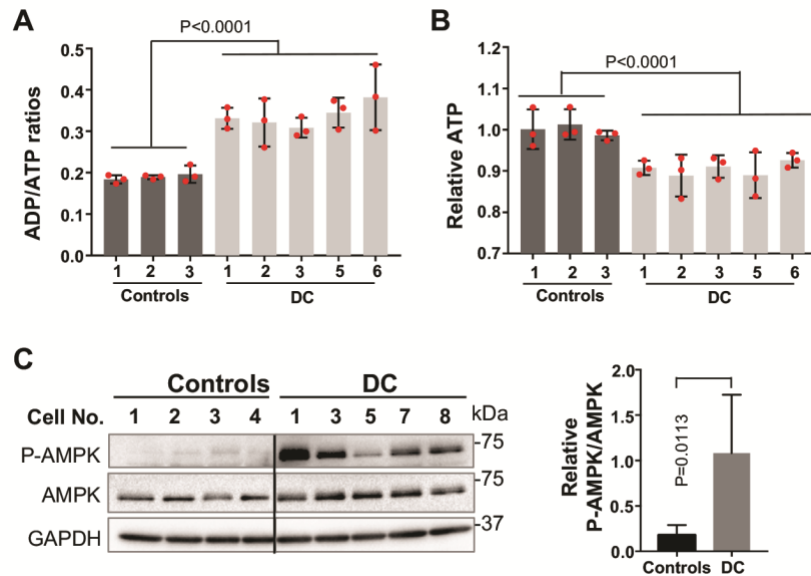


Appendix for “Re-equilibration of imbalanced NAD metabolism ameliorates the impact of telomere dysfunction”

Chongkui Sun, Kun Wang, Amanda J. Stock, Yi Gong, Tyler G. Demarest, Beimeng Yang, Neelam Giri, Lea Harrington, Blanche P. Alter, Sharon A. Savage, Vilhelm A. Bohr, and Yie Liu

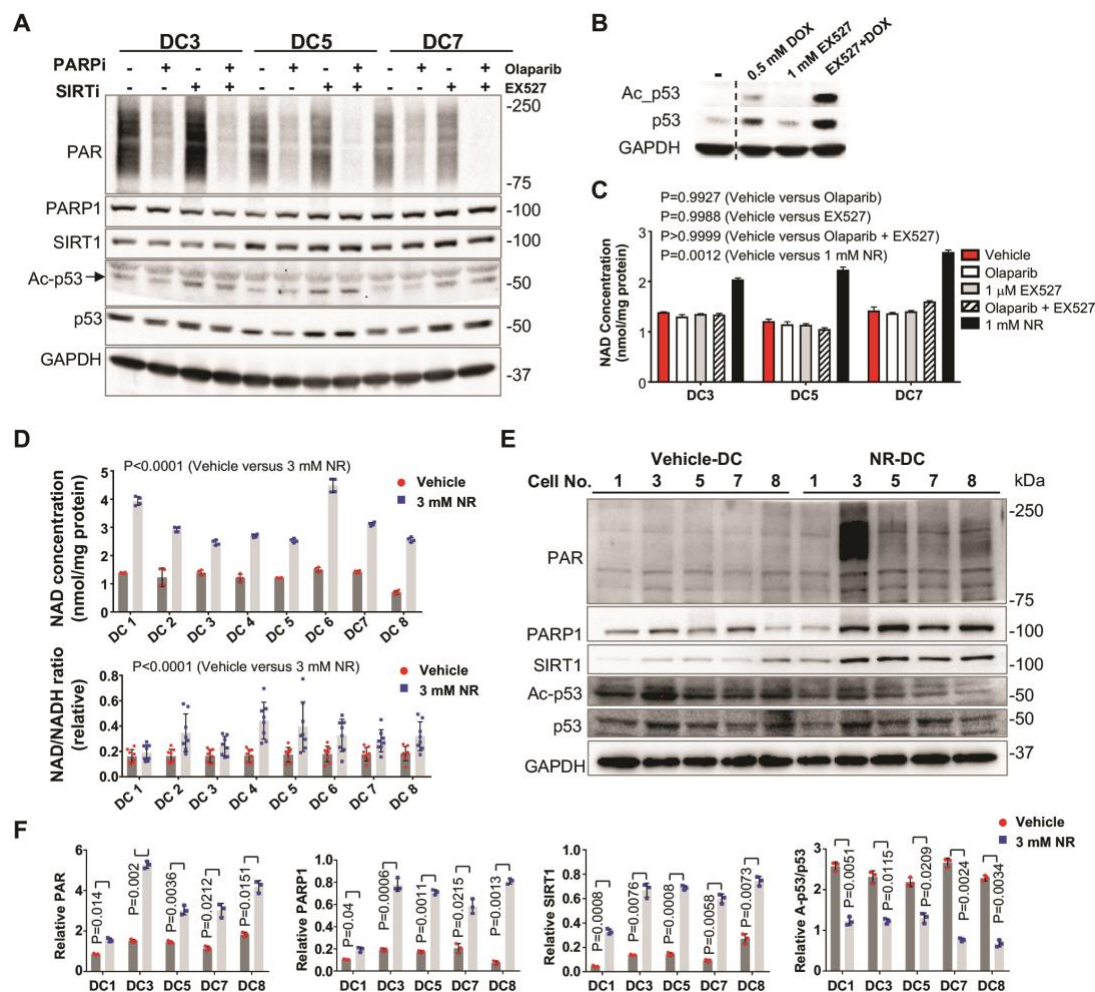
Table of contents:

1. Appendix Figures S1-S8
2. Appendix Supplementary Methods
3. Appendix Table S1
4. Appendix Table S2



Appendix Figure S1. DC fibroblasts exhibit energy deficit.

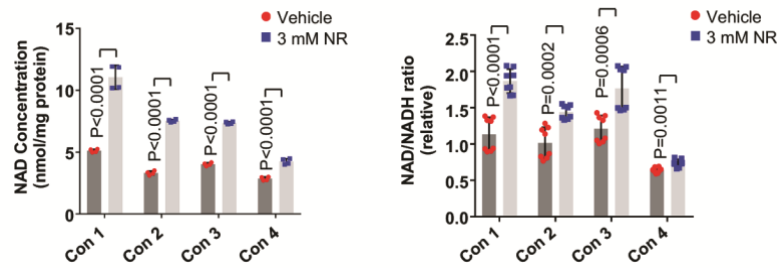
- A,B** ADP/ATP ratios and relative ATP levels in DC and age-matched healthy control fibroblasts. The relative values of each sample were normalized to the controls, and are presented as mean $\pm$ SD of three replicates. Student's *t* test was performed on DC cells versus controls.
- C** Immunoblots (left) and quantification (right) of the expression of AMPK and pAMPK in DC and control fibroblasts. Protein levels are normalized to GAPDH. Quantification values are presented as mean $\pm$ SD of four controls versus five DC samples. Student's *t* test was performed on DC cells versus controls.



Appendix Figure S2. NAD-dependent PARP PARylation and SIRT1 deacetylation activity does not contribute to NAD decline in DC fibroblasts.

