Loss of the p53 transactivation domain results in high amyloid aggregation of the Δ40p53 isoform in endometrial carcinoma cells

Nataly Melo dos Santos¹, Guilherme A. P. de Oliveira²,³, Murilo Ramos Rocha¹, Murilo M. Pedrote², Giulia Diniz da Silva Ferretti², Luciana Pereira Rangel²,⁴, José A. Morgado-Diaz¹, Jerson L. Silva²*, Etel Rodrigues Pereira Gimba¹,²,⁵*

¹Instituto Nacional de Câncer (INCA), Coordenação de Pesquisa, Programa de Oncobiologia Celular e Molecular, Rio de Janeiro, Brazil;  
² Instituto de Bioquímica Médica Leopoldo de Meis, Instituto Nacional de Ciência e Tecnologia de Biologia Estrutural e Bioimagem, Centro Nacional de Biologia Estrutural e Bioimagem, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil;  
³ Department of Biochemistry and Molecular Genetics, University of Virginia, Charlottesville, VA, 22908-0733, United States of America.  
⁴, Faculdade de Farmácia, Universidade Federal do Rio de Janeiro (UFRJ); Rio de Janeiro, Brazil;  
⁵Universidade Federal Fluminense, Instituto de Humanidades e Saúde (IHS), Departamento de Ciências da Natureza, Rio de Janeiro, Brazil;

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*To whom correspondence should be addressed: Etel Rodrigues Pereira Gimba and current email address: etelgimba@id.uff.br; Tel.: +55-21-3207-6599 and Jerson L. Silva, email: jerson@bioqmed.ufrj.br

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ABSTRACT

Dysfunctional p53 formation and activity can result from aberrant expression and subcellular localization of distinct p53 isoforms or aggregates. Endometrial carcinoma (EC) is a cancer type in which p53 status is correlated with prognosis, and TP53 mutations are a frequent genetic modification. We aimed here to evaluate the expression patterns of different p53 isoforms and their contributions to the formation and subcellular localization of p53 amyloid aggregates in both EC and endometrial non-tumor cell lines. We found that full-length (fl) p53 and a truncated p53 isoform, Δ40p53, resulting from alternative splicing of exon 2 or alternative initiation of translation at ATG-40, are the predominantly expressed p53 variants in EC cells. However, Δ40p53 was the major p53 isoform in endometrial non-tumor cells. Immunofluorescence assays revealed that Δ40p53 is mainly localized to cytoplasmic punctate structures of EC cells, resembling solid-phase structures, similar to those found in neurodegenerative pathologies. Using light-scattering kinetics, circular dichroism (CD) and transmission electron microscopy, we noted that the p53 N-terminal transactivation domain (TAD) significantly reduces the aggregation of the wild-type p53 DNA-binding domain, confirming the higher aggregation tendency of Δ40p53, which lacks this domain. The differential aggregation properties of p53 isoforms in EC cells may open up new avenues in the development of therapeutic strategies that preferentially target specific p53 isoforms to prevent p53 amyloid aggregate formation.

INTRODUCTION

Endometrial carcinoma (EC) is the most common gynecological malignancy in the Western world. It is traditionally classified as type I and type II tumors (1), which is based on clinical, endocrine, histopathological, genetic, as well as molecular features. More recently, the Cancer Genome Atlas Research (TCGA) network classified them into four molecular subgroups (2). Mutations at TP53 are present in 5-12% of all type I EC cases, as opposed to type II EC, in which TP53 mutations are the most common genetic modification (3). It has also been described that p53 overexpression in EC tumors is strongly predictive of recurrent EC and mostly not correlated with TP53 mutations (4). Moreover, it has been revealed that EC tumors presenting extensive copy-number alterations and TP53 mutations are highly aggressive (2). Thus, TP53 genetic modifications are key genetic features in EC biology.

The human TP53 gene encodes a nuclear protein that generally behaves as a tumor suppressor and responds to stress conditions to induce cell-cycle arrest, senescence, or programmed cell death (5). The roles of p53 are controlled by transcriptional and translational mechanisms, protein stability, and subcellular localization. In particular, the regulation of p53 subcellular localization depends on factors that influence its nuclear import and export, subnuclear localization, and cytoplasmic tethering and sequestration (6). Notably, cytoplasmic expression provides additional roles to p53, such as modulating apoptosis via a transcription-independent action (7), autophagy, metabolism, oxidative stress, and drug response (8). Cytoplasmic inclusions of p53 have also been correlated with sequestration of p53 as large protein amyloid aggregates (9).

