The amyloid precursor protein (APP) intracellular domain regulates translation of p44, a short isoform of p53, through an IRES-dependent mechanism

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\begin{abstract}
p44 is a short isoform of the tumor suppressor protein p53 that is regulated in an age-dependent manner. When overexpressed in the mouse, it causes a progeroid phenotype that includes premature cognitive decline, synaptic defects, and hyperphosphorylation of tau. The hyperphosphorylation of tau has recently been linked to the ability of p44 to regulate transcription of relevant tau kinases. Here, we report that the amyloid precursor protein (APP) intracellular domain (AICD), which results from the processing of the APP, regulates translation of p44 through a cap-independent mechanism that requires direct binding to the second internal ribosome entry site (IRES) of the p53 mRNA. We also report that AICD associates with nucleolin, an already known IRES-specific trans-acting factor that binds with p53 IRES elements and regulates translation of p53 isoforms. The potential biological impact of our findings was assessed in a mouse model of Alzheimer’s disease. In conclusion, our study reveals a novel aspect of AICD and p53/p44 biology and provides a possible molecular link between APP, p44, and tau.
\end{abstract}

1. Introduction

Owing to a combination of alternative promoter usage and alternative initiation of translation, the TP53 gene generates at least 12 different proteins (Bourdon, 2007; Bourdon et al., 2005). The full-length product (known as p53) has been extensively studied for its tumor-suppressor activity. In contrast, the shorter N-terminal-truncated isoforms, Delta40p53, Delta133p53, and Delta160p53, are relatively new and their biological functions are largely unknown. Delta40p53 (referred to as p44 thereafter) lacks the first 39 amino acids of the full-length protein. Transgenic mice overexpressing p44 (p44\textsuperscript{+/+}) develop a progeroid phenotype that includes premature cognitive decline, synaptic defects, and hyperphosphorylation of the microtubule-binding protein tau (Maier et al., 2004; Pehar et al., 2010). The aberrant phosphorylation of tau is because of the ability of p44 to induce transcription of several tau kinases, including dual-specificity tyrosine-regulated kinase 1x (Dyrk1x), glycogen synthase kinase-3β (GSK3β), and cyclin-dependent kinase 5 (Cdk5) regulatory partners, Cdkp35 and Cdkp39 (Pehar et al., 2014). Importantly, haploinsufficiency of Mapt, the gene encoding tau, can rescue the synaptic impairments displayed by p44\textsuperscript{+/+} mice (Pehar et al., 2010). Finally, p44 appears to be activated in an age-dependent manner in the brain (Pehar et al., 2014), suggesting a possible association with the cognitive decline associated with aging.

It is also worth stressing that hyperphosphorylation of tau has been linked to different forms of age-associated tauopathies and to the memory defects that characterize different mouse models of neurodegenerative diseases (Kurz and Perneckzy, 2009;
Lee et al., 2001). Hyperphosphorylation and increased aggregation of tau in neurofibrillary tangles are also essential features of Alzheimer’s disease (AD) pathology, the most common form of dementia in the elderly (Pehar and Puglielli, 2012).

The amyloid precursor protein (APP) is a type I membrane protein that is tightly linked to the pathogenesis of AD. More than 25 pathogenic mutations have been found in the APP gene (Loy et al., 2014; Tanzi, 2012), and they all cause an autosomal-dominant form of AD. A sequence variant that confers strong protection against AD has also been identified (Jonsson et al., 2012). The causative role of APP (and its proteolytic derivatives) in AD neuropathology is further supported by studies performed in mouse models of the disease (Duyckaerts et al., 2008; Ghosal et al., 2009; Gotz et al., 2004). Finally, downregulation of the Mapt gene in APP mouse models of AD can rescue the memory loss associated with the disease, suggesting a possible role of tau downstream of APP (Roberson et al., 2007; Santacruz et al., 2005).

2. Materials and methods

2.1. Animals and cells

APP695/Swe, p53−/−, and APP intracellular domain transgenic (AICDtg) mice were described before (Gilberto et al., 2008; Maier et al., 2004; Pehar et al., 2010). APP−/− mice were from The Jackson Laboratory (stock number 004133). Intact frozen brains and 32 months of age) were obtained from the National Institute on Aging (NIA) Rodent Tissue Bank. Animals were maintained in accordance to the guidelines for the ethical care and treatment of animals from the Institutional Animal Care and Use Committee of the University of Wisconsin-Madison and the Madison Veterans Administration Hospital. On the day of the experiment, mice were killed and brains were immediately processed for analysis. Euthanasia was performed according to the NIH Guide for the Care and Use of Laboratory Animals.

Down syndrome (DS) fibroblasts were obtained from Coriell Cell Repository or from biopsies with approval from the University of Wisconsin Human Subjects Institutional Review Board. Induced pluripotent stem cells (iPSCs) were generated and characterized as previously described (Weick et al., 2013). iPSCs were differentiated into neurons following established protocols (Johnson et al., 2007; Li and Zhang, 2006; Zhang et al., 2001). SH-SYSY (human neuroblastoma) cells were cultured as described before (Constantini et al., 2006).

