

The human Δ Np53 isoform triggers metabolic and gene expression changes that activate mTOR and alter mitochondrial function

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Summary

A naturally occurring p53 isoform that lacks 39 residues at the N-terminus (denoted Δ Np53), when expressed with wild-type p53 (Wtp53), forms mixed Δ Np53:Wtp53 tetramers and causes accelerated aging in mice. Cellular alterations specific to Δ Np53:Wtp53 have been difficult to assess because Δ Np53 and Wtp53 coexpression results in tetramer heterogeneity, including formation of contaminating Wtp53 tetramers. Based on the p53 tetramer structure, we expressed Δ Np53 and Wtp53 as a single transcript that maintained tetramer architecture, ensuring a 2:2 Δ Np53:Wtp53 stoichiometry. As expected, Δ Np53:Wtp53 tetramers were stable and transcriptionally active *in vitro* and in cells, largely mimicking the function of Wtp53 tetramers. Microarray analyses, however, revealed about 80 genes whose expression was altered twofold or more in Δ Np53:Wtp53 cells. Moreover, global metabolomic profiling quantitated hundreds of biochemicals across different experiments (Wtp53, Δ Np53:Wtp53, plus controls). When evaluated collectively, these data suggested altered mTOR signaling and mitochondrial function—each canonical regulators of longevity—in cells expressing Δ Np53:Wtp53 vs. Wtp53. Increased levels of free amino acids, increased expression of *IRS-1*, and decreased expression of *INPP5D/SHIP-1* suggested activated mTOR signaling in Δ Np53:Wtp53 cells; this was confirmed upon comparative analyses of several mTOR pathway intermediates. We also observed changes in mitochondrial function in Δ Np53:Wtp53 cells, which correlated with increased *MARS2* expression and increased levels of carnitine, acetyl CoA, ATP, and Krebs cycle intermediates. Finally, increased levels of succinate and 2-hydroxyglutarate indicate potential epigenetic means to propagate Δ Np53:Wtp53-induced gene expression changes to cell progeny. This may be especially important for aging, as biological effects manifest over time.

Key words: *DOT1L*; urea cycle; polyamines; glutathione; 2HG; Epigenetic.

Introduction

The role of p53 in mammalian aging is best exemplified by mouse studies with p53 truncation mutants (Tyner *et al.*, 2002; Maier *et al.*,

2004). Both artificial and naturally occurring p53 isoforms that lack the N-terminal p53 activation domain, when expressed together with wild-type p53 (Wtp53), cause a progeroid phenotype that includes early-onset osteoporosis, memory loss, and premature death (Tyner *et al.*, 2002; Maier *et al.*, 2004; Pehar *et al.*, 2010). The molecular mechanisms by which Δ Np53 cause accelerated aging remain incompletely understood. It is clear, however, that Δ Np53 requires Wtp53 to mediate its physiological effects; mice expressing only Δ Np53 do not age rapidly (Maier *et al.*, 2004). Because p53 binds DNA as a tetramer, the formation of mixed Δ Np53:Wtp53 heterotetramers is believed to mediate the accelerated aging phenotype (Courtois *et al.*, 2002). Although Δ Np53 has been well studied using knockin mouse models, Δ Np53 has not been thoroughly studied in human cells. Moreover, global gene expression and metabolic changes induced by Δ Np53:Wtp53 have not been assessed in human cells or any model organism. One potential reason for this is that coexpression of Δ Np53 and Wtp53 will confound analyses due to heterogeneity, including formation of ‘contaminating’ Wtp53 tetramers. As one consequence, it has been difficult to define cellular changes that are specifically dependent upon Δ Np53:Wtp53, because a means to examine a single entity with fixed Δ Np53:Wtp53 stoichiometry was not evident. For these reasons, the underlying mechanisms that link Δ Np53 and its progeroid phenotype remain incompletely understood, even in model organisms.

To further define the molecular pathways impacted by Δ Np53 expression in human cells, we expressed 2:2 Δ Np53:Wtp53 tetramers (i.e., two copies of Δ Np53 and two copies of Wtp53 per tetramer) in p53-null H1299 cells. Based on the p53 tetramer structure (Okorokov *et al.*, 2006), we implemented a strategy that expressed Δ Np53 and Wtp53 as a single transcript that maintained the native tetramer architecture, ensuring a 2:2 Δ Np53:Wtp53 stoichiometry. These Δ Np53:Wtp53 tetramers were stable and transcriptionally active *in vitro* and in cells, largely mimicking the function of Wtp53 tetramers. Microarray analyses identified 84 genes whose expression was up- or down-regulated twofold or more in Δ Np53:Wtp53 cells compared with Wtp53. This set of genes included many with established roles in longevity and metabolism. We next completed global metabolomic profiling, which identified and quantitated hundreds of biochemicals across different experiments (Wtp53, Δ Np53:Wtp53, plus controls). When evaluated collectively, the gene expression and metabolomics data provided clear predictions (validated via pathway analyses) about cellular processes disrupted by Δ Np53:Wtp53 expression. Among the most striking were mitochondrial function and the mTOR pathway, each well-established regulators of longevity. Biochemical and cellular assays confirmed a hyperactive mTOR pathway and altered mitochondrial function in cells expressing Δ Np53:Wtp53 (vs. Wtp53). We also uncovered metabolites whose increased levels could contribute to heritable epigenetic changes upon Δ Np53:Wtp53 expression. Because Δ Np53 is a naturally occurring isoform whose levels can increase with cell stress, the Δ Np53-induced changes identified here could play a role in normal human aging.

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Results

Expression and purification of 2:2 Δ Np53:Wtp53 tetramers

The p53 activation domain (p53AD) resides in the N-terminus of the protein. Two separate activation motifs have been identified, with residues 14–26 comprising p53AD1, and residues 41–57 comprising p53AD2. Thus, the Δ Np53 isoform lacks p53AD1 but retains p53AD2. The structure of the native p53 tetramer indicates that p53 assembles as a dimer of dimers, with the C-terminus of one p53 polypeptide interacting with the N-terminus of a second p53 protein (Okorokov *et al.*, 2006). The juxtaposition of the N- and C-termini suggested that a flexible linker could tether two p53 monomers to yield a 1:1 Δ Np53:Wtp53 dimer (Fig. S1). Implementing the strategy outlined in Fig. 1A, we expressed a tethered Δ Np53:Wtp53 protein as a single transcript in insect cells. Purification of p53 tetramers from these cells matched that of Wtp53 tetramers; in particular, Δ Np53:Wtp53 migrated similar to the native p53 tetramer on a size-exclusion column (Fig. 1B). Silver stain and Western blot analysis (Fig. 1C) revealed a molecular weight of approximately 100 kDa, consistent with a tethered Δ Np53:Wtp53 dimer. For functional comparisons, we purified Wtp53 tetramers and tetramers consisting of only Δ Np53 proteins (Fig. 1D).