Somatic TP53 mutations are the most frequent in most human cancers and eliminate its tumor suppressor functions as well as promote oncogenic properties (5). It is often stated that 50% of cancers have mutated or inactivated p53. However, the real number is probably much higher when the involvement of the entire p53 pathway in tumorigenesis is considered. In tumors in which TP53 is not mutated, p53 itself or its signaling can be inactivated by post-transcriptional and post-translational modifications, subcellular localization, and interaction with other proteins (10). In tumor cells, a dysfunctional p53 protein often presents an aberrant misfolded and inactive conformation, which accumulates and aggregates to form amyloid-like oligomers and fibrils, related to impairment of p53 roles (11-13). Accordingly, p53 aggregation may be a crucial step in tumor progression. Our group and others have suggested that the formation of mutant p53 aggregates is associated with loss-of-function, dominant-negative, and gain-of-function effects and that these features seem to be correlated to its prion-like behavior (11-15). Intriguingly, wild-type (wt) p53 aggregation has also been reported in high-grade serous ovarian carcinoma (HGSOC) cancer cells ex-
hibiting cancer stem cell properties, which was associated with p53 loss-of-function and platinum resistance (16). Thus, it seems that not only mutant, but also wt p53 can undergo aggregation and dysfunction.

The human TP53 gene expresses at least twelve p53 isoforms, as a result of distinct molecular mechanisms such as alternative splicing, alternative promoter, or alternative initiation codon usage (17) (Fig. 1). Each p53 variant can be combined to three distinct C-terminal forms (α, β, and γ). In the C-terminus domain, the α isoforms contain an oligomerization domain (OD), whereas the β and γ isoforms include novel amino acid residues instead of an OD. The fl-p53, p53β, and p53γ proteins contain the conserved N-terminal transactivation domain (TAD). Δ40p53 isoforms are truncated proteins resulting from alternative splicing of exon 2 and/or alternative initiation of translation at ATG-40. The Δ40p53 protein lacks the conserved N-terminal TAD1, but still contains TAD2. Δ133p53 isoforms are truncated p53 proteins with the entire TAD and part of the DBD deleted. Translation is initiated at ATG-133. These isoforms can modulate p53 transcriptional activity in the absence of genomic alterations and are capable of altering p53 function, besides being differentially expressed in tumor and non-tumor cells (18). Most of these p53 variants share a common DNA-binding domain (DBD) with three different N-terminal domains. By convention, p53α corresponds to the canonical or full-length (fl)-p53. In addition to these isoforms, Δp53 variants lack N-terminal domains (18). Although these p53 isoforms have been hypothesized to aggregate differently in cancer cells (19), experimental data showing the contribution of p53 isoforms to protein oligomer aggregation are still lacking. The N-terminal transactivation domain (TAD) is intrinsically disordered and has been recently shown to interact with the DNA-binding surface of the DBD through primarily electrostatic interactions, resulting in inhibition of binding of non-specific DNA (20).

Given the key contributions of TP53 genetic modifications in EC etiopathogenesis, the p53 aggregation potential, and related functional roles of p53 isoforms, this work aimed to evaluate the expression profile of some p53 isoforms in EC tumor and endometrial non-tumor cell lines. We then investigated the aggregation potential and subcellular localization of differentially expressed p53 isoforms, mainly focusing on fl-p53 and Δ40p53 variants. Moreover, we also compared the in vitro aggregation properties of recombinant p53 constructs to test whether the intrinsically disordered p53 TAD modulates its DBD aggregation.

RESULTS

Transcriptional profile of p53 isoforms in endometrial carcinoma – We investigated the expression of five p53 isoforms: fl-p53 (p53), p53β, p53γ, Δ40p53, and Δ133p53 (Fig. 2 and Fig S1) in EC tumor (Ishikawa, RL95-2, AN3CA, and KLE) and non-tumor cell lines (E6/E7/TERT and EM42), and determined their putative subcellular localization and presumed aggregation potential.

We found that transcripts coding for the tested p53 isoforms are differentially expressed between EC tumor and non-tumor cell lines (Fig. 2 and Fig. S1). Markedly, fl-p53 followed by Δ40p53, are the major p53 isoforms expressed in EC cells (Fig. 2A-D). Especially in cell lines representative of type I EC tumors (Ishikawa, RL95-2, and AN3CA), Δ40p53 was expressed at higher levels than the remaining shorter p53 variants (Fig. 2). Δ40p53 variant was also the major isoform expressed in EC non-tumor cells (Fig. 2E and F).