2.2. Human brain tissue

Brain tissue from patients with late-onset AD and age-matched controls was kindly provided by the Brain Bank of the Neuropathology Core of the Wisconsin Alzheimer’s Disease Research Center (established by grant P50-AG033514 from NIH/NIA). The use of human brain tissue was approved by the University of Wisconsin-Madison Institutional Review Board in accordance with the US federal regulations (as defined under 45 CFR 46.102(f)).

2.3. Western blot

Protein extraction and Western blot procedures were described before (Constantini et al., 2006, 2007; Jonas et al., 2008, 2010; Pehar et al., 2010; Peng et al., 2014). The following antibodies were used: p53 (monoclonal-DO1; cat. n. ab1101; Abcam); p53 (monoclonal-PAb240; cat. n. 227-020; Ancell); H3 (polyclonal; cat. n. 07-690; Millipore); APP-C-terminal/AICD (polyclonal; cat. n. ABS352; Millipore); Fe65 (monoclonal; cat. n. 05-758; Millipore); green fluorescent protein (GFP) (monoclonal; cat. n. 32-160; Poway); Nucleolin (monoclonal; cat. n. sc-8031; Santa Cruz; monoclonal; cat. n. ab13541; Abcam); and PTBP1 (polyclonal; cat. n. sc-16549; Santa Cruz).

2.4. Nuclear fraction

Cells were suspended in 400-μL of cold buffer A (10-mM HEPES pH 7.9, 10-mM KCl, 0.1-mM EDTA, 1-mM DTT with complete protease and phosphatase inhibitors) and incubated on ice for 15 minutes. 10% of 25 μL NP-40 was added and centrifuged for 30 seconds at 11,000 g at 4 °C. Supernatant was collected as cytosolic fraction. Pellets were resuspended in 50 μL of cold buffer B (20-mM Heps pH 7.9, 400-mM KCl, 1-mM EDTA, 1-mM EGTA, 1-mM DTT with complete protease and phosphatase inhibitors) and centrifuged at 11,000 g for 5 minutes at 4 °C. Supernatant was collected as nuclear fraction.

2.5. Real-time PCR

Real-time polymerase chain reaction (PCR) was performed as described by Pehar et al. (2014). Primers for DYRK1A, GSK3β, CDK5, CDK5P35, and CDK5P39 are reported in Pehar et al. (2014). Primers for p44 “short” messenger-ribonucleic acid (mRNA) were Forward-5′-TGGAATCCATTGGAAGGCGCAG-3′; Reverse-5′-TTGGCAAAAACATCTTGTGAGGGC-3′.

2.6. Immunostaining

Immunostaining procedures were described before (Pehar et al., 2010; Peng et al., 2014). The following primary antibodies were used for tau phosphorylation: mouse anti-PHF-Tau (clone AT8; 10 μg/mL; Innogenetics-AutogenBioclear) and rabbit anti-phospho-S356-Tau (polyclonal; 1:100; Abcam). Secondary antibodies and imaging were described before (Pehar et al., 2010).

2.7. Polyosome preparation

Cells were homogenized on ice in polysome lysis buffer (1-mM KCl, 2-mM MgCl2, 10-mM Tris pH 7.6, 2-mM DTT, 100 units/mL RNasin, and protease inhibitor cocktail). After centrifugation at 10,000 g for 10 minutes, supernatants were layered over a cushion of polysome lysis buffer containing 30% sucrose and immediately centrifuged at 130,000 g for 2.5 hours. The polyribosomal pellets were resuspended in NT2 buffer (50-mM Tris-HCI, pH 7.4, 150-mM NaCl, 0.05% NP40, 200 units/mL RNasin, and protease inhibitor cocktail).

2.8. RNA-binding protein immunoprecipitation assay

RNA-binding protein immunoprecipitation assay was used to isolate target mRNAs from polysomes. Polyribosome pellets, isolated as above, were used to immunoprecipitate AICD:mRNA complexes. Specifically, an anti-C-terminal APP antibody (Millipore) was incubated with protein-G Sepharose beads overnight; beads-antibody complexes were then incubated with polyribosome lysate at 4 °C for 4 hours. Target mRNAs were purified by proteinase K digestion and then isolated with the RNeasy Plus Mini Kit (Qiagen). One microgram of mRNAs was reverse transcribed to cDNA and detected by PCR according to the manufacturer’s protocol (Qiagen). The following primers were used: p53/p44 forward-5′-GACCGCCAGTCGATCCCTCT-3′; p53/p44 reverse-5′-TCGTTGGACCCGCAAGC-3′; lymphoid enhancer factor-1 (LEF1) forward-5′-TTGAGTCCCCGTCCTCTTGGT-3′; LEF1 reverse-3′-CTGGAGATTGGTCCCTTTC-3′.
reverse-5’-TGG CTCCTGCTCCTTTCTCTGTTC-3’. LEF1 primers were from Tsai et al. (2014).