Δ Np53:Wtp53 tetramers are functionally equivalent to Wtp53 *in vitro*

To compare the activity of the 2:2 Δ Np53:Wtp53 tetramers (hereafter called Δ Np53:Wtp53) with Wtp53 tetramers, we used an *in vitro* transcription system reconstituted from purified human factors (Knuesel *et al.*, 2009). An outline of the assay is shown in Fig. 2A. Transcription activation at the native HDM2 promoter was assessed in the absence of p53, or in the presence of Wtp53 tetramers, Δ Np53 tetramers, or Δ Np53:Wtp53 tetramers. As expected, transcription activation required mediator and Wtp53 (Fig. 2B, compare lanes 1–3); Δ Np53 tetramers, which lack p53AD1 but retain p53AD2, were unable to activate

transcription (Fig. 2B, lanes 4–6). By contrast, the Δ Np53:Wtp53 tetramers could activate HDM2 transcription equally well compared with Wtp53 (Fig. 2B, lane 7). This result demonstrated that Δ Np53:Wtp53 had an inherent activity equal to Wtp53, at least in this cell-free reconstituted transcription assay.

Evaluation of Δ Np53:Wtp53 in cells

To establish whether Δ Np53:Wtp53 tetramers would bind a p53-regulated promoter in cells, we expressed either Wtp53 or Δ Np53:Wtp53 in p53-null H1299 cells and performed chromatin immunoprecipitation assays (ChIP). As shown in Fig. 2C, both Wtp53 and Δ Np53:Wtp53 tetramers bound the p21 (*CDKN1A*) promoter at the consensus p53-binding sites. Because Wtp53 and Δ Np53:Wtp53 tetramers each contain four intact DNA-binding domains, these data were not unexpected. Note that the p53 antibody (DO-2) used for ChIP targets residues 10–16, which are absent in Δ Np53. Consequently, the occupancy of Δ Np53:Wtp53 appears reduced because 50% fewer epitopes are present. Next, we used RT-qPCR to measure p21 (*CDKN1A*) expression. The p21 gene represents one of only a few genes thus far identified as differentially expressed (reduced expression relative to Wtp53) in cells expressing both Δ Np53 and Wtp53 (Powell *et al.*, 2008; Ungewitter & Scrbale, 2010). In agreement, we observed a decrease in p21 expression, relative to Wtp53, in cells expressing Δ Np53:Wtp53 (Fig. 2D). We also examined p21 expression in H1299 cells expressing matched levels of both Δ Np53 and Wtp53 (i.e., as separate transcripts). Whereas the stoichiometry of p53 tetramers could not be controlled with such experiments, p21 expression in these ' Δ Np53 + Wtp53' cells showed a decrease relative to Wtp53 that was similar to Δ Np53:Wtp53 cells (Fig. 2D).

Δ Np53:Wtp53 induces a gene expression program slightly distinct from Wtp53

Having confirmed that tetramers assembled from Δ Np53:Wtp53 were stable, could bind p53-regulated promoters in cells, and were transcriptionally active *in vitro* and in cells, we next assessed their impact on global gene expression patterns. We used p53-null H1299 cells, which do not express any p53 isoform at detectable levels. Expression of p53 (Wtp53 or Δ Np53:Wtp53) was achieved by transfection using a vector that enabled bicistronic expression of green fluorescent protein (GFP); this allowed p53-expressing cells to be sorted by GFP fluorescence. Prior to harvesting RNA for microarray analysis, we confirmed that p53 protein levels (Wtp53 or Δ Np53:Wtp53) were matched by quantitative Western blot (Fig. S2A). Also, at the time of the analysis, each cell population (Wtp53, Δ Np53:Wtp53, or controls) displayed similar cell cycle distribution, as revealed by flow cytometry (Fig. S2B); relative levels of apoptosis and senescence were measured in each cell population, and only live cells were isolated (Fig. S3).

Microarray analysis was completed for four different samples: mock transfection (reagent only), empty vector (reagent plus GFP vector), Wtp53, and Δ Np53:Wtp53. The gene expression profile for cells expressing Wtp53 (compared with mock or empty vector controls) yielded predictable results, and gene set enrichment analysis (GSEA) of the genes differentially expressed in Wtp53 cells identified canonical p53-regulated processes, as expected (Fig. S4). The overall gene expression profiles of Wtp53 vs. Δ Np53:Wtp53 were similar when compared with control (GFP vector only) cells, as shown by the Venn diagrams in Fig. S5 (Supporting information) as well as the GSEA (Fig. S4). These similarities were not unexpected, as Δ Np53:Wtp53

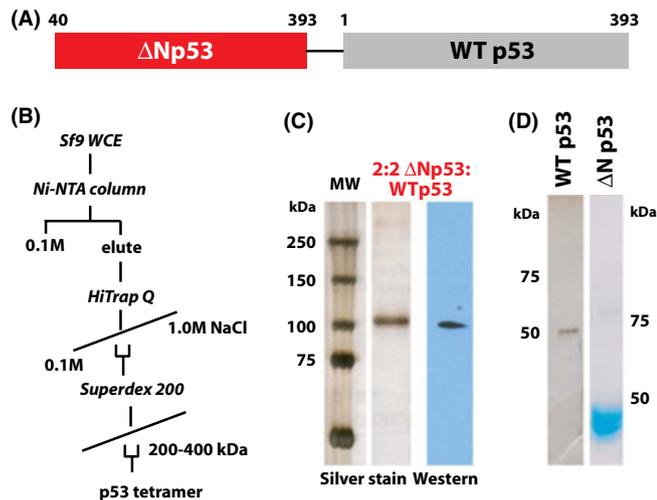


Fig. 1 Expression and purification of Δ Np53:Wtp53 tetramers with 2:2 stoichiometry. (A) Schematic of Δ Np53:Wtp53. See also Fig. S1. To allow conformational flexibility, the linker was 14 residues in length (sequence: GGGGENLYFQGGGG) and contained a TEV cleavage site. (B) Purification protocol used for isolation of Wtp53, Δ Np53:Wtp53, and Δ Np53 tetramers. (C) Silver stain gel and p53 Western blot for purified Δ Np53:Wtp53. (D) Silver-stained and Coomassie-stained gel for purified Wtp53 and Δ Np53.

