ES = effect size
- ALS = amyotrophic lateral sclerosis
- CRC = colorectal cancer
- ATM = serin/threonine protein kinase
- GSEA = gene set enrichment analysis
- APP = amyloid precursor protein
- WWOX = WW domain contains oxidoreductase
- DDR = DNA damage response pathway
- ROS = reactive oxygen species
- ATR = ataxia telangiectasia kinase
- CDK = cyclin-dependent kinase
- CHK1/ CHK2 = checkpoint kinases 1 and 2
- DNAPK = DNA-dependent protein kinase
- PARP = poly-ADP-ribosome protease
- BRCA 1/ BRCA2 = breast cancer type 1 and 2 susceptibility protein
- NF-kB = nuclear factor kappa-light-chain-enhancer of activated B cells
- Mdm2 = murine double minute 2 protein
- Mdmx = murine double minute X protein
- RING = really interesting new gene
- ApoE = apolipoprotein E
- PFTα = pifithrin
- Tau-KO = knocked-out for Tau

- mTOR = mechanistic target of rapamycin kinase
- 3T3DM = transformed Balb/c 3T3 line
- C2H2C4 = HC subclasses, order of cysteine and histidine
- MARCH7 = membrane associated ring-CH-type finger 7
- PROTAC = proteolysis-targeting chimera
- DMEM = Dulbecco's modified eagle medium
- FBS = fetal bovine serum
- NEAA = non-essential amino acids
- CRISPR-Cas9 = Clustered Regularly Interspaced Short Palindromic Repeats-Cas9 gene editing
- gRNA = guide RNA
- PCR = polymerase chain reaction
- shRNA = short hairpin RNA
- Tau-KD = knocked-down for Tau
- Tau-WT = wild-type for Tau protein
- GFP = green fluorescent protein
- LDH = lactate dehydrogenase
- MTS = 3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethonyphenol)- 2-(4-sulfophenyl)-2H-tetrazolium
- PBS = phosphate-buffered saline
- RIPA = radioimmunoprecipitation assay buffer
- RT = room temperature
- RT-qPCR = quantitative reverse transcription PCR
- mRNA = messenger RNA
- DSBs = double strand breaks
- h = hours
- min = minutes
- IP = immunoprecipitation
- WB = western blot
- IF = immune fluorescence
- ReBiL = recombinant bimolecular luciferase complementation
- TriFC = tripartite fluorescent complementation
- BiFC = bipartite fluorescent complementation
- DMSO = dimethyl sulfoxide
- MTB = microtubule binding domain
- Luc = luciferase
- MOPS = 3-(N-morpholino)propanesulfonic acid
- MetOH = methanol
- FACS = fluorescent-activated cell sorting

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7. List of publication and other activities

Publications in International Peer-Reviewed Journals

'Phosphorylation of nuclear Tau is modulated by distinct cellular pathways'

Scientific Reports - December 2018

Giorgio Ulrich, Agnese Salvadè, Paul Boersema, Tito Calì, Chiara Foglieni, **Martina Sola**, Paola Picotti, Stèphanie Papin & Paolo Paganetti

https://www.nature.com/articles/s41598-018-36374-4

' Tau affects P53 function and cell fate during the DNA damage response'

Communication Biology – May 2020

Martina Sola, Claudia Magrin, Giona Pedrioli, Sandra Pinton, Agnese Salvadè, Stèphanie Papin & Paolo Paganetti

https://www.nature.com/articles/s42003-020-0975-4

'Tau seed in extracellular vesicles induce Tau accumulation in degradative organelles of cells'

DNA and cell biology - September 2021

Giona Pedrioli, Marialuisa Barberis, Claudia Magrin, Diego Morone, Ester Piovesana, Giorgia Senesi, **Martina Sola**, Stèphanie Papin & Paolo Paganetti

https://www.liebertpub.com/doi/abs/10.1089/dna.2021.0485

Posters at National and International Congress

- BeNeFri Workshop – February 2019
- BeNeFri Workshop – February 2020
- BeNeFri Workshop – February 2021
- ExoDay Workshop – October 2019
- Neurex – March 2019
- 9° Giornata della Ricerca della Svizzera Italiana – June 2019
- 10° Giornata della Ricerca della Svizzera Italiana – June 2021
- Synapsis Forum – September 2018
- Synapsis Forum – September 2021 (Oral Presentation)
- PhD Day – October 2019
- PhD Day – October 2020
- PhD Day – October 2021 (Oral Presentation)
- ESN Milan – September 2019
- CSH America – December 2020

Awards : Best Poster presentation, Synapsis Forum, Bern (CH), September 2021