2.9. RNA-protein pull down

RNA pull down was performed with the Pierce Magnetic RNA-Protein Pull-Down Kit (cat. n. 20,164; Pierce/Thermo Fisher Scientific). First and second internal ribosome entry sites (IRES) of p53 fragments were cloned by PCR with primer sets as follows: first IRES: forward-5’-GACATTAATACGACTCACTA-TAGGGCGTCCAGGGAGCAGGTA-3’ (including T7 promoter); reverse-5’-CAGTGACCCGGAAGGCA-3’; second IRES: forward-5’-GACATTAATACGACTCACTAGGGAGCCGCAGTCAGATCCTA-3’ (including T7 promoter); reverse-5’-TGCTTGGGACGGCAAGG-3’. RNA probes were synthesized in vitro with the T7 RNA polymerase (Promega). Fifty picomoles of RNA were labeled with biotin by using the RNA 3’ End Desthiobiotinylation kit (cat. n. 20,163; Pierce). Labeled RNA was conjugated to streptavidin magnetic beads and incubated with cytosol at 4 °C overnight. Unbound proteins were removed by washing; bound proteins were eluted by boiling for 10 minutes in sodium dodecyl sulfate (SDS) sample buffer (Invitrogen) and then analyzed by Western blotting. As negative control, we used a fragment corresponding to the 3’ end of the p53 mRNA that was generated with the following primers: forward-5’-GACATTATACGACTCACTATAGGGTTTACATTCTGCAAGCACATCT; reverse-5’-ACAGGTGGCAGCAAAGT.

2.10. Luciferase assay

An mRNA construct containing the green fluorescent protein (GFP), a hairpin structure, and the 2 IRES elements
of p53 was generously obtained from Dr Fahraeus (Bourougaa et al., 2010). The GFP/hairpin component with the second IRES of the p53/p44 mRNA was inserted into a promoterless pGL4.18 plasmid (Promega) with the following primers: forward-5'CTAACTGGCCGGTACCTAATACGACTCACTATAGGGAGACC-3'0, reverse-5'CCGGATTGCCAAGCTTTGCTTGGGACGGCAAGG-3'0. SY5Y and SY5Y-APP cells were transfected with the above construct, and luciferase activity was determined 36 hours after transfection as described by Ko and Puglielli (2007) and Pehar et al. (2014). Co-transfected Renilla luciferase was used for transfection efficiency.

2.11. Electrophysiology

Mice were rapidly decapitated and the brain was removed and submerged in ice-cold cutting saline (CS [in mM]: 110 sucrose, 60 NaCl, 3 KCl, 1.25 NaH2PO4, 28 NaHCO3, 0.5 CaCl2, 7 MgCl2, 5 glucose, and 0.6 ascorbate). The hippocampi were sectioned transversely in a Vibratome (Vibratome) into 400-μM slices immersed in ice-cold CS. After sorting, slices were allowed to recover for 45 minutes at room temperature in 50:50 CS:artificial cerebro spinal fluid (ACSF [in mM]: 125 NaCl, 2.5 KCl, 1.25 NaH2PO4, 25 NaHCO3, 2 CaCl2, 1 MgCl2, and 25 glucose), and a further 45 minutes in 100% ACSF at room temperature before being transferred into an interface chamber (Fine Science Tools) bathed in 100% ACSF (1 e 1.5 mL/min) at 32 °C (Warner Instrument LLC, in Google Corporation, TC-324B) for 90 minutes. All solutions were carb-oxygenated (95/5, O2/CO2). Enameled bipolar platinum-tungsten (92:8 Pt:Y) stimulating electrodes were placed at the border of Area CA3 and Area CA1 along the Schaffer-Collateral pathway. Field excitatory postsynaptic potentials (fEPSPs) are recorded from the stratum radiatum, with ACSF filled recording electrodes (1–2 MΩ). Baseline synaptic transmission
was assessed for each individual slice by applying gradually increasing stimuli (0.5–15 V, 25 nA–1.5 μA, A-M Systems Model 2200 Stimulus Isolator) to determine the relationship between fiber volley and fEPSP slopes (input:output). All subsequent experimental stimuli were set to an intensity that invoked an fEPSP slope half that of the maximum recorded slope. Synaptic efficacy was continually monitored (0.05 Hz). Every 2 minutes, 4 sweeps were averaged; the fEPSPs were amplified (A-M Systems Model 1800), digitized (Digidata 1322B, Molecular Devices), and then analyzed (pCLAMP, Molecular Devices).

2.12. Statistical analysis

Data analysis was performed using GraphPad InStat and GraphPad Prism statistical software (GraphPad Software). Data were expressed as mean ± standard deviation (sd) and were

![Graph](image-url)
analyzed using Student’s t-test or one-way analysis of variance followed by Tukey-Kramer multiple comparisons test. Differences were declared statistically significant if \( p < 0.05 \).