Protein expression of p53 isoforms in endometrial carcinoma – We also characterized the protein expression levels of the fl-p53 and Δ40p53 isoforms using immunoblot in these same cell lines (Fig. S2). Because specific antibodies against Δ40p53 cannot be produced, its protein levels were determined only indirectly with a combination of anti-p53 antibodies. Δ40p53 expression was evaluated using the anti-p53 monoclonal antibodies DO-1, DO-7, and 1801, as described before (17). These preliminary findings showed that when fl-p53 expression was analyzed using the DO-1 or DO-7 antibodies, which only detect the fl-p53 variant (with molecular weight around 53 kDa), higher levels of fl-p53 were observed compared to those found in the non-tumor cell lines E6/E7/TERT and EM42 (Fig. S2A and S2B), as has been generally observed at the transcriptional level (Fig. S1). Similar data have also been found using the 1801 anti-p53 antibody, which detects both the fl-p53 (53 kDa) and the Δ40p53 (47 kDa) variants (Fig. S2C), as previously described (17). Notably, in non-tumor cells, lower levels of the Δ40p53


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protein were observed when compared to EC tumor cells, even though the corresponding gene transcript was the most abundant in non-tumor cells (Figs. 2, Fig. S2 and Fig. S1). Therefore, transcriptional and supporting protein analysis of these p53 isoforms provide evidence that mainly fl-p53 and Δ40P53 display differential expression patterns in EC tumor and non-tumor cells and that the transcript levels of these isoforms display a tendency to correspond to the respective protein levels.

Aggregation potential of p53 isoforms in endometrial carcinoma – We next considered whether fl-p53 and Δ40p53 contribute differently to the formation of p53 amyloid aggregates and on the isoforms subcellular localization. We performed immunofluorescence analysis of E6/E7/TERT, KLE, and AN3CA cells using the A11 amyloid oligomer-specific antibody, DO-7, and 1801 anti-p53 antibodies (Fig. 3A).

Differential staining patterns between these two antibodies would indicate Δ40p53-specific staining in these cells. A11 antibody staining (green) exhibited a diffuse pattern throughout the cells (Fig. 3A, G-L). As shown by the DO-7 staining pattern, fl-p53 presented a differential staining pattern shifting from a predominantly cytoplasmic staining in E6/E7/TERT EC non-tumor cells to a both cytoplasmic and mainly nuclear staining pattern in KLE and AN3CA EC cells (Fig. 3A, M, O, and Q). Notably, as evidenced by 1801 antibody staining, we found a very particular pattern of p53 punctate cytoplasmic structures in all three cell lines analyzed (mostly in EC tumor cells), in addition to a nuclear p53 staining in KLE and AN3CA EC cells (Fig. 3A, O-R and U-X). Taking into consideration the different affinities of DO-7 and 1801 antibodies (Fig. S2) for fl-p53 and Δ40p53, respectively, we might infer that their differential staining patterns possibly reflect Δ40p53-specific staining. A concentration of puncta was observed in the regions between cells and also under the plasma membrane (Fig. 3A, T, V, X, and insets). Through co-localization evaluation and correlation analysis, we further assessed the interaction between p53 isoforms and amyloid aggregates. Although the Δ40p53 staining patterns were similar among the cell lines analyzed, we found a higher number of 1801-stained puncta and a higher degree of co-localization between 1801- and A11-stained amyloid aggregates in EC cells (Fig. 3A, T, V, X, and insets; quantification is shown in Fig. 3B). However, scattered cytoplasmic and nuclear DO-7-stained patterns were mainly observed in non-tumor and EC tumor cell lines, respectively (Fig. 3A, M, O, Q, S, U, W, and insets).

The transactivation domain of p53 prevents its aggregation – As an attempt to explain what triggers the higher amyloid aggregation of the Δ40p53 isoform in EC cells, we hypothesized whether the missing N-terminal residues of this isoform (residues 1-40) from the TAD would favor aggregation. To do so, we recombinantly expressed a construct containing p53 residues 1-320 comprising the p53 TAD, residues 1-61 and the DBD (Fig. 4A, red). As previously shown, we used the p53 DBD construct (residues 94-312) as a standard for p53 aggregation (11) and (Fig. 4A, blue). After purification of both constructs (Fig. 4B) and analytical size exclusion chromatography verification (Fig. 4C), we measured the time course of both proteins by light scattering at physiological temperature (Fig. 4D). Notably, the construct containing the full-length TAD and the DBD region revealed negligible scattering when compared to the DBD alone, suggesting that no higher-order aggregates were formed. Ultracentrifugation (UC) after incubating both constructs at physiological temperature for 40 min showed aggregation for the DBD and a clear solution for the DBD construct containing the TAD segment (Fig. 4E). Additionally, the aggregation of the DBD construct revealed a weak thioflavin T binding (ThT) against UV light (Fig. 4F). To further explore the aggregation potential of the TAD within the longer construct, we increased the temperature of both constructs from 25˚C up to 75˚C using a constant rate of 1˚C/min. By monitoring the far-UV circular dichroism (CD) spectra at 25˚ and 75˚C, the DBD revealed the typical behavior of secondary structure lost followed by aggregation and protein precipitation (Fig. 4G). Indeed, precipitation was clearly visible at the end of the measurement similarly to what was shown in Fig. 5E. Surprisingly, the TAD.DBD maintained a clear solution even after achieving 75˚C and the CD spectrum was consistent with increased content of β-strands (Fig. 4H). These observations support the formation of soluble oligomers, later confirmed by negative staining transmission.
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electron microscopy (Fig. 4I). Altogether, the data shows how the TAD negatively impacts the aggregation behavior of p53. It slows down the kinetics of p53 aggregation in such a way it allows structural reorganization to form ordered oligomeric species.