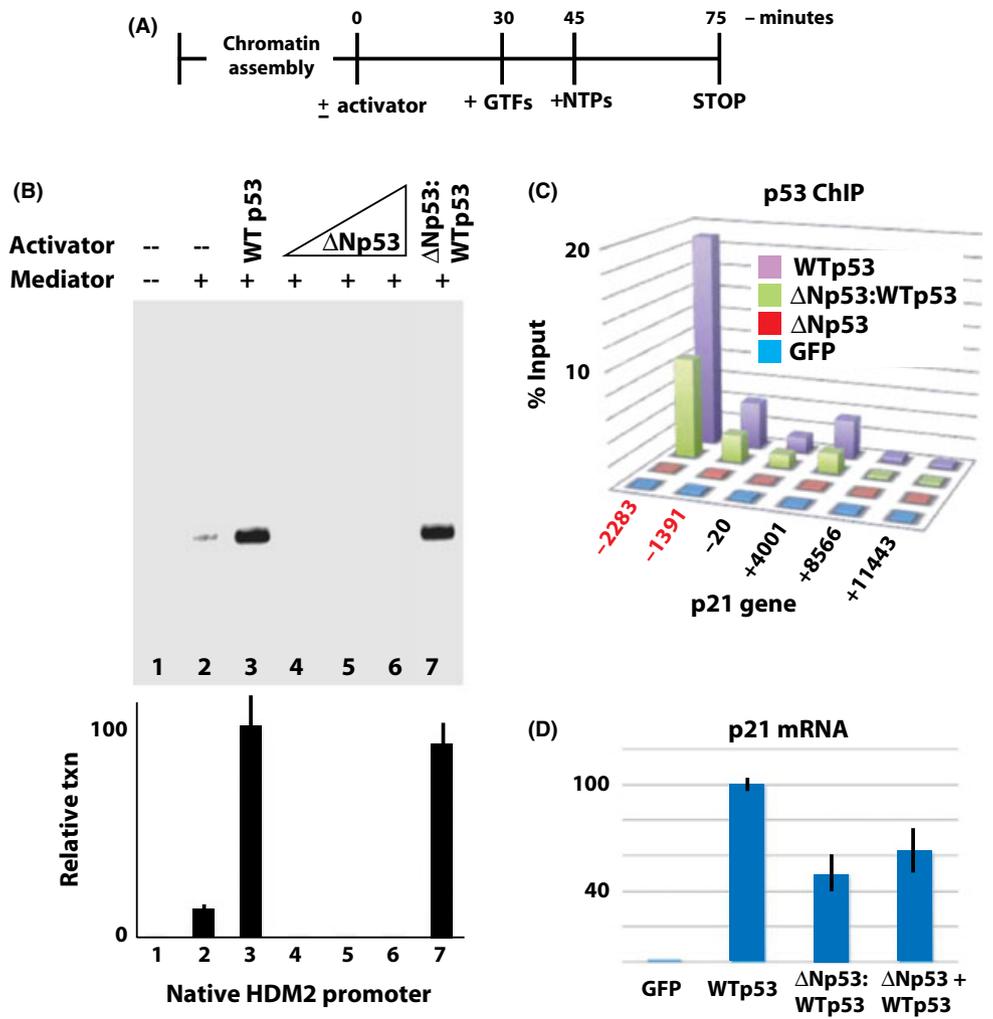


Fig. 2 Like WTp53, Δ Np53:Wtp53 tetramers bind p53 target genes and are transcriptionally active *in vitro* and in cells. (A) Schematic of *in vitro* transcription reaction. GTFs refer to purified TFIIA, TFIIB, TFIID, TFII E, TFIIF, TFIIH, RNA polymerase II, and mediator (Knuesel *et al.*, 2009). (B) Reconstituted transcription. Activators and mediator added as indicated. Representative data shown; plot summarizes results (mean and standard error) from multiple experiments ($n = 3, 4, 6, 2, 2, 2, 6$ for lanes 1–7). Note that Δ Np53 tetramers were titrated over a 200-fold range, with no evidence of transcription activation. (C) ChIP data (vs. p53) across the *CDKN1A/p21* locus in H1299 cells. (The p53 antibody targeted residues 10–16; this epitope is absent in Δ Np53.) Binding sites for p53 are highlighted in red. The six probe locations are shown relative to the transcription start site. (D) Expression of the *CDKN1A/p21* gene was probed by RT–qPCR in H1299 cells expressing WTp53, Δ Np53:Wtp53, or Δ Np53 + Wtp53. Cells expressing GFP vector only shown as a negative control. Note that Δ Np53 + Wtp53 refers to separate expression of Δ Np53 and Wtp53.

tetramers retain all four DNA-binding domains and only differ in two of four activation domains. About 80 genes, however, showed greater than twofold differences in expression in Δ Np53:Wtp53 cells (up or down, compared with WTp53). These genes are shown in Tables 1 and 2. Several hundred genes showed altered expression at levels 1.5-fold or greater in Δ Np53:Wtp53 cells, compared with WTp53. These genes are shown in Table S1 and S2 (Supporting information). The expression levels of numerous genes listed in Tables 1 and 2 were probed independently using qRT–PCR. These experiments verified the microarray results (Fig. S6).

We also used qRT–PCR to probe expression of numerous genes in Saos-2 cells. Like H1299 cells, Saos-2 cells are p53-null. Because p53-induced gene expression patterns are not identical in different cell types, we expected to observe some differences in transcription response (Espinosa, 2008). As shown in Fig. S7 (Supporting information), most, but not all, genes probed by qRT–PCR showed similar changes in expression levels in Saos-2 cells compared with H1299 cells.

The peptide linker itself does not alter biological function of p53

Collectively, the data in Figs 1 and 2 indicated that the flexible peptide linker inserted between Δ Np53 and WTp53, which was based upon the p53 tetramer structure (Okorokov *et al.*, 2006), did not affect the

biological activity of Δ Np53:Wtp53 tetramers. To further address whether the linker itself might impact p53 function, we expressed tethered WTp53 tetramers (i.e., WTp53:Wtp53) in p53-null H1299 cells. In other words, WTp53 dimers were expressed as a single transcript using the same linker sequence used for Δ Np53:Wtp53. Microarray analyses were then completed in which we compared global gene expression changes induced by ‘standard’ WTp53 vs. its tethered counterpart (i.e., WTp53:Wtp53). These and other data, summarized in Fig. S8A–J (Supporting information), indicate that the peptide linker itself does not appear to direct gene expression changes or otherwise alter WTp53 function, at least within the duration of these experiments.

In agreement with these findings, we performed qRT–PCR experiments in H1299 cells coexpressing equal levels of Δ Np53 and WTp53 (i.e., Δ Np53 + WTp53). As anticipated, and in agreement with data for p21 (Fig. 2D), gene expression from these ‘ Δ Np53 + WTp53’ cells was largely similar to Δ Np53:Wtp53 cells (Figs S9 and S10). Importantly, in cases where differences were observed, these could be attributed to tetramer heterogeneity (e.g., WTp53 tetramers) resulting from Δ Np53 and WTp53 coexpression (see legend, Figs S8, S9 and S10).

Global metabolomics analyses

Whereas many genes up- or down-regulated in Δ Np53:Wtp53 cells are directly or indirectly linked to aging, a significant fraction are involved in

Table 1 Genes up-regulated twofold or more in H1299 cells expressing Δ Np53: WTp53, relative to WTp53