3. Results

3.1. APP can induce the expression of p44

As mentioned above, p44\(^{+/+}\) transgenic mice display hyperphosphorylation of tau, synaptic deficits, and memory impairment (Pehar et al., 2010). When engineered to overexpress APP, the animals develop a severe form of neurodegeneration that affects memory-forming and memory-retrieving areas of the brain (Pehar et al., 2010). While analyzing the phenotype of p44\(^{+/+}\) and p44\(^{+/+};\text{APP}_{695/\text{swe}}\) mice, we noticed that the distribution pattern, as well as magnitude of changes, of tau hyperphosphorylation in these 2 different animal models was very similar (Pehar et al., 2010). Recently, we reported that p44 regulates tau phosphorylation by controlling transcription of tau kinases Dyrk1A, GSK3\(\beta\), Cdk5, p35, and p39 (Pehar et al., 2014). To assess whether the similarities previously observed at the histological levels were caused by similar biochemical events, we determined mRNA levels of the above kinases in the hippocampus of p44\(^{+/+}\) and p44\(^{+/+};\text{APP}_{695/\text{swe}}\) mice. Again, the results showed a strikingly similar outcome (Fig. 1A).

The above results would suggest that the changes in tau metabolism, as well as tau kinases, in the single- and double-transgenics are caused by p44 alone and are not influenced by the APP transgene. Alternatively, they could also suggest that APP acts—at least in part—through p44 itself and that the overexpression of transgenic APP in p44\(^{+/+}\) mice is unable to further stimulate a pathway that is already saturated by the overexpression of its down-stream target (in this case p44; see Fig. 1B).

To discriminate between the above 2 possibilities, we analyzed the expression profile of p53 isoforms in human neuroblastoma (SH-SY5Y) cells following overexpression of human APP. It is already known that the different p53 isoforms distribute differently between the cytosol and the nucleus (Bourdon et al., 2005); specifically, p53 is found in both compartments, whereas p44 is preferentially found in the nucleus (Pehar et al., 2014). Therefore, we separated nuclear from cytosolic proteins and analyzed them separately.

Fig. 1C shows that overexpression of APP caused a marked increase in the levels of both full-length p53 and p44. The upregulation of p44 was only evident in the nuclear fraction, whereas the upregulation of p53 was evident in both the nuclear and cytosolic fractions. Interestingly, APP overexpression also affected the levels of Delta133p53\(\alpha\) (Fig. 1C), another short and naturally occurring p53 isoform that is preferentially found in the nucleus (Pehar et al., 2014). In contrast, no effect was observed with Delta133p53\(\gamma\) (Fig. 1C), which is known to distribute to the cytosol (Bourdon et al., 2005).

When analyzed on SDS-polyacrylamide gel electrophoresis, human p44 migrates with a molecular mass of ~47 kDa, which is...
similar to p53β, a C-terminal truncated isoform of p53 (Bourdon et al., 2005). Because the antibody (PAb240) used in Fig. 1C cannot differentiate between the 2 isoforms, we decided to use antibody D01, which binds to the N-terminal 20-25 aa region that is missing in p44 (Fig. 1D) and recognizes p53β but not p44 (Bourdon, 2007). Fig. 1E shows that the band labeled as p44 in Fig. 1C was not observed when we used antibody D01. A similar behavior was observed with Delta133p53α. Finally, we confirmed the migration profile of both p53 and p44 in SH-SY5Y cells following transient overexpression (Fig. 1F).

To further confirm the results obtained with APP overexpressing cell lines, we analyzed the levels of p44 in the brain tissue of patients with late-onset AD. Tissue was processed to isolate nuclei and then analyzed by Western blot. Fig. 2A shows a very modest (not significant) or no increase in the levels of p44 in patients with AD. The analysis of post-mortem brain tissue is hampered by several confounding factors, which include the long duration of the disease, the degeneration that affects the brain tissue, and, finally, the intrinsic subject variability (discussed later). To compensate for these potential confounding factors, we decided to analyze

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**Fig. 5.** Haploinsufficiency of p53/p44 can rescue some of the phenotypic features caused by the overexpression of APP in the mouse. (A) Quantitative real-time PCR determination of indicated kinases in the hippocampal formation. Animals (males) were 2.5-month-old when analyzed. All values are mean (n = 12) ± sd. *p < 0.05; **p < 0.005. (B) Immunostaining with anti-phospho-tau antibodies shows reduced labeling in APP695/swe;p53−/− mice. Two different antibodies were used: AT8 (against pSer202 and pThr205) and S356 (against pSer356). Mice (males) were ~1-year-old when analyzed. (C) Long-term potentiation induction in hippocampal slices. Mice (males) were 2.5-month-old when analyzed. APP695/swe mice display deficits in the late component of long-term potentiation. These deficits are rescued by p53/p44 haploinsufficiency. **p < 0.005. Abbreviations: APP, amyloid precursor protein; PCR, polymerase chain reaction.
the nuclear levels of p44 in human neurons developed from iPSCs established from skin fibroblasts mosaic for DS (trisomy 21) that yielded isogenic control and trisomy 21 iPSCs. It is worth remembering that patients with DS have higher propensity of developing a clinical and pathological phenotype that resembles AD (Lott and Dierssen, 2010; Nelson et al., 2011; Zigman, 2013). This increased risk for AD has been linked to the extra copy of the APP gene, which is located on chromosome 21. Therefore, DS neurons were used in this study because—in light of the trisomy—they naturally overexpress APP. Analysis of differentiated neurons from DS (trisomy 21) and control iPSCs revealed a marked increase in the levels of p44 in the nucleus of DS neurons (Fig. 2B and C), supporting the results obtained with APP overexpressing cell lines (Fig. 1C). It is worth stressing that all DS iPSCs lines used here had a third copy of APP. Finally, control and DS neurons appeared morphologically similar (Fig. 2B; see also, Weick et al., 2012). Importantly, p44 levels were unchanged in non-differentiated iPSCs and only slightly increased in skin fibroblasts (data not shown), suggesting that the APP-mediated upregulation of p44 is neuron specific.