**DISCUSSION**

This work aimed to evaluate the expression patterns and aggregation properties of p53 isoforms in EC and endometrium non-tumor cells and the correlation of these patterns to p53 aggregation potential in these cells. We found that although the fl-p53 transcript is the predominantly expressed p53 transcript in EC cells, followed by Δ40p53, the latter is the major component of p53 amyloid aggregates in EC cells. Once Δ40p53 was the major component of p53 cytoplasmic aggregates in EC cells, we propose herein that Δ40p53 is a key modulator of p53 tumor suppression and oncogenic activities in EC cells (Fig. 5), as has been shown in hepatocellular carcinoma, melanoma and breast tumors (21), in which this isoform presents a dominant-negative role over fl-p53. Our results also suggested that Δ40p53 may be mainly sequestered in cytoplasmic amyloid aggregates, similar to those previously reported to modulate p53-mediated tumor suppressor roles or its oncogenic activities (22). The presence of these structures in cellular projections between adjacent cells suggest that they can be transported from cell to cell, as reported elsewhere (11,12,15). The Δ40p53 may modulate p53 aggregation properties and EC progression in which p53 genetic alterations are related to a poor prognosis (2).

Few studies have described the expression patterns of N-terminally truncated p53 isoforms, including the Δ40p53 variant, in human carcinomas and corresponding non-tumor samples. The Δ40p53 isoform lacks the first 40 amino acids encoding the first TAD of p53, the activating phosphorylation sites, and the Mdm2-binding site, the primary regulator of p53 degradation (23). This isoform can result from alternative translation initiation in exon 4 or from alternative splicing of intron 2 and is typically expressed during early embryogenesis, associated with less differentiated and proliferative cells, and generally not expressed in the corresponding adult tissues (21).

The ability of the p53 TAD to inhibit aggregation of the DBD (Fig. 4) could be an explanation for the high Δ40p53 propensity to form amyloid aggregates in the cytoplasm of EC cells. As recently shown by Krois and co-authors (20), the p53 TAD would directly interact within segments of the p53 DNA-binding site through electrostatic interactions. These authors proposed that these interactions are dynamically transient and would modulate the binding of p53 to DNAs (20). It is noteworthy that the p53 DNA-binding motif is not in the vicinity of the previously reported aggregated prone p53 segment (14,15) (Fig. 4J). However, we showed by molecular dynamic simulations that the p53 DBD presents labile segments with enhanced tendency of exposed backbone hydrogen bonds (BHBs), a condition that may explain its structural instability at near-physiological temperature and the average-weighted population of molten globule-like species prone to aggregation (24,25). With that, we tempted to speculate that transient interactions of the TAD within the DNA-binding motif may help to provide overall p53 structural integrity narrowing the distribution of molten globule-like species, the ones previously shown to be pre-amyloidogenic.

Our findings supported the formation of soluble oligomers by TAD(DBD), later confirmed by negative staining transmission electron microscopy (Fig. 4I), implying that TAD negatively impacts the aggregation behavior of p53.

In the context of cancer, our group recently overviewed the pathological aggregation of misfolded p53 in malignant tumors. It has been found that p53 mutants exert a dominant-negative regulatory effect on wild-type (WT) p53, by converting the wild-type p53 into aggregated species, acquiring a gain-of-function (GoF) phenotype and the loss of its tumor-suppressor roles. This prion-like behavior of oncogenic p53, as observed for neurogenerative diseases, provides an explanation for its dominant-negative and GoF properties, including the high metastatic potential of cancer cells carrying p53 mutations (11-13). In the context of our current data, isoforms that lack the TAD domain could then modify p53 aggregation properties, similarly to p53 oncogenic mutants, possibly presenting dominant-negative and GoF properties.