Gene name	Fold CHG	P value	Description
CDKL2	3.6808	0.0085	Kinase; development
CHAC1	2.8481	0.0009	cation transport, unfolded protein response
TMEM229A	2.8415	0.0285	transmembrane protein, putative TF
DUSP5P	2.7321	0.0016	pseudogene
ZNF443	2.7007	0.0208	zinc-finger protein
RAB27B	2.6512	0.0008	GTPase, exosome secretion pathway
PDE5A	2.6027	0.0211	cGMP phosphodiesterase; purine metab.
MARS2	2.4967	0.0095	Met tRNA synthetase, mitochondrial
ZBTB42	2.4794	0.03	linked to metabolic syndrome
LOC643923	2.4794	0.0351	
PLK3	2.406	0.0152	Kinase., mitosis, Insulin/PI3K/mTOR pathway
DUSP10	2.3839	0.0072	Phosphatase; MAPK signaling
ANKRD36B	2.308	0.0075	ankyrin repeat domain
IRF1	2.2921	0.0003	TF; immune function
IFFO2	2.2501	0.0028	intermediate filament
C6orf14	2.2449	0.0067	
HIST1H3H	2.2397	0.0191	histone H3
IRS1	2.214	0.0013	Insulin/PI3K/mTOR pathway
ZNF799	2.2089	0.0138	zinc-finger protein
ZBTB1	2.2089	0.0004	cell cycle
CNKSR3	2.1685	0.0002	sodium transport; MAPK pathway
SNX33	2.1685	0.003	phospholipid binding
DOT1L	2.1585	0.0057	H3K79 methyltransferase; cell cycle/diff.
C3orf59	2.1386	0.0037	
ZNF354B	2.1238	0.0138	zinc-finger protein
JMY	2.1092	0.0045	p53 cofactor; cell motility
MAP3K1	2.1092	0.0047	Kinase; Insulin signaling; differentiation
ANKZF1	2.0849	0.0032	binds p97 AAA ATPase
DHRS13	2.0801	0.0296	glucose dehydrogenase
VLDLR	2.0801	0.0013	FA metabolism
SNORD44	2.0801	0.0044	snoRNA
SEPTIN6	2.0705	0.0011	cytokinesis, GTPase
DENND3	2.0658	0.002	GTP-GDP exchange, reg. MAPK signaling
P2RY1	2.061	0.0441	ATP/ADP-gated receptor
WHAMML1	2.0562	0.0089	pseudogene
LRCH1	2.0515	0.0094	CHO transporter
CYBRD1	2.0467	0.0148	heme binding/iron uptake, reductase
LN2	2.042	0.0017	ligand of numb protein X, Notch signaling
HIST1H2BF	2.0373	0.0084	histone H2B
ANGPTL4	2.0373	0	glucose/FA metab, mitochondrial function
BHLHE41	2.0326	0.0039	TF; circadian rhythm, differentiation
PLEKHF1	2.0326	0.0263	phosphatidylinositol signaling
ANKRD36	2.0279	0.002	ankyrin repeat domain
MGC16384	2.0232	0.0072	
PTCH1	2.0232	0.0007	sonic hedgehog receptor; Insulin signaling
FGF5	2.0232	0.0165	MAPK/insulin signaling, differentiation
FAT4	2.0139	0.0016	cell-cell adhesion
NFIL3	2.0046	0.02	TF, circadian rhythm

basic metabolic processes (Tables 1 and 2). Given the major role that p53 plays in regulating cell metabolism (Vousden & Ryan, 2009; Feng & Levine, 2010), we compared the metabolomes of H1299 cells expressing WTp53 or Δ Np53:WTp53. The metabolomics analysis utilized an analytical platform that combines gas chromatography (GC) and liquid chromatography (LC) mass spectrometry (GC-MS and LC-MS/MS) techniques (Reitman *et al.*, 2011).

Six biological replicate samples were prepared for metabolic profiling. In addition to WTp53 and Δ Np53:WTp53, we evaluated mock and GFP

Table 2 Genes down-regulated at least twofold in H1299 cells expressing Δ Np53: WTp53, relative to WTp53

Gene name	Fold CHG	P value	Description
GDF9	0.163	0.0001	cytokine; growth/differentiation
UGT2B7	0.1826	0.0062	UDP-glucuronyltransferase
CLCA2	0.2333	0.0066	chloride channel; secretion
SERPINB5	0.2541	0.0003	Maspin; cell motility
WDR69	0.2973	0.0045	dynein assembly factor
AREG	0.3064	0.006	cytokine; EGR family
SLC2A2	0.3157	0.0014	glucose xporter; CHO metabolism
LCE1C	0.3231	0.0139	late envelope protein; differentiation
DISP1	0.3479	0.0138	hedgehog signaling; differentiation
PALMD	0.361	0.0188	palmelphin
CRYAB	0.3686	0.0009	chaperone; ER-assoc. degradation
GPR56	0.3772	0.006	G-protein receptor; differentiation
INPP5D	0.3833	0.0001	Insulin/PI3K/mTOR pathway
CKS2	0.3959	0.027	CDK reg protein; Insulin signaling
PLK1	0.3978	0.0456	Kinase; reg. AURKA; mitosis
AURKA	0.4043	0.024	Kinase; reg. PLK1; reg. p53; mitosis
PTH2R	0.4108	0.0099	parathyroid hormone receptor
KLHL30	0.4156	0.0157	kelch-like 30
SATL1	0.4156	0.0015	spermidine acetyltransferase
NCF2	0.4166	0.0102	NADPH OX complex; differentiation
CENPA	0.4166	0.039	Centromere; reg. by AURKA; mitosis
GDF15	0.4253	0.0006	cytokine; growth/differentiation
SLA	0.4263	0.0021	immune function
PLA2G10	0.4569	0.0159	FA metabolism; arachidonic acid
LOC285679	0.4569	0.0366	
KIF2C	0.458	0.0439	kinesin; cenfomere-associated
SLC46A1	0.459	0.0003	folate/heme transporter
CLEC17A	0.4633	0.0064	CHO binding; cell-cell signaling
NEK2	0.4676	0.0248	Kinase; mitosis; centrosome assoc.
PADI3	0.473	0.011	Arg de-aminase; urea cycle
BDKRB1	0.473	0.0041	Bradykinin receptor; cell motility
CCDC99	0.4841	0.0367	mitosis
KIF20A	0.4863	0.0395	kinesin
CEP55	0.4875	0.0427	centrosome;cytokinesis;reg. by PLK1
BEND2	0.4908	0.0014	BEN domain
KIF4A	0.4943	0.0324	kinesin; chromosome dynamics

vector only transfected H1299 cells. Because cell cycle changes or changes in cell state (e.g., apoptosis or senescence) could potentially alter metabolite levels, we probed samples to ensure viability and that each cell population was similar in cell state and cell cycle distribution (Figs S2B and S3). As with microarray analyses, we also confirmed that p53 levels were consistent and comparable in WTp53 vs. Δ Np53:WTp53 samples by quantitative Western blot (Fig. S2A). A statistical summary of the biochemicals identified in each sample is shown in Table S3 (Supporting information); a complete list of the metabolites identified, including statistical parameters and absolute or relative quantitation, is provided in Table S4 (Supporting information).

Metabolic changes associated with WTp53 support roles in tumor suppression and metabolism

Relative to control cells (WTp53 vs. GFP vector; Table S3), 142 biochemicals were categorized as significantly altered in H1299 cells expressing WTp53 ($P \leq 0.05$). As anticipated, the metabolic alterations induced by WTp53 support its known roles as a metabolic regulator and tumor suppressor (Vousden & Ryan, 2009). Although the primary focus

of this study was how the Δ Np53 isoform altered Wtp53 function, we highlight the metabolic changes induced by Wtp53 in Table S5 (Supporting information).

Metabolic changes associated with Δ Np53:Wtp53 vs. Wtp53

Compared with Wtp53, the levels of 94 biochemicals were altered ($P \leq 0.05$) in cells expressing Δ Np53:Wtp53 (Table S3). Many intriguing results emerge from these data, and several key findings are highlighted in Fig. 3. As shown in Fig. 3A, amino acid levels were uniformly increased in Δ Np53:Wtp53 cells, compared with Wtp53. Moreover, derivatives of these amino acids (e.g., *N*-acetylated forms) were more abundant in Δ Np53:Wtp53 cells (Table S4). Figure 3B shows a subset of biochemicals whose levels were increased or decreased in Δ Np53:Wtp53 cells, including carnitine and its derivatives, TCA/Krebs cycle intermediates, and glutathione metabolites.

Increased amino acids are known to activate the mTOR pathway, which acts as a nutrient sensor and regulates life span in yeast, worms, flies, and mammals (Laplante & Sabatini, 2012). Moreover, increased levels of TCA intermediates and carnitine (Fig. 3B), which is critical for transporting fatty acids within mitochondria, suggested alterations in

mitochondrial function in cells expressing Δ Np53:Wtp53. As with the mTOR pathway, mitochondrial function is strongly linked to mammalian aging (Vendelbo & Nair, 2011).