Next, we analyzed the levels of p44 in mice overexpressing APP (APP<sup>695/swv</sup>) and lacking (APP<sup>−/−</sup>) APP. Analysis of the nuclear fraction of APP<sup>695/swv</sup> mouse brains showed a consistent and statistically significant increase in the levels of p44 (Fig. 2D). However, no change in p44 levels was observed in APP<sup>−/−</sup> animals (Fig. 2D), suggesting that although APP can induce the expression of p44, it is not essential for its baseline levels. Finally, we determined the nuclear levels of p44 in transgenic mice overexpressing AICD alone (AICD<sup>Tg</sup>; Giliberto et al., 2008). The results revealed a significant upregulation (Fig. 2E).

In conclusion, when taken together, the results displayed in Figs. 1 and 2 indicate that APP can induce the expression of p44. These results were obtained in a variety of experimental systems, which included cell lines overexpressing APP, mice overexpressing APP and AICD, and human trisomy 21 iPSCs-induced neurons that express a third copy of APP. Therefore, the similar behavior of p44<sup>−/−</sup> and p44<sup>+/+</sup>;APP<sup>695/swv</sup> mice (see Fig. 1A and Pehar et al., 2010) could be explained by the fact that the overexpression of transgenic APP in p44<sup>−/−</sup> mice is unable to further stimulate a pathway that is already saturated by the overexpression of its downstream target. Under these conditions (mice overexpressing transgenic p44), further expression of APP (and induction of endogenous p44) is ineffective (see Fig. 1B).

### 3.2. AICD regulates translation of p53/p44 through an IRES-dependent mechanism

Next, we decided to determine the molecular mechanism responsible for the APP-mediated upregulation of p44. The different p53 isoforms are generated by alternative splicing, alternative promoter usage, or alternative initiation of translation (Bourdon, 2007). Specifically, p44 preferentially originates from alternative initiation of translation of the full-length p53 mRNA (reviewed in Scorable et al. 2005). The alternative translation of the p53 mRNA is achieved through 2 IRESs that regulate cap-independent translation of full-length p53 and p44 (see Fig. 3A). The first IRES, mediating translation at codon 1, is in the 5′-untranslated region of the mRNA and leads to translation of full-length p53; in contrast, the second IRES, mediating translation at codon 40 (p44), extends into the protein coding region and leads to translation of p44 (Ray et al., 2006). In addition to the above IRES-dependent alternative initiation of translation, p44 can also originate from a shorter mRNA generated by alternative splicing of intron 2 (Ghosh et al., 2004).

To discriminate between the 2 possibilities, we initially determined the levels of the short, p44-specific mRNA in SH-SY5Y cells overexpressing APP. However, no changes were observed (Fig. 3B), suggesting an IRES-dependent mechanism. This was somehow expected because the above mRNA appears to be limited to certain cellular situations and does not seem to be the preferential form of regulation of p44 levels (Bourdon, 2007; Scorable et al., 2005). Therefore, we decided to assess whether APP, or its proteolytic derivatives, could regulate IRES-dependent translation of the p53 mRNA.

APP undergoes proteolytic processing, which results in at least 3 major fragments: the N-terminal ectodomain, which is secreted in the extracellular milieu; the Aβ peptide, which tends to aggregate in extracellular amyloid plaques; and the C-terminal tail (also known as AICD), which is found in the cytosol and nucleus. AICD is known to act as a transcriptional activator and/or regulator (Cao and Sudhof, 2001; Pardossi-Piquard et al., 2005; reviewed in Beckett et al., 2012; Pardossi-Piquard and Checler, 2012). Therefore, we decided to focus our attention to AICD.

We initially determined the distribution profile of AICD. In fact, we reasoned that if AICD regulates translation of the p53 mRNA, then it must be found in messenger-ribonucleoprotein (mRNA) complexes and in the cytosol. Consistent with its biological functions, AICD was found in both the cytosol and the nucleus (Fig. 3C). Importantly, AICD was also found in polysomes containing highly purified mRNPs particles that were isolated from the cytosolic fraction (Fig. 3C). Next, we assessed whether the AICD found in the cytosol and polysome fractions was tightly bound to the p53 mRNA in the mRNP complexes. For this purpose, we performed an RNA-binding protein immunoprecipitation assay. Specifically, we purified polysomes from the cytosol of APP overexpressing SH-SY5Y cells and used them to immunoprecipitate AICD:mRNA complexes. The immunoprecipitated complexes were then digested with proteinase K to eliminate the protein component; the free mRNA was retrotranscribed and amplified by PCR. Primers used for the assay covered the second IRES located between the +1 and the +120 codons of the p53 mRNA (see Fig. 3A). The results indicate that AICD is bound to the p53 mRNA in native mRNP complexes (Fig. 3D). The band corresponding to the p53 mRNA was not resolved when the polysomes were immunoprecipitated with an anti-myc antibody (Fig. 3D), thus providing specificity to our results. As additional control we used the same AICD:mRNA complexes and tried to expand the IRES element of the LEF1 mRNA (Jimenez et al., 2005; Tsai et al., 2014). The results show that AICD does not bind to the IRES element of LEF1 and, therefore, does not appear to behave as a promiscuous IRES-binding protein.