We recently found that RNA can modulate the aggregation of p53 DBD and fl-p53 (26). Low RNA:protein ratio resulted in more aggregation than high RNA:protein ratios. This
could further explain the higher tendency of Δ40p53 to aggregate in the cytoplasm where there is lower concentration of RNA. It has been recently proposed that prion-like RNA binding proteins, such as TDP-43 or FUS, form solid pathological aggregates in the cytoplasm because low RNA/protein ratios promote phase separation into liquid droplets (27). This could be the case of Δ40p53 which lacks part of the TAD, preventing interaction with non-specific nucleic acids (20). Recent molecular dynamics data also corroborate to our findings, in which it has been reported that the Δ133p53β and Δ160p53β isoforms are much less stable than the wt p53β. Moreover, these authors demonstrated that Δ133p53β dimer could form relatively stable complex with p53-specific DNA (28).

Several lines of evidence have shown the dominant-negative behavior of N-terminally truncated p53 and p73 isoforms against its full-length counterparts (23,29,30). Dominant-negative events are frequently explained when mutated or truncated proteins interfere by somehow in the activities of the full-length protein. To explain that, proposed hypothesis include promoter competition and heterocomplex formation (31). It was previously shown that Δ40p53 is able to form hetero-tetramers with wt p53 and suppress the MDM2-mediated p53 degradation (32). Further, Δ40p53 was shown to activate gene expression through the second TAD domain (residues 43-63) and has shown dominant-negative effect toward wt p53 inhibiting p53 transcriptional activity and the p53-mediated apoptosis (23,32). It is worth to mention that the p53 TAD domain is an essential element for either p53 tumor suppressor activities and regulation by post-translational modifications. Upon DNA damage, a cascade of phosphorylation occurs within the TAD including Ser15, Thr18, and Ser20, a situation that facilitates p53-MDM2 dissociation and p300/CTB binding for transcriptional activity (33,34). Indeed, because the first TAD is lacking in Δ40p53, a profound deregulation effect is expected in these cells, especially concerning changes in transcriptional activity and the potential dominant-negative activity toward the full-length protein. Based upon the RNA, p53, and Δ40p53 levels observed in our study we tempted to speculate these are possible mechanisms occurring in EC cells. More interesting, the presence of Δ40p53 aggregates in these cells rises relevant questions whether gain-of-function effects would also participate in this kind of tumor. These are important aspects awaiting further investigation.

Our immunofluorescence data indicate that mainly cytoplasmic p53 amyloid oligomers may be prevalent in the EC cell lines and that these aggregates mostly contain the Δ40p53 isoform. As stated before, fl-p53 and Δ40p53 form heterocomplexes. Because Δ40p53 lacks an Mdm2-binding site, these heterocomplexes escape Mdm2-mediated degradation and therefore can accumulate (35). These characteristics are also consistent with our Δ40p53 results, which is the second major p53 variant in EC cells and the dominant variant in endometrial non-tumor cells, being the main component at p53 amyloid aggregates in these cells. Moreover, it has been described that Δ40p53 possess a p53 conformation that is associated with a more active state, and that Δ40p53 can also alter the posttranslational modification profile of fl-p53 (36). Posttranslational modifications at the N-terminus of fl-p53, for instance, might increase the recruitment of transcriptional co-activators such as p300 and PCAF, and thus be responsible for increased promoter-binding capacity of the heterocomplexes. Thus, we suggest that mainly cytoplasmic and also nuclear amyloid aggregates containing mostly the Δ40p53 variant could be a strategy to efficiently modulate p53 tumor suppressor and oncogenic activities in EC cells. Cytoplasmic structures mainly containing Δ40p53 amyloid aggregates could be related to dysfunctional p53 related to EC progression, a hypothesis that should be validated by further studies. In addition, physical interaction between the Δ40p53 and amyloid aggregates and the differential binding relative to fl-p53 should be further validated by other approaches, such as immunoprecipitation.

Therefore, we provide the first experimental evidence that distinct p53 isoforms can differently contribute to misfolded p53 aggregate states in tumor cells (Fig. 5), especially in EC cells, and possibly other tumors in which p53 is functionally deregulated. Our in vitro work (Fig. 4) clarifies the high propensity of Δ40p53 aggregation in EC cells (Fig. 4J). Targeting mutant p53 aggregation has been proposed as a novel strategy against cancer, especially to nullify the devastating gain-of-function effects. Our data support the notion that the Δ40p53 variant can be specifically targeted as a strategy to prevent formation of
p53 amyloid aggregates in which this isoform is the major component.

**EXPERIMENTAL PROCEDURES**

**Cell lines and culture conditions**

Endometrium tumor (Ishikawa, RL-95-2, AN3CA, e KLE) and non-tumor (E6/E7/TERT e EM42) cell lines were kindly provided by Dr. Tim Hui-Ming Huang and Dr. Ya-Ting Hsu (University of Texas, San Antonio, USA). Cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) or DMEM/F12 1:1 (Nutrient F-12 Ham, Thermo Fisher Scientific, Waltham, MA, USA) containing 10 % fetal bovine sera (FBS) and 1 % penicillin and streptomycin.