Given the number of genes and metabolites altered in Δ Np53:Wtp53 cells relative to cells expressing Wtp53, pathway analyses were completed as an additional means to evaluate the data. Results are summarized in Tables S6–S9 (Supporting information) and further implicate mTOR and mitochondrial function in Δ Np53:Wtp53 cells. Therefore, we decided to probe Δ Np53:Wtp53 cells for changes in mitochondrial function and mTOR signaling.

Altered mitochondrial function in Δ Np53:Wtp53 cells

Gene expression (e.g., *MARS2*, *CYBRD1*) and metabolic changes (Fig. 3B) suggested altered mitochondrial function in Δ Np53:Wtp53 cells (Xu *et al.*, 2010; Bayat *et al.*, 2012). Carnitine is critical for transporting fatty acids within mitochondria; subsequent beta-oxidation of fatty acids generates acetyl CoA, NADH and FADH₂. NADH and FADH₂, in turn, provide electrons that drive mitochondrial electron transport and oxidative phosphorylation (Fig. 4A). The increase in carnitine and its intermediates suggested that mitochondrial function (in particular, ATP production) might be altered in cells expressing Δ Np53:Wtp53. Gene expression changes may contribute to the increased carnitine levels in Δ Np53:Wtp53 cells; for example, the lysine methyltransferase *DOT1L* and the iron import protein *CYBRD1* were up-regulated over twofold (Fig. 4A).

To determine whether mitochondrial function might be altered in H1299 cells expressing Δ Np53:Wtp53, we measured mitochondrial membrane potential using MitoTracker Red. As shown in Fig. 4B, cells expressing Δ Np53:Wtp53 display increased MitoTracker staining, indicative of a greater potential difference across the inner mitochondrial membrane. The apparent increase in membrane potential did not result from an increased number of mitochondria in Δ Np53:Wtp53 cells, because no significant changes in mitochondrial DNA content were observed in cells expressing Wtp53 vs. Δ Np53:Wtp53 (Fig. 4C). Together, these data confirm that an increase in membrane potential accompanies Δ Np53:Wtp53 expression.

Mitochondrial membrane potential is generated via electron transport, which establishes a proton gradient that helps drive ATP synthesis. The increased membrane potential (Fig. 4B) suggested that ATP production might be enhanced in Δ Np53:Wtp53 cells. To assess ATP levels, we implemented a quantitative, luciferase-based assay. This analysis revealed an ~50% increase in ATP production in cells expressing Δ Np53:Wtp53 relative to Wtp53 (Fig. 4D), consistent with the increased mitochondrial membrane potential shown in Fig. 4B. Collectively, the data in Fig. 4 (see also Fig. S11) indicate an activity for Δ Np53:Wtp53 that alters mitochondrial function in a manner distinct from Wtp53.

Up-regulation of mTOR pathway in Δ Np53:Wtp53 cells

The across-the-board elevation in amino acids (Fig. 3A) suggested the mTOR pathway was activated in Δ Np53:Wtp53 cells. Amino acids are known to activate mTOR via an elaborate mechanism involving lysosomes and a set of Rag GTPases (Laplante & Sabatini, 2012). Hyperactive mTOR signaling was further supported by the gene expression data, which indicated increased expression of *IRS-1* and decreased expression of *INPP5D/SHIP-1* in Δ Np53:Wtp53 cells, compared with Wtp53 (Tables 1 and 2). As shown schematically in Fig. 5A, *IRS-1* activates *PI3K* to generate phosphoinositol (3,4,5) triphosphate;

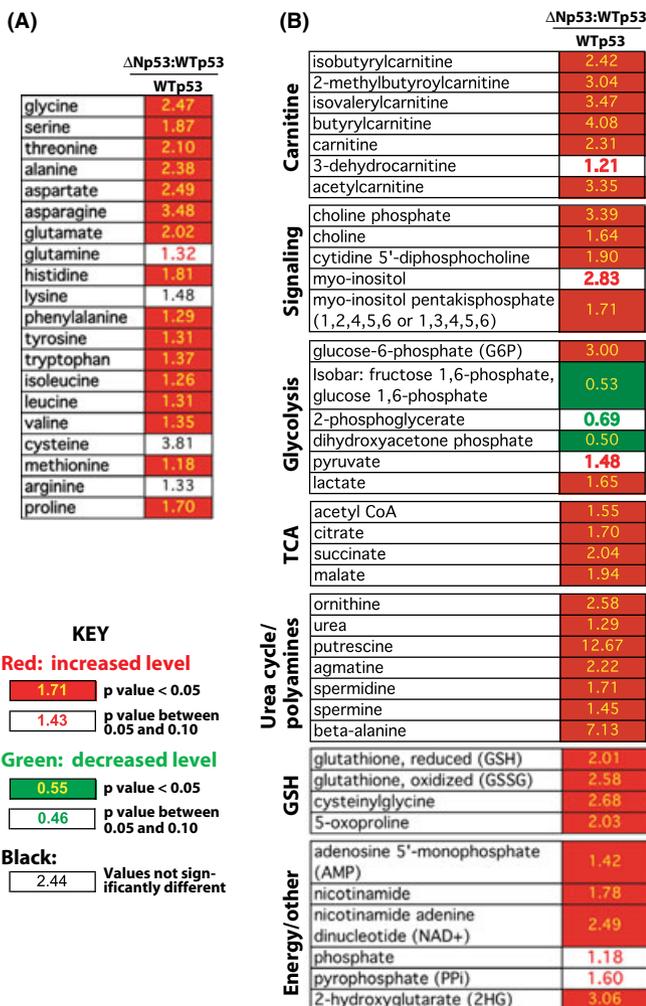


Fig. 3 Summary of metabolomics data. (A) Relative levels of amino acids in Δ Np53:Wtp53 cells vs. Wtp53. (B) Relative levels of select metabolites (Δ Np53:Wtp53 vs. Wtp53).