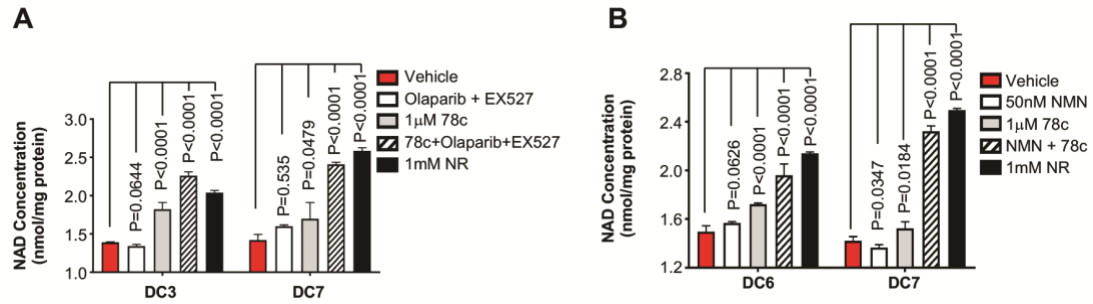
- A** Immunoblots of the expression of indicated proteins in DC fibroblasts treated with the PARP1 inhibitor Olaparib (400nM) and the SIRT1 inhibitor EX 527 (1 μ M) for 24 hours.
- B** The effect of the SIRT1 inhibitor EX527 on the deacetylation of p53. BJ fibroblasts were exposed to the DNA damaging agent, doxorubicin and tested for acetylated p53 (Ac-p53) compared with total levels of p53. The “-” symbol indicates no treatment.
- C, D** Intracellular NAD levels and NAD/NADH ratio in DC fibroblasts treated with vehicle, PARP1 and/or SIRT1 inhibitors, and NR. All values are presented as mean $\pm$ SD of three replicates in C and four and eight replicates for NAD levels and NAD/NADH ratio, respectively in D.
- E, F** Representative immunoblots of the indicated proteins in DC fibroblasts treated with vehicle or 3 mM NR. Quantification of the indicated proteins are presented as mean $\pm$ SD from three immunoblots.

Data information: One-way ANOVA (C) and Student’s *t* test (D and F) were performed on each DC line in indicated conditions.



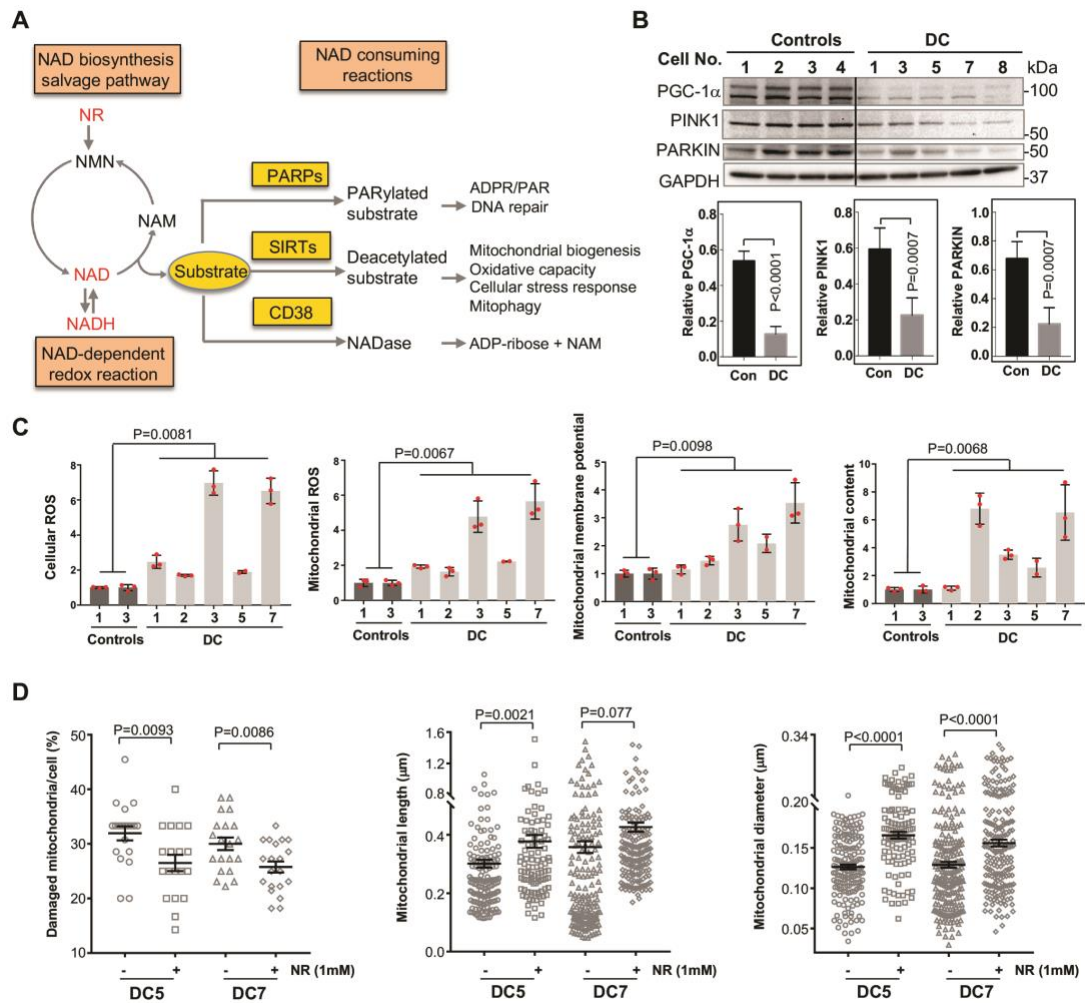
Appendix Figure S3. Effects of NR supplementation on the NAD⁺ metabolites in age-matched healthy control fibroblasts.