**Quantitative real-time PCR (qRT-PCR) and oligonucleotide sequences**

The transcript levels of each p53 isoform was determined using qRT-PCR. Total RNA was extracted using RNeasy mini kit (Qiagen, Hilden, Germany) and cDNA synthesis was performed using 1 µg of total RNA. Oligonucleotides primers used for these assays and amplification conditions are shown on Table 1.

**Immunoblotting**

Cell line pellets were washed in phosphate buffered saline (PBS) and then incubated with lysis buffer (1 % Triton X-100; 150 mM NaCl; 10 mM Tris pH 7.4; 1 mM EDTA; 1 mM EGTA pH 8.0; 0.2 mM Na3VO4; 0.2 mM NP-40). 100 µg of protein lysates were resolved using SDS-PAGE electrophoresis and then transferred to nitrocellulose membranes. Anti-p53 antibodies DO-1, DO-7, and 1801 (Santa Cruz Biotechnology, Dallas, Texas, USA) were used at 0.8 µg/mL and incubated overnight. Because Δ40p53 lacks the 40 N-terminal amino acids (Fig. 1, region TAD 1), it can be distinguished from fl-p53 using a combination of the anti-p53 monoclonal antibodies DO-1 or DO-7 and 1801, which bind to this region. The ECL detection reagent (Amersham ECL Prime Western Blotting Detection Reagent – GE, Little Chalfont, United Kingdom) was used to detect antigen-antibody complexes. Membranes were imaged on the ChemiDoc MP Imaging System (BioRad).

**Immunofluorescence**

Cells (5 × 10^5 per chamber) were seeded into 12-chamber tissue culture slides. The next day, cells were rinsed with ice-cold PBS, fixed with 4 % paraformaldehyde, permeabilized with 0.5 % Triton X-100, and blocked in 5 % BSA. Cells were subjected to immunofluorescence staining with either with DO-7 or 1801 anti-p53 antibodies (1:400) and anti-A11 antibody (1:400) and incubated overnight at 4°C. Cells were then incubated with goat anti-mouse IgG/Alexa 568-labeled anti-rabbit (1:800) or Alexa 488-labeled anti-mouse (1:800) secondary antibodies (Thermo Fisher Scientific, Waltham, MA, USA) at room temperature for 1 h, and finally stained with 4’,6-diamidino-2-phenylindole (DAPI). Cells were then examined using fluorescence confocal microscopy (FV10i-O, Olympus, Tokyo, Japan) and analyzed using FV10-ASW and ISI softwares.

**Protein expression and purification**

The pET15b vectors containing the His-tagged constructs of p53 DBD (Addgene, #24866) and p53 TAD.DBD (Addgene, #24864) were heat-shock transformed into E.coli BL21 (DE3) pLysS strain (Invitrogen, #C6060-03), grown at 37°C in LB to an OD600nm between 0.8-1.2, and induced with 1 mM IPTG for 16-20 h at 25°C. Cell lysis (mass from a 2L of culture) was performed with 45 mL of lysis buffer composed by 50 mM Tris-Cl (pH 7.4), 150 mM NaCl, 2.5 mM Tris(2-carboxyethyl) phosphine hydrochloride (TCEP, Sigma, #646547) (buffer A), and supplemented with 1 mg/mL of lysozyme (Sigma, #L6876), and 1 tablet of a protease inhibitor cocktail (Sigma, #S8830). Sonication was performed with cycles of 1 min on and 1 min off during 30-45 min. After centrifugation at 45,000 g, 15 min at 4°C, soluble fractions were loaded onto a column of Ni-NTA agarose (Qiagen, #30210) coupled to an ÄKTA system. Weakly-bound proteins were washed out using buffer A + 25 mM of imidazole followed by a linear gradient of 150 mL and a target concentration of 500 mM imidazole. Peak fractions were directly loaded onto a Superdex 75 16/600 PrepGrade (GE Healthcare, #28-9893-33) that was previously equilibrated with phosphate buffer saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, and 1.8 mM KH2PO4). The concentration of eluted proteins was estimated by absorbance at 280 nm using a
molar extinction coefficient of 17,420 M⁻¹.cm⁻¹ (p53 DBD) and 34,545 M⁻¹.cm⁻¹ (p53 TAD,DBD). Protein aliquots were stored at -80°C after addition of 5% glycerol.

**Light scattering and ultracentrifugation**

Kinetics monitoring the light scattering of constructs (5 μM) were performed using an ISS-PC1 spectrofluorimeter (ISS, Inc.) with excitation and emission at 320 nm at 37°C and gentle agitation. Ultracentrifugation was performed at 45,000 g for 15 min, 4°C (Beckman Coulter, Optima TLX) after incubation of 20 μM of constructs at 37°C under 600 rpm agitation (Thermo Scientific, #13687711). Thioflavin T at 25 μM was mixed to assess binding.