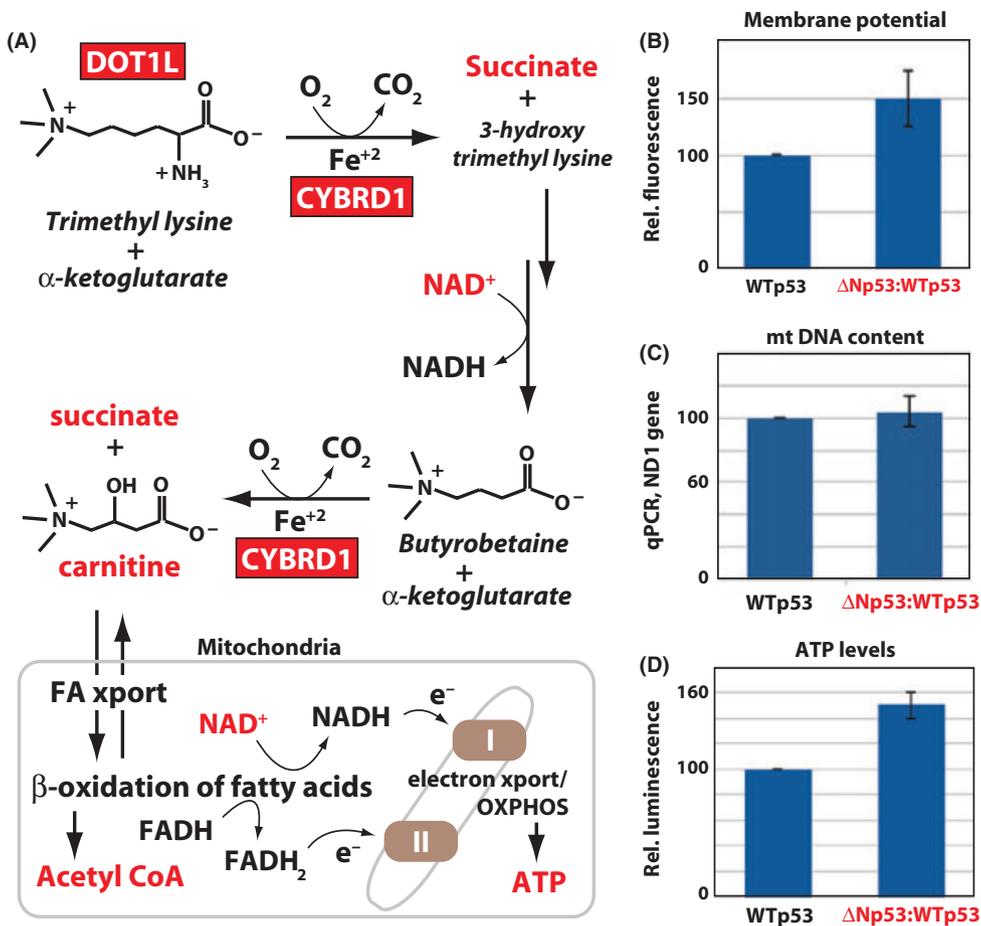


Fig. 4 Altered mitochondrial function in cells expressing Δ Np53:WTp53 relative to WTp53. (A) Simplified schematic of carnitine biosynthesis and beta-oxidation of fatty acids in mitochondria. Red boxes indicate genes up-regulated in Δ Np53:WTp53 cells, and red font denotes biochemicals whose levels are increased in Δ Np53:WTp53 cells, relative to WTp53. (B) Mitochondrial membrane potential in H1299 cells expressing WTp53 or Δ Np53:WTp53. Plot shows mean and standard error of MitoTracker Red fluorescence intensities from three independent experiments. Data were normalized to WTp53. (C) Mitochondrial DNA copy number (as determined by real-time PCR) in H1299 cells expressing WTp53 or Δ Np53:WTp53. Data were normalized to *CDKN1A/p21* genomic DNA. (D) Relative ATP levels (normalized to WTp53) in H1299 cells. Plot shows mean and standard error from three independent experiments. See also Fig. S11.

this serves to activate AKT kinase, an upstream activator of mTOR. Similarly, decreased expression of the 5-position inositol phosphatase *INPP5D* would promote phosphoinositol (3,4,5) triphosphate signaling by limiting its breakdown to phosphoinositol (3,4) diphosphate. In addition, reduced expression of *LYN* (which activates *INPP5D*) in Δ Np53:WTp53 cells (Table S2) could contribute to mTOR activation through a variety of mechanisms (Gardai *et al.*, 2002; Baran *et al.*, 2003).

To test whether the mTOR pathway was up-regulated in Δ Np53:WTp53 cells, we probed for well-established markers of mTOR activation, including phospho-S6 kinase, phospho-4EBP-1,2,3, and phospho-mTOR. Levels of the phosphorylated intermediates were compared with total mTOR and CCNC, as shown in Fig. 5B. Phospho-S6 kinase, phospho-4EBP-1,2,3, and phospho-mTOR were uniformly increased in Δ Np53:WTp53 H1299 cells, compared with cells expressing WTp53 (Fig. 5B). We next evaluated levels of mTOR pathway intermediates in a different p53-null cell line, Saos-2. As shown in Fig. 5C, phosphorylated mTOR, phospho-S6K, and phospho-4EBP-1,2,3 were each increased in Saos-2 cells expressing Δ Np53:WTp53 compared with WTp53. Taken together, the data in Fig. 5 (see also Fig. S12) demonstrate that Δ Np53:WTp53 expression activates the mTOR pathway in human cells. This outcome is opposed to WTp53, which suppresses mTOR signaling (Feng & Levine, 2010).

Discussion

The p53 transcription factor is expressed in most cell types, plays important roles throughout development, and helps sustain basic cellular

functions, such as stress response. Whereas myriad factors influence aging, this study focused on the naturally occurring human Δ Np53 isoform, whose expression causes accelerated aging in mice (Tyner *et al.*, 2002; Maier *et al.*, 2004). To more thoroughly understand the distinct activities of human Δ Np53, we focused on differences relative to WTp53. Our goal was not to distinguish direct from indirect effects of Δ Np53, but rather to gain insight into the cellular pathways altered by its expression. A key feature of this study was analysis of Δ Np53:WTp53 tetramers with fixed 2:2 stoichiometry; this circumvented confounding issues associated with Δ Np53 and WTp53 coexpression and allowed clear delineation between WTp53 and Δ Np53:WTp53. Another key feature was the combination of gene expression and metabolomics data, which allowed better assessment of pathways altered by Δ Np53:WTp53. On their own, gene expression data correlate poorly with organismal aging. Age-associated changes in gene expression are heterogeneous and vary with cell or tissue type (Bahar *et al.*, 2006; Somel *et al.*, 2006). Also, age-associated gene expression changes are not consistent across model organisms (Zahn *et al.*, 2007). Signaling and metabolic pathways, however, are more consistently linked to aging across different cell types and even model organisms (Vijg & Campisi, 2008). Collectively, our data suggest that in human cells, Δ Np53:WTp53 helps orchestrate changes in basic cellular processes that contribute to aging. In fact, mitochondrial function and mTOR signaling are linked to longevity in organisms as distinct as mammals and yeast (Vendelbo & Nair, 2011; Laplante & Sabatini, 2012).

We would like to point out some advantages and limitations of our study. Whereas the metabolomics analyses complemented the gene