Intracellular NAD levels and NAD/NADH ratio in the vehicle and NR treated fibroblasts derived from age-matched healthy donors (Con). All values are presented as mean $\pm$ SD of four and eight replicates for NAD levels and NAD/NADH ratio, respectively. Student's *t* test was performed for individual pairs with indicated conditions.



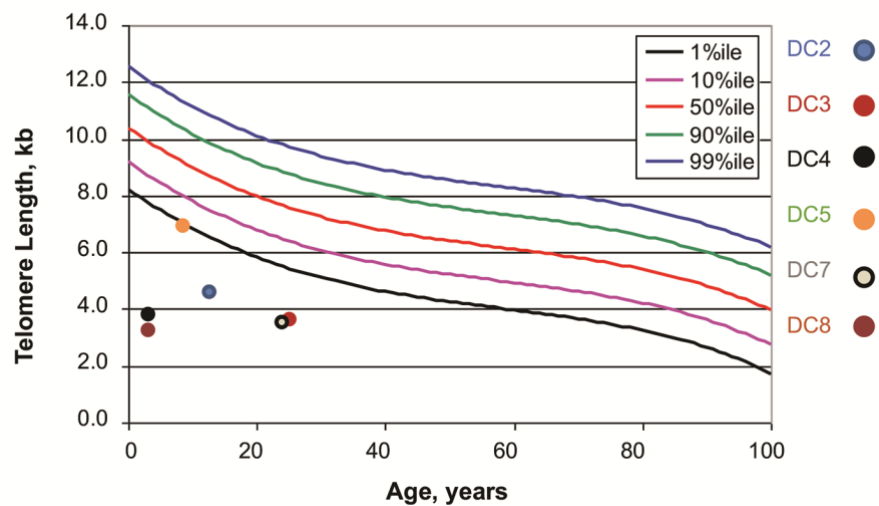
Appendix Figure S4. Effects of the PARP1, SIRT1, and CD38 inhibitors on intracellular NAD levels in DC fibroblasts.

Intracellular NAD levels in DC fibroblasts treated with vehicle, the PARP1 inhibitor (0.5 μM olaparib), the SIRT1 inhibitor (1μM EX527), the CD38 inhibitor (1 μM 78c), and 1 mM NR for 48 hours. All values are presented as mean ± SD of three replicates. One-way ANOVA was performed on DC fibroblasts in indicated conditions.