To further confirm the above results, we assessed whether AICD was able to bind to the p53 mRNA in vitro. Specifically, we generated an mRNA construct corresponding to either the IRES1 or IRES2 area and then cross-linked it to biotin at the 3′ end. The biotinylated mRNA probe was used to pull down AICD from the cytosol. After purification with streptavidin, the mRNA:protein complex was digested with RNAses prior to SDS—polyacrylamide gel electrophoresis and immunoblotting with an anti-AICD antibody. The AICD fragment was successfully pulled down from the cytosol of both DS fibroblasts and SH-SY5Y<sub>APP</sub> cells (Fig. 3E and F). Interestingly, Fe65, which is known to associate with AICD and potentiate its transcription–regulatory activity (Pardossi-Piquard and Checler, 2012), was not found in the pull down (Fig. 3E). These findings might indicate that Fe65 is not required for AICD translation-regulatory activities. As internal control for the pull-down experiment, we also used a probe corresponding to the 3′end of the p53 mRNA. The results indicate that the AICD does not simply bind any mRNA probe (Fig. 3E and F; see lanes labeled as 3′end); binding is specific for IRES structures.
Finally, we used a bicistronic mRNA construct with a hairpin structure inserted at the 3′ of the GFP and immediately before the second IRES (IRE2) of the p53 mRNA, which controls translation of p44. IRES2, was—in turn—placed upstream of a luciferase-reporter system (see Fig. 3G). The hairpin element was used to minimize cap-dependent ribosomal read-through. Therefore, in this system, translation of GFP is cap-dependent, whereas translation of the luciferase system is IRES-dependent. When the bicistronic mRNA construct was transfected into SH-SY5Y cells, we detected successful translation of GFP (Fig. 3H and I) and luciferase activity (Fig. 3J). However, only the translation of the luciferase-reporter system was significantly affected by the overexpression of APP (Fig. 3H–J). The above findings indicate that AICD can bind to the second IRES of the p53 mRNA and regulate translation of p44 through a cap-independent mechanism.

When taken together, the above results indicate that AICD is able to regulate translation of p53/p44 through a cap-independent-mechanism that involves direct binding of the IRES structures on the p53 mRNA. Importantly, our results were obtained with different strategies, all of which included internal controls.

When the DNA binding properties of AICD were first described, it was shown that AICD acts as part of a complex that includes the adaptor protein Fe65 and the histone acetyltransferase Tip60 activity (Cao and Sudhof, 2001). Therefore, we decided to investigate whether the translational properties of AICD also required additional cofactors. Specifically, we used the IRES1 and IRES2 mRNA probes described in Fig. 3E and F to pull-down potential IRES-specific trans-acting factors (ITAFs) that bind to p53 IRES elements. Mass spectrometry of the pull down identified 2 proteins: nucleolin (UniProt/Swiss-Prot: P193338) and poly(pyrimidine tract)-binding protein 1 (PTBP1; UniProt/Swiss-Prot: P26599). Both proteins had previously been shown to be bound to the IRES elements of the p53 mRNA and regulate translation of p53 isoforms (Grover et al., 2008, 2009; Takagi et al., 2005). To confirm the mass spectrometry results, we repeated the mRNA:protein pull down and assessed the presence of the proteins by immunoblotting. The results show successful pull down for both nucleolin and PTBP1 (Fig. 4A and B). Again, an mRNA probe corresponding to the 3′ end of the p53 mRNA was used to confirm specificity (Fig. 4A and B; see lanes labeled as 3′end). Next, to explore functional association with AICD, we performed co-immunoprecipitation experiments in DS fibroblasts. Immunoprecipitation of AICD successfully pulled-down nucleolin but not PTBP1 (Fig. 4C). Nucleolin is known to act as a “negative” regulator of IRES-dependent translation (Takagi et al., 2005), whereas the AICD appears to act as a “positive” regulator (see Fig. 3). To assess whether age-associated changes in the expression profile of nucleolin contribute to the age-associated upregulation of p44 that we previously described (Pehar et al., 2014), we assessed levels of nucleolin in the frontal cortex of wild-type/non-transgenic mice. The results show a significant downregulation of nucleolin as a result of normal aging (Fig. 4D).

When taken together, the above results suggest that functional association between AICD and nucleolin may affect translation of the p53 mRNA in an age-dependent manner (discussed later).