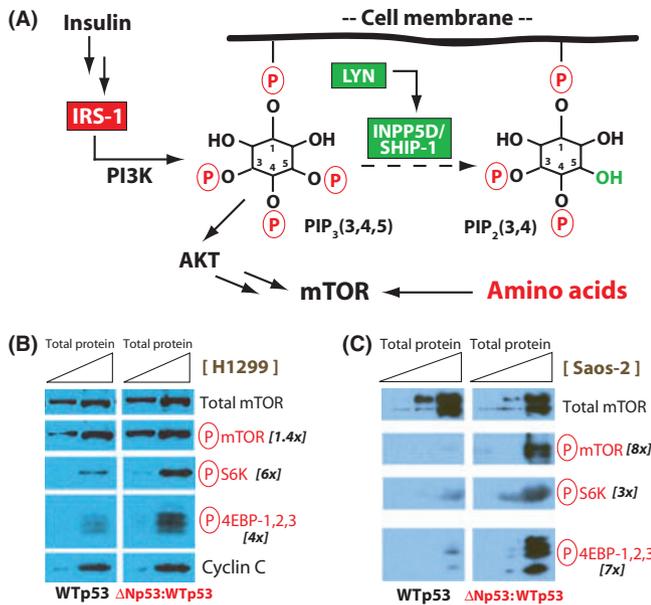


Fig. 5 The mTOR pathway is activated in cells expressing Δ Np53:WTp53. (A) Simplified schematic of signaling events that regulate the mTOR pathway. Red boxes indicate genes up-regulated in Δ Np53:WTp53 cells, and green boxes indicate genes down-regulated. Note the LYN kinase activates INPP5D (Gardai *et al.*, 2002; Baran *et al.*, 2003), and both gene products were down-regulated in Δ Np53:WTp53 cells. Amino acid levels were uniformly increased in Δ Np53:WTp53 cells, relative to WTp53. (B) Western blot data from H1299 cells showing increased levels of mTOR kinase targets (red font) in Δ Np53:WTp53 cells, compared with WTp53. mTOR substrates included phospho-mTOR (S2448), phospho-p70 S6 Kinase (S371), and phospho-4E-BP1/2/3 (T37/46). Each experiment (WTp53 or Δ Np53:WTp53) loaded the same amount of total protein (7.9 and 31.7 μ g, respectively), with additional loading controls (total mTOR, CCNC) shown. Quantitation (fold change) is indicated at right. (C) Western experiments completed as described in B, except using p53-null Saos-2 cells. Total protein loaded in each lane was 0.9, 3 and 9 μ g. Quantitation (fold change) shown at right. See also Fig. S12.

expression data and provide a wealth of information, they do not offer a fully comprehensive view of cellular alterations that accompany Δ Np53:WTp53 expression. The total number of chemically distinct metabolites in human cells exceeds a few thousand (Wishart *et al.*, 2009), whereas we identified several hundred in this study. We also cannot delineate the cellular location of each metabolite, or its lifetime within each cell. Furthermore, noncoding RNAs can affect gene expression and cell physiology, and it remains unclear how Δ Np53:WTp53 affects expression of noncoding RNA genes. The effect of Δ Np53:WTp53 vs. WTp53 on H1299 cell growth, cell cycle, or senescence was essentially identical; however, a \sim 10% difference was observed in annexin V staining (early apoptosis). This could make some contribution to gene expression and metabolic differences between Δ Np53:WTp53 and WTp53 cells; however, our analyses focused on large-scale changes (typically twofold or greater) such that it is unlikely a major contributing factor. Consistent with this, phenotypic changes (e.g., mitochondrial membrane potential or mTOR signaling) were insensitive to removal of annexin V-positive cells and were consistent at 24 or 41 h following Δ Np53:WTp53 or WTp53 expression. We acknowledge that the gene expression and metabolic changes induced by Δ Np53:WTp53 have similarities with aging studies in model organisms. Aging is controlled by a complex array of genetic, epigenetic, and environmental factors, however, and the data shown here focus only on Δ Np53:WTp53-dependent changes. Thus, our results will not correlate with all aging studies completed in

model organisms. Moreover, the bulk of our analysis was completed in a single cell type. Although this has obvious advantages (isogenic; identical culture conditions) and avoids physical and physiological heterogeneity inherent in young vs. old comparisons, the Δ Np53:WTp53-induced changes identified here may not be identical in distinct cell types or contexts.

Analysis of Δ Np53:WTp53 tetramers as a single entity

The cryo-EM structure of the WTp53 tetramer has provided unprecedented insight about its structural organization (Okorokov *et al.*, 2006) and revealed a straightforward means to link Δ Np53 and WTp53 as a single transcript while preserving p53 tetramer architecture. As expected, the flexible peptide linker—which was longer than necessary to enable conformational variability—did not notably affect the biological activity of p53, as tethered versions of WTp53 (i.e., WTp53:WTp53) mimicked phenotypic and gene expression changes induced by native WTp53 tetramers. Similarly, Δ Np53:WTp53 formed stable tetramers, bound p53 target sequences in cells, activated transcription similar to WTp53 *in vitro*, and displayed similar activity to coexpressed Δ Np53 + WTp53 in cells. It has been postulated that translation of p53 generates homodimers, followed by post-translational tetramer formation (Nicholls *et al.*, 2002). Based on this model, the structural organization of 2:2 Δ Np53:WTp53 tetramers would be slightly distinct, with one WT dimer merging with one Δ Np53 dimer. Whether this assembly mechanism predominates in cells remains unclear, in part because few studies have directly addressed this question, likely due to its enormous practical and technical challenges. The similar gene expression changes induced by Δ Np53:WTp53 compared with Δ Np53 + WTp53, however, suggest that either these potential architectural distinctions have little functional consequence, or that the linked Δ Np53:WTp53 tetramers evaluated here represent a predominant tetramer architecture.

Expression of linked Δ Np53:WTp53 dimers to ensure a 2:2 tetramer stoichiometry offered several advantages over coexpression of Δ Np53 and WTp53 (i.e., Δ Np53 + WTp53). Because Δ Np53:WTp53 stoichiometry cannot be controlled with separate coexpression (even when each protein is present at similar levels), up to five distinct entities could form, ranging from zero to four copies of Δ Np53 within any given tetramer. The strategy employed here avoided this heterogeneity, and ensured the presence of a single entity in which the Δ Np53:WTp53 stoichiometry was kept constant. Equally important, our strategy ensured that gene expression and metabolomics data would not be confounded by the presence of contaminating WTp53 tetramers that result from Δ Np53 and WTp53 coexpression. Thus, the gene expression and metabolomics comparison between Δ Np53:WTp53 and WTp53 completed here enabled a clear contrast that would simply not be possible with any series of coexpression studies.

Neomorphic function of human Δ Np53

Mutations can cause a gain- or loss-of-function in a transcription factor that modify its effect on global gene expression patterns; such 'neomorphic' mutations are well documented for p53 (Freed-Pastor & Prives, 2012). In general, the effects of Δ Np53:WTp53 vs. WTp53 in H1299 cells were largely indistinguishable. Only a subset of genes were differentially expressed, and only minor changes, if any, were observed upon assessing cell growth, apoptosis, cell cycle, or senescence. Potentially, Δ Np53:WTp53 could gain functions that promote growth; however, this was not evident based on our observations with cultured

cells or from the gene expression and metabolomics data. Based upon our comparative analyses with WTp53, alterations in mTOR signaling and mitochondrial function appear to represent neomorphic outcomes of Δ Np53:WTp53 expression. Whereas WTp53 is a negative regulator of mTOR signaling (Feng & Levine, 2010), this function appears to be lost with Δ Np53:WTp53. Up-regulation of the mTOR pathway appears to result—at least in part—from altered gene expression (*IRS-1*, *INPP5D*) and metabolic changes (amino acids) in cells expressing Δ Np53:WTp53.

Potential mechanisms that underlie the mitochondrial changes (e.g., increased membrane potential) can also be deduced from the metabolic and gene expression data. In addition to carnitine and its role in mitochondrial fatty acid transport, we observed an up-regulation of *MARS2*, the mitochondrial tRNA^{met} synthetase, which suggests enhanced translation of mitochondrial proteins in Δ Np53:WTp53 cells. Because translation initiates with the Met codon, it has been proposed that increased levels of tRNA^{met} up-regulate translation (Marshall *et al.*, 2008). Increased translation of mitochondrial proteins, in particular those involved in electron transport and oxidative phosphorylation, could result in the observed increase in membrane potential and ATP. Mitochondrial function is also sensitive to iron. In Δ Np53:WTp53 cells, we observed increased levels of glutathione and its derivatives, along with altered expression of the heme transporter *SLC46A1* and the iron reductase/iron import protein *CYBRD1*. Glutathione is essential for regulating iron metabolism (Kumar *et al.*, 2011), and increased levels of glutathione metabolites, coupled with changes in iron import-export factors, suggest that iron homeostasis might be disrupted in Δ Np53:WTp53 cells. Mitochondrial function is highly dependent upon iron, in part because many proteins and protein complexes important for electron transport contain iron–sulfur clusters. In fact, glutathione is required for proper biogenesis of iron–sulfur clusters (Lill, 2009).