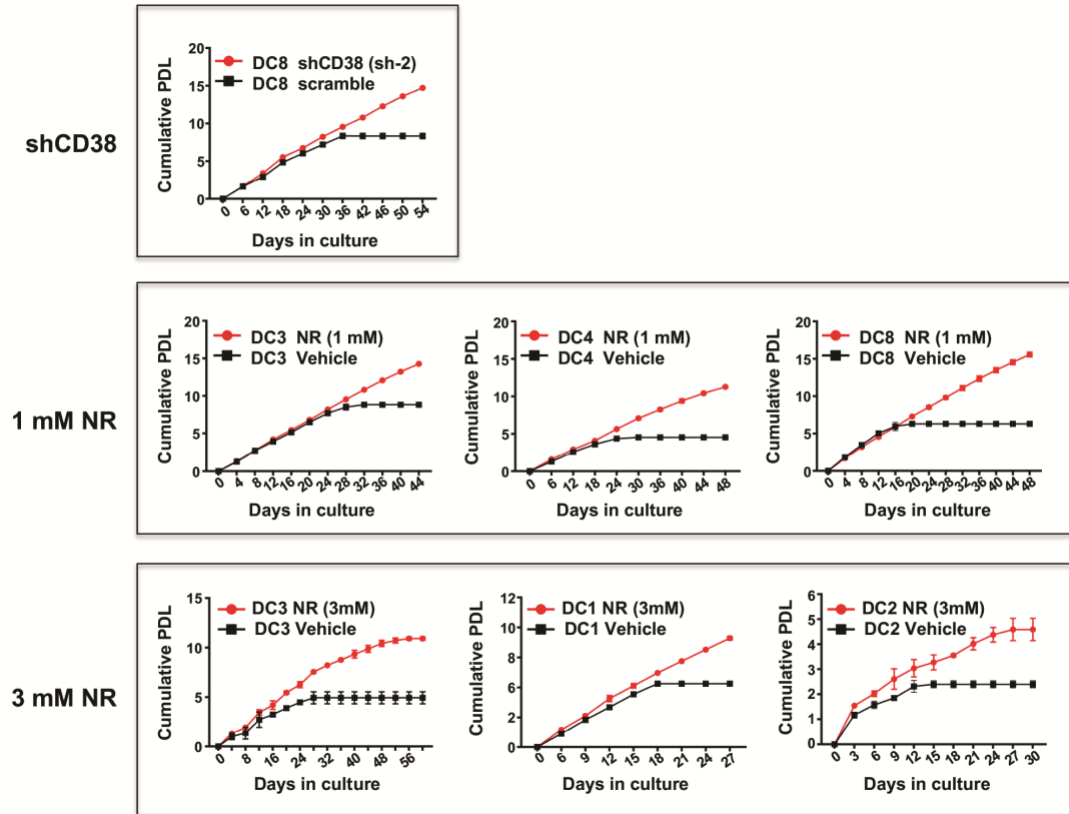
Appendix Figure S5. DC fibroblasts are defective in the NAD⁺-SIRT1-mitochondrial axis.

- A** An overview of NAD metabolism and NAD biosynthesis and consuming enzymes. The activity of NAD consuming enzymes, such as PARPs, SIRT1s, or CD38 consume NAD⁺, which is involved in indicated cellular functions. NAD and NADH are also involved in redox reactions.
- B** Immunoblots and quantification of the expression of indicated proteins in DC and age-matched healthy control (Con) fibroblasts. Protein levels are normalized to GAPDH, and mean (± SD) quantification values of 4 controls vs 5 DC samples are shown. P values on the basis of Student's *t* test.
- C** Various mitochondrial parameters in DC and control fibroblasts measured by flow cytometry. Each data point is represented as mean ± SD of three replicates. Student's *t* test was performed on DC cells versus controls.
- D** Quantifications of percentage of damaged mitochondria, mitochondrial length, and mitochondrial diameter of DC fibroblasts treated with vehicle or 1 mM NR by transmission electron microscopy analysis. A minimum of 200 mitochondria counted per group. All data are represented as mean ± SD. Student's *t* test was performed on DC cells in indicated conditions.