**Size exclusion chromatography**

For analytical size exclusion chromatography, we used a Superdex 200 10/300 column (GE Healthcare, #17-5175-01). Runs were performed in PBS at a flow rate of 0.7 mL/min and the absorbance was monitored at 280 nm using a ÄKTA Prime System (GE Lifesciences). The column was previously calibrated using thyroglobulin, 670 kD; γ-globulin, 158 kD; ovalbumin, 44 kD; myoglobin, 17 kD; and vitamin B12, 1.35 kD (Bio-Rad, #151-1901).

**Circular dichroism**

CD was carried out on a JASCO spectropolarimeter (J-1500). Far-UV spectra were recorded from 260 to 200 nm at 25 and 75°C using 0.2-nm steps. Mean residue ellipticity [Θ] MRE in degrees.cm².dmolt⁻¹ were calculated using the equation, [Θ]/l.c.10.n, where Θ is the measured ellipticity in millidegrees, n is the number of peptide bonds in the primary sequence, l is the path length in cm, and c is the molar concentration. A quartz cell of 2 mm path length was used with protein samples of 10 μM in PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 1.8 mM KH₂PO₄).

**Transmission electron microscopy**

Images were obtained for TAD,DBD after increasing the temperature from 25 to 75°C at 1°C/min. Before grid preparation, samples were left for an hour at room temperature to settle. Samples were applied to previously discharged carbon films on 200-mesh copper grids (EMS, catalog no. CF200-cu) for 1 min, gently dried with filter paper, and stained with 2% uranyl acetate for 5 s. Negatively stained images were acquired on a Philips Tecnai microscope operated at 80 kV at 46,000 magnification. As DBD samples precipitated heavily, grids were not suitable for imaging.
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FOOTNOTES

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Δ40p53 isoform explains p53 aggregation

FIGURE LEGENDS

FIGURE 1: Schematics of the p53 isoform domains. The canonical p53 (p53α) or full-length (fl-p53 or p53) isoform has two transactivation domains (TAD1, aa 1–42, and TAD2, aa 43–63), a proline-rich domain (PR, aa 64–92), a DNA-binding domain (DBD, aa 102–306), a nuclear localization domain (NLS, aa 316–325), an oligomerization domain (OD, aa 307–355), and a negative-regulation domain (NEG, aa 364–393). The predicted molecular weight (MW) in KD of each isoform is shown on the right of each studied isoform. Above the fl-p53 structure are indicated, in red color, the domain regions to which the DO-1, DO-7, and 1801 anti-p53 antibodies bind. aa, amino acid; kDa, kilo Dalton; ATG: translation initiation codon used to initiate each N-truncated p53 protein isoform. Adapted from (17).

FIGURE 2. Transcript levels of p53 isoforms in endometrial tumor and non-tumor cell lines. Relative transcript levels of fl-p53 (p53), p53β, p53γ, Δ40p53, and Δ133p53 isoforms were determined using quantitative real-time PCR (qRT-PCR) and isoform-specific oligonucleotide pairs (shown on Table 1). β-actin gene was used as a normalization control. Data are representative of three independent experiments using duplicates in each assay. We tested four tumor endometrial cell lines, Ishikawa (A), RL95-2 (B), AN3CA (C), and KLE (D), and two endometrial non-tumor cell lines, E6/E7/TERT (E) and EM42 (F). The expression levels of fl-p53 (p53) were used as a reference to calculate the relative expression level of each p53 isoform.

FIGURE 3. The Δ40p53 isoform is a major component of cytoplasmic amyloid aggregates in endometrial carcinoma (EC) cell lines. (A) Immunofluorescence analysis of E6/E7/TERT (endometrial non-tumor cell line), KLE and AN3CA (endometrial tumor cell lines) stained with A11, DO-7, and 1801 anti-p53 primary antibodies and secondary goat anti-mouse IgG/Alexa 546 and goat anti-mouse IgG/Alexa 488 antibodies. A11 anti-amyloid oligomer antibody (green) was used to detect amyloid oligomer aggregates, and DO-7 and 1801 anti-p53 antibodies were used to detect, respectively, fl-p53 and Δ40p53 (magenta) isoforms. Cells were counterstained with DAPI to identify nuclei (blue). Scale bar represents 10 μm. (B) Quantification of co-localization between A11 and anti-p53 antibodies. Quantification of co-localization between A11 (green) and DO-7 (magenta) or A11 (green) and 1801 (magenta) antibodies in E6/E7/TERT, KLE, and AN3A cell lines are shown, respectively, in the left and right bar graphs. Dots represent one quantified image with at least 5-10 cells (n > 15 cells for all studied conditions). Images in 3B are the same images in 3A without the nuclei (DAPI staining). Immunofluorescence images from which the quantification was performed are shown below each bar. Scale bar: 10 μm. Statistical significance was set at *P < 0.05 or **P < 0.005 using non-parametric Kruskall Wallis and Dunn test. Images were visualized using a confocal microscope (FV10i-O, Olympus, Tokyo, Japan).