Given the well-studied roles of the mTOR pathway and mitochondrial function in longevity, our results with Δ Np53:WTp53 are consistent with its accelerated aging phenotype in mice and implicate the naturally occurring Δ Np53 isoform as a potential regulator of human aging.

Potential links between Δ Np53 and normal physiological aging

A growing number of studies indicate that single metabolites are sufficient to trigger phenotypic changes in cells and within an organism. To cite a few examples, acetate can correct insulin signaling defects (Shin *et al.*, 2011), and choline can promote atherosclerosis (Wang *et al.*, 2011). The metabolic changes induced by Δ Np53:WTp53 expression are striking and encompass a limited set of pathways. We observed that amino acids, urea cycle intermediates, polyamines, and glutathione metabolites were significantly increased in cells expressing Δ Np53:WTp53, relative to WTp53. Numerous studies have linked these same metabolites to aging and longevity in mammalian model organisms; however, far fewer studies have addressed the metabolic changes that accompany human aging. A metabolomic study of human plasma isolated from healthy old vs. young donors shows clear similarities to our metabolomic comparison of Δ Np53:WTp53 and WTp53 (Lawton *et al.*, 2008). As seen in Δ Np53:WTp53 cells, amino acid levels were uniformly increased in old vs. young human plasma samples, as were polyamines, carnitine, TCA cycle, and urea cycle metabolites. Potentially, these shared features could reflect a distinct metabolic signature in aged human cells that is rapidly adopted upon Δ Np53 expression. Much additional work will be needed to rigorously test this hypothesis.

Among the various models for mammalian aging, a common theme includes stochastic, heritable changes that gradually alter gene expression patterns and metabolic pathways (Martin, 2009). Such mechanisms appear to represent a fundamental requirement for aging, as biological effects manifest over time. Because Δ Np53 is a naturally occurring isoform, its levels could change with age. Existing gene expression data have limited value, however, as Δ Np53 levels are controlled primarily by alternate translation (Candeias *et al.*, 2006; Ray *et al.*, 2006). In mice, relative levels of Δ Np53 change with developmental state (Ungewitter & Scoble, 2010), and several reports indicate that human Δ Np53 protein levels change during cell stress (Courtois *et al.*, 2002; Candeias *et al.*, 2006; Bourougaa *et al.*, 2010). The links to cell stress imply that transient increases in Δ Np53 levels can occur throughout the human life span.

Could transient increase of Δ Np53 levels have chronic, long-term consequences? We note two potential mechanisms by which transient Δ Np53 expression could initiate a self-reinforcing cascade, with possible implications for aging. First, it is established that ER stress increases relative levels of Δ Np53 vs. WTp53 in human cells by favoring alternate translation initiation from an IRES within the WTp53 mRNA (Bourougaa *et al.*, 2010). Increased levels of 2-hydroxyglutarate (2HG) can independently induce an ER stress response (Sasaki *et al.*, 2012). Because 2HG is elevated in Δ Np53:WTp53 cells, Δ Np53 could promote its own translation by inducing ER stress. Second, we observed increased levels of succinate and 2HG in cells expressing Δ Np53:WTp53, as well as increased expression of the histone H3K79 methyltransferase *DOT1L*. This suggests that DNA methylation and histone H3K79 methylation patterns may be globally altered by Δ Np53 expression. Because 2HG or succinate can competitively inhibit demethylase enzymes that use alpha-ketoglutarate as a cofactor, increased 2HG and succinate favor a general retention of methyl marks on both histones and DNA (Xu *et al.*, 2011; Xiao *et al.*, 2012). DNA methylation and histone H3K79 methylation are epigenetic regulators of transcription that may enable even transient Δ Np53 expression to 'lock in' gene expression patterns that propagate to cell progeny. Indeed, whereas both H3K79 methylation and DNA methylation marks are reversible, DNA methylation also appears to be heritable (Bonasio *et al.*, 2010). Future studies will focus on whether Δ Np53:WTp53 alters patterns of DNA methylation and/or H3K79 trimethylation genome-wide, and how this might contribute to the geriatric pathology of Δ Np53.

Accession numbers

Microarray data were deposited in GEO (<http://www.ncbi.nlm.nih.gov/geo/>) under the accession number GSE37271.

Experimental Procedures

In vitro transcription

In vitro transcription reactions and purification of factors for the reconstituted transcription system were completed as described (Knuesel *et al.*, 2009).

Microarray analyses

Total RNA from FACS-sorted cells was extracted with the RNeasy kit (Qiagen) and was analyzed at the Genomics and Microarray Shared Resource (University of Colorado Cancer Center) for transcriptional profiling. Per standard protocol, 100–150 ng of starting total RNA was

converted to cDNA with the Ambion WT Expression kit, according to the manufacturer's protocol. After standard labeling, each sample was hybridized to Affymetrix GeneChip Human Gene 1.0 ST array, followed by examination with an Affymetrix GeneChip Scanner 3000.

Global metabolic profiling

Metabolomic profiling analysis was performed by Metabolon (Durham, NC) as described (Reitman *et al.*, 2011).

Real-time quantitative polymerase chain reaction

See SI; primer sequences are shown in Table S10 (Supporting information).

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Author contributions

Conceived and designed the experiments: SL and DT. Performed the experiments: SL, EK, and DT. Analyzed the data: SL, EK, and DT. Wrote the paper: SL and DT.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Fig. S1 Schematic of Δ Np53:Wtp53.

Fig. S2 Representative Western blot and cell cycle data.

Fig. S3 Assessment of apoptosis, growth, and senescence

Fig. S4 GSEA of microarray data.

Fig. S5 Venn diagrams of gene expression data.

Fig. S6 qRT-PCR validation of microarray data.

Fig. S7 qRT-PCR data from Saos-2 cells.

Fig. S8 Evaluation of Wtp53:Wtp53 tetramers.

Fig. S9 qRT-PCR data comparing Δ Np53:Wtp53 with Δ Np53 and Wtp53 coexpressed at equal levels.

Fig. S10 Comparison of Δ Np53-induced gene expression changes in H1299 cells.

Fig. S11 Additional data for mitochondrial function.

Fig. S12 Additional data for mTOR pathway.

Table S1 Genes up-regulated 1.5-fold or greater in H1299 cells expressing Δ Np53:Wtp53, compared with Wtp53.

Table S2 Genes down-regulated 1.5-fold or greater in H1299 cells expressing Δ Np53:Wtp53, compared with Wtp53.

Table S3 Overview of metabolomics data.

Table S4 Metabolomics data.

Table S5 Summary of metabolic changes induced by Wtp53.

Table S6 Pathway analysis of gene expression data: GO Processes and GO Metabolic Networks.

Table S7 Pathway analysis of gene expression data: GO Pathways.

Table S8 Pathway analysis of Metabolomics data.

Table S9 Merged Pathway analysis of gene expression and metabolomics data.

Table S10 Primer sequences used for cloning and qRT-PCR.

Data S1 Experimental procedures.