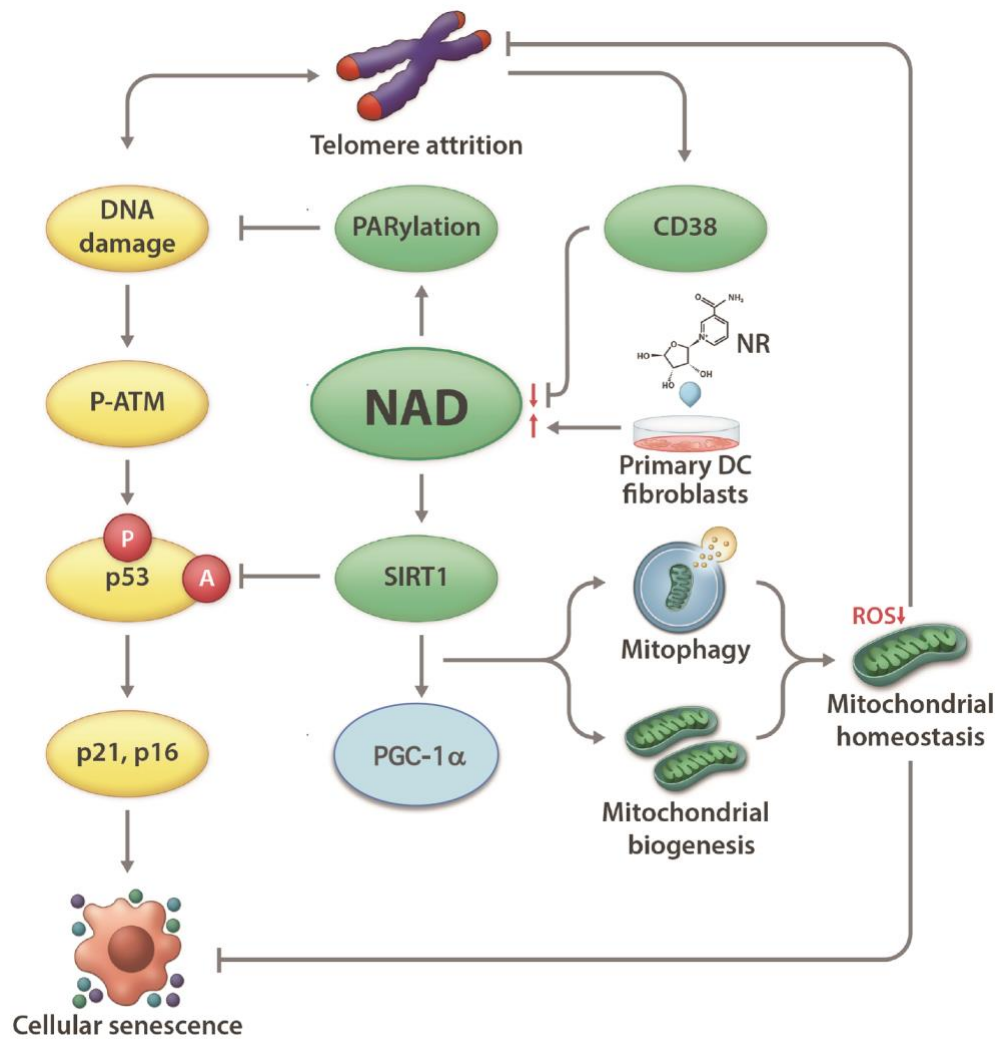
Appendix Figure S6. Telomere length measurement in DC peripheral blood mononuclear cells, related to Appendix Table S1.

Telomere lengths of study participants as determined by flow cytometry with fluorescence *in situ* hybridization using a FITC-telomere 5'-(CCCTAA)₃-3' probe. Legend indicates colors corresponding to patients on the graph.



Appendix Figure S7. NR supplementation and CD38 knockdown improve the proliferative capacity of DC fibroblasts in vitro.

Cumulative population doubling analysis of the proliferation of the scrambled and CD38 knockdown DC fibroblasts or DC fibroblasts treated with vehicle and 1 or 3 mM NR. All values are represented as mean $\pm$ SD of three replicates.



Appendix Figure S8. Telomere dysfunction contributes to dysregulated NAD⁺-metabolism, DNA damage, mitochondrial dysfunction, and cellular senescence of DC cells.