3.3. Downregulation of p53/p44 rescues the APP phenotype

To assess the biological significance of our findings, we analyzed mice overexpressing APP (APP695/swe). The animals displayed increased mRNA levels of Dyn1,1A, GSK3β, p35, and p39 (Fig. 5A) as well as hyperphosphorylation of tau (Fig. 5B). The upregulation of the above kinases was already evident at 2.5 months of age. These changes were all rescinded by the haploinsufficiency of Tp53, the gene that encodes all p53 isoforms (Fig. 5A and B). APP695/swe mice display defects in the postsynaptic component of long-term potentiation (reviewed in Duyckaerts et al. 2008). These defects were also rescued by the haploinsufficiency of Tp53 (Fig. 5C).

When taken together, the above studies suggest that some of the changes induced by the overexpression of APP, specifically those impinging on tau, might depend on the induction of p44. Tp53 encodes both full-length p53 and p44; therefore, p53- and p44-specific events cannot be truly differentiated in the above p53+/− model. However, overexpression of p44 alone (p44+/− mice) caused altered metabolism of tau and synaptic deficits (Pehar et al., 2010), whereas overexpression of full-length p53 (Super-p53 mice) did not (Garcia-Cao et al., 2002). Therefore, it is likely that the changes induced by the genetic disruption of Tp53 are directly linked to p44 itself or to the imbalance in p44:p53 expression caused by AICD.

4. Discussion

In the present study, we show that AICD, the cytosolic tail of APP, can bind to the IRES elements of the p53 mRNA and regulate translation of p44, the longevity-assurance isoform of p53. Specificity of binding and translational-regulatory activity were established with a combination of in vitro and in vivo strategies. We also show that AICD functionally associates with nucleolin, an already known ITAF that binds to the IRES elements of the p53 mRNA (Grover et al., 2008, 2009). Finally, the possible biological implications of our findings were assessed in a mouse model of AD. Combined with already published data (Pehar et al., 2010, 2014), our results provide a possible molecular link between APP, p44, and tau (discussed below).

4.1. IRES elements and protein translation

The initiation of protein translation in eukaryotes is typically mediated by a cap- and 5′end-dependent mechanism. Specifically, the “ribosomal scanning machinery,” which includes the 40S ribosomal subunit and the initiation factor complex, recognizes and binds to the 5′end (methylated) cap structure of the mRNA. Translation starts when the “scanning machinery” reaches the initiation codon (Voet and Voet, 2011). IRES elements are highly structured internal segments of the mRNA that allow cap- and 5′end-independent binding of the “ribosomal scanning machinery.” They were initially identified on virus mRNAs. In eukaryotes, they ensure protein translation under conditions that are characterized by a partial block of cap-dependent translation (i.e., ER stress, mitosis, and so forth) (reviewed in Baird et al., 2006). IRES elements across mRNAs tend to have limited sequence homology and are most typically characterized by their secondary and tertiary structure. Comparative sequence homology and minimum free energy structure modeling have been used to search for IRES elements across the mRNA database; however, they have not been able to correctly predict a common structural signature (Wu et al., 2009). As a result, the identification of IRES structures has mostly been driven by direct testing of individual mRNAs through bicistronic constructs (Baird et al., 2006). IRES-dependent translation is regulated and enhanced by mRNA binding proteins known as ITAFs. In general, ITAFs are found associated with mRNP complexes and “free” in the cytosol. Some ITAFs also participate in processing and export of mRNAs from the nucleus and, as a result, can also be found in nuclear fractions (Baird et al., 2006). Finally, ITAFs with both transcription- and translation-regulatory functions have also been identified (Baird et al., 2006).
4.2. AICD binds to IRES elements on the p53 mRNA

The first evidence that AICD is released in the cytosol after γ-cleavage of APP was reported by Passer et al. (2000). Soon after, Cao and Sudhof (2001) showed that a functional complex of AICD, Fe65, and Tip60 had DNA binding activity. The transcriptional properties of AICD have now been confirmed by several groups (reviewed in Beckett et al. 2012; Pardossi-Piquard and Checler, 2012). Targeted genes include APP, BACE1, and NEP, among others (reviewed in Beckett et al. 2012; Pardossi-Piquard and Checler, 2012). Previous work from Alves da Costa et al. (2006) showed that AICD can induce transcription of the p53 mRNA. Here, we report yet another surprising feature of AICD biology, specifically, its ability to bind to IRES structures on the translating mRNA of p53. This activity seems to be Fe65 independent and results in increased translation of p44, a short isoform of p53 with “longevity-assurance” activity. Whether AICD can bind to other IRES structures is currently unknown and it remains to be assessed. Direct analysis of known IRES structures (http://www.iresite.org/IRESWeb.php) did not reveal specific features that would make p53 IRES elements unique. Therefore, it is possible that AICD might regulate translation of other mRNAs. However, as stressed above, no prediction model has been able to find a common IRES or ITAF signature (Baird et al. 2006). As such, identification of other putative targets will require direct testing.