FIGURE 4. The transactivation domain is a negative regulator of p53 aggregation. (A) Schematics of the TAD.DBD (red) and DBD (blue) constructs recombinantly expressed in this work. (B) 20% SDS-PAGE stained with Coomassie brilliant blue (R-250) showing purified proteins. (C) Analytic size exclusion chromatography of both constructs monitored by the absorbance at 280 nm. (D) Time course measuring the light scattering of p53 TAD.DBD and DBD at 37°C. Results are shown as the avg. of three independent experiments. (E) Ultracentrifugation (UC) tubes showing the DBD aggregation and a clear solution of the TAD.DBD construct. (F) UV-light illumination of thioflavin T (ThT)-bound DBD aggregates after incubation at 37°C for 40 min and UC. ThT fluorescence was quantified and is expressed as the avg. +/- s.e.m (n=3). Far-UV circular dichroism spectra of (G) DBD and (H) TAD.DBD at room temperature and 75°C. (I) Transmission electron microscopy of TAD.DBD species formed after heating the sample to 75°C. (J) Atomic model representation of the TAD (PDB code: 2L14, residues 13-61) and the DBD (PDB code: 2FEJ, residues 94-297) to illustrate the negative regulation of the TAD segment to the p53 DBD aggregation. The amyloidogenic segment of the DBD is colored in red. PR, proline region; OD, oligomerization domain;
NEG, negative-regulatory domain. Curly bracket shows the p53 DNA-binding motif in which the TAD potentially interacts according to (11).

**FIGURE 5.** Δ40p53 isoform in EC cells. Scheme shows Δ40p53 as a modulator of p53 tumor suppression and oncogenic activities in EC cells.
Table 1. Oligonucleotide sequence for p53 isoforms

| P53 isoform | Forward primer | Reverse primer |
|-------------|----------------|----------------|
| p53α/(fl-p53) | 5’-ATG GAG CAG CCG CAG TCA GAT-3’ | 5’-AAT GTC AGT CAG GCC CTT CTG TC-3’ |
| p53β | 5’-GCG AGC ACT GCC CAA CA-3’ | 5’-GAA AGC TGG TCT GGT CCT GAA-3’ |
| p53γ | 5’-ACT AAG CGA GCA CTG CCC AA-3’ | 5’-GTA AGT CAA GTA GCA TCT GAA GGG TG-3’ |
| Δ40p53 | 5’-TCC CTG GAT TGG CAG CC-3’ | 5’-TGG TGG GCC TGC CCT T-3’ |
| Δ133p53 | 5’-TGA CTT TCA ACT CTG TCT CCT TCC T-3’ | 5’-GGC CAG ACC ATC GCT ATC TG-3’ |
| β-actin | 5’-GTG GGG CGC CCC AGG CAC CA-3’ | 5’-CTC CTT AAT GTC ACG CAC GAT TTC-3’ |

Relative expression levels were calculated using the ΔΔCT method. Cell cycling conditions were as follows: initial incubation at 50°C for 2 min, followed by 94°C for 3 min and 34 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min, followed by a final incubation at 72°C for 10 min. Melting curve analysis was performed at 95°C for 5 s, 50°C for 5 s, and 95°C for 5 s.
Δ40p53 isoform explains p53 aggregation

FIGURE 1
Δ40p53 isoform explains p53 aggregation

FIGURE 3

(A) E6/E7/TERT    KLE    AN3CA

DAPI

A    B    C    D    E    F

A11

G    H    I    J    K    L

p53

M    N    O    P    Q    R

Merge

S    T    U    V    X    Z

Inset

D07    1801

(B)

D07    1801

Graph 1

Graph 2

ALU / D07    ALU / 1801

Graph 3

Graph 4
FIGURE 4

\[ \Delta 40p53 \text{ isoform explains } p53 \text{ aggregation} \]
Δ40p53 isoform explains p53 aggregation

FIGURE 5
Loss of the p53 transactivation domain results in high amyloid aggregation of the Δ40p53 isoform in endometrial carcinoma cells

Nataly Melo dos Santos, Guilherme A. P. de Oliveira, Murilo Ramos Rocha, Murilo M. Pedrote, Giulia Diniz da Silva Ferretti, Luciana Pereira Rangel, José A. Morgado-Díaz, Jerson L. Silva and Etel Rodrigues Pereira Gimba

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