The 2 IRES elements on the p53 mRNA have been shown to regulate translation of p53 and p44 under different cellular conditions (Grover et al. 2009). Specific ITAFs have also been identified. They include, p53 itself, PTBP1, ribosomal protein L26, and nucleolin (Grover et al., 2008, 2009; Mosner et al., 1995; Takagi et al., 2005). Our work provided evidence that AICD is a novel p53 ITAF. Interestingly, immunoprecipitation of AICD was also able to pull-down nucleolin, suggesting functional interaction between the 2 proteins. The interaction appears to be specific because PTBP1 was not pulled down with the AICD. The biological significance of the AICD—nucleolin interaction remains to be fully dissected. However, as mentioned above, nucleolin seems to be a “negative” ITAF (Takagi et al., 2005), whereas, such as PTB, AICD appears to be a “positive” factor (present study). In our study, we also show that nucleolin levels in the brain decrease in an age-dependent manner. Unfortunately, the AICD peptide displays a very short half-life (reviewed in Nalivaeva and Turner, 2013) impeding direct and definitive assessment of its endogenous levels throughout age. However, both expression and β-processing of AICD appear to be regulated in an age-dependent manner (reviewed in Nalivaeva and Turner, 2013). Because AICD is stoichiometrically released with the Aβ peptide as a result of the β/γ processing of APP, we could expect a parallel increase in intracellular levels of AICD. Obviously, as mentioned above, because of the intrinsic difficulties in assessing endogenous levels of AICD in the brain, the above scenario remains speculative. Of interest is the fact that the expression of p44 is also regulated (at least in the brain) in an age-dependent manner (Pehar et al., 2014). Perhaps AICD and nucleolin interact to regulate p53 translation in a coordinated manner. It is likely that molecular dissection of their interaction will yield important information.

4.3. AICD-p44 and possible impact for aging, AD, and age-associated tauopathies

Although mostly known for its tumor-suppressor activities, TP53 encodes a large family of proteins retaining different domains and/or motifs of the full-length protein (Bourdon, 2007; Bourdon et al., 2005). The different isoforms result from a combination of alternative promoter usage and alternative initiation of translation (Bourdon, 2007; Bourdon et al., 2005). The alternative translation of p53 and p44 is tightly regulated by several ITAFs. As mentioned above, p53 itself can regulate its own translation by binding to the first IRES element (reviewed in Scrable et al., 2005). In fact, full-length p53 binds to the stem-loop element of its own mRNA and, by doing so, prevents translation of codon 1 while allowing translation of codon 40. This blockage can be released by the Mdm2 E3 ligase, which regulates the degradation of p53 itself (Murray-Zmijewski et al., 2008), thereby reactivating translation of codon 1. The blockage is reinstanted when p53 levels increase again, because of either increased translation or reduced degradation. Importantly, Mdm2 also regulates degradation of p53 by binding to the same N-terminal region of p53 that is missing in the Δ40p53 isoform (Yin et al., 2002). In conclusion, the expression of the p53 isoforms appears to be tightly regulated by a complex array of molecular events.

The p53 isoforms are likely to retain some of the functions elicited by the full-length protein. However, they are also likely to elicit biological events that are either unique or different. Specifically, p44 appears to possess “longevity-assurance” activity. When overexpressed in the mouse (p44+/+), it causes a progeroid phenotype and reduced life span (Maier et al., 2004). Importantly, the phenotype requires co-expression of the full-length protein (Maier et al., 2004), suggesting that the ratio (or functional interaction) of the different p53 isoforms, rather than the individual proteins, determine the biological outcomes. When engineered to overexpress APP, p44+/+ mice display an accelerated form of AD-like neuropathology (Pehar et al., 2010) offering a possible link between aging and AD.

Although highly suggestive, the impact of our findings for AD neuropathology remains to be fully determined. Because p44 can regulate transcription of several tau kinases (Pehar et al., 2014) and, therefore, affect the phosphorylation status of tau, we could envision a molecular pathway that connects AICD, p44, and tau (present study and Pehar et al., 2014, 2010). This pathway could be implicated in AD and in other forms of tauopathies associated with aging. Assessment of p44 levels in the brain of patients with late-onset AD did not yield striking results. However, AD is a heterogeneous disease and not all patients with AD are identical. For example, patients with AD with a duplication of the APP gene have been identified; they express levels of APP mRNA that are in the same range of patients with DS (trisomy 21) (Israel et al., 2012). Because differentiated neurons of patients with DS display increased levels of p44, we could conceive that patients with AD that display either duplication of APP or clear upregulation of APP β processing will share similar features. Interestingly, studies have shown that patients with late-onset AD (who do not have a duplication of the APP gene) express increased levels of a p53 isoform that migrates with the molecular mass of p44 on polyacrylamide gels (Uberti et al., 2006, 2008). It is also worth stressing that transgenic mice over-expressing AICD (alone or with Fe65) develop some of the features that characterize AD (Ghosal et al., 2009; Giliberto et al., 2008). These include abnormal activation of tau kinases and abnormal phosphorylation of mouse tau as well as synaptic deficits and increased neuronal susceptibility to exogenous stress (Ghosal et al., 2009; Giliberto et al., 2008).

In conclusion, because of the impact of the role that p44 plays in longevity and cognitive-related events, our findings might offer novel insights on an important topic of biomedical research.

Disclosure statement

The authors have no actual or potential conflicts of interest.
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