Defective NAD metabolism is attributed by increased CD38 NADase activity in human and mouse models with critically short telomeres/telomere dysfunction. Decline in intracellular NAD levels limits the NAD-consuming activities of SIRT1 and poly (ADP-ribose) polymerase. SIRT1 participates in the deacetylation of p53 and in the activation of PGC-1 α , which together have been implicated in the regulation of the DNA damage response and mitochondrial homeostasis. PARP1 plays a critical role in genome maintenance and DNA repair. NAD consumption by PARPs is mostly increased during genotoxic stress due to PARylation. NR supplementation and CD38 inhibition improve PARylation and SIRT1 activities and their related pathways in mitochondrial homeostasis and telomeric DNA damage response and repair, consequently mitigating phenotypes associated with cellular senescence.

Supplemental Information

Mice and human cell lines

Tert heterozygous mice were bred to produce *Tert*^{-/-} mice. The *Tert*^{-/-} mice were designated as generation 1 (G1), and the progeny of G1 cousins defined as G2, and so on. At G3, *Tert*^{-/-} mice become infertile. All animal experiments were carried out according to the “Guide for the Care and Use of Laboratory Animals” (National Academy Press, USA, 1996), and were approved by the Institutional Animal Care and Use Committee of the National Institute on Aging. The animals in each genotype group were randomly sampled.

Primary skin fibroblasts were obtained from the Coriell Cell Repository and the National Cancer Institute’s Institutional Review Board approved study, “Cancer in Inherited Bone Marrow Failure Syndromes” (ClinicalTrials.gov identifier NCT00027274, <https://marrowfailure.cancer.gov>). Study participants or their legal guardian provided informed consent and were evaluated at the National Institutes of Health Clinical Center. Genetic testing was performed in CLIA-approved laboratories. The experiments confirmed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report. A547 is a human lung cancer cell line (ATCC, male).

Antibodies

The primary antibodies are listed as follows: p-ATM (#13050, Cell Signaling), p-p53 (#9284, Cell Signaling), p53 (sc-126, Santa Cruz), p21 (#610234, BD Biosciences), p16 (#92803, Cell Signaling), p-AMPK (#2535, Cell Signaling), AMPK (#2532, Cell Signaling), PGC-1 α (#ab77210, Abcam), PINK1 (#BC100-494, Novus Biologicals), PARKIN (#H00005071-M01, Novus Biologicals), γ H2AX (#sc101696, Santa Cruz), PAR (#4336-BPC-100, Trevigen), PARP1 (#9542, Cell Signaling), SIRT1 (#sc74504, Santa Cruz; #sc15404, Santa Cruz), acetylated-p53 (#2525 and 2570, Cell Signaling), CD38 (#ab108403, #ab216343, Abcam, #AF4947, R&D), NMNAT-1 (#98354, Cell Signaling), NMNAT-2 (#ab110040, Abcam), NMNAT-3 (#ab71904, Abcam), NAMPT (#61122, Cell Signaling), β -tubulin (#ab6046, Abcam), and GAPDH (#AC027, AB Clonal).

Appendix Table SI. Information on primary fibroblasts derived from patients with dyskeratosis congenita (DC) and healthy controls.

Cell line ID*	Study ID	Gene	Mutation(s)	Gender	Age at fibroblast collection	Telomere length (kb)^	Age at telomere length measurement
DC1	GM01774	<i>DKC1</i>	201_203delICTT	Male	7	/	/
DC2	NCI-106-1	<i>DKC1</i>	p.K314R	Male	13	4.3	13
DC3	NCI-167-3	<i>DKC1</i>	R322Q (c.965 G>A)	Male	27	3.7	26
DC4	NCI-256-1	<i>DKC1</i>	p.T49M (c.146C>T)	Male	4	3.9	3
DC5	NCI-422-1	<i>TERT</i>	c.1990 G>A (p.Val664Met) and c.2455 C>T (p.Arg819Cys)	Male	9	6.9	9
DC6	NCI-345-1	<i>RTEL1</i>	p.His1148Thrfs*12 (c.3442del) and p.Pro17Ser (c.49C>T)	Male	8	ND+	/
DC7	NCI-74-6	<i>TINF2</i>	p.K280E (c.838 A-G)	Male	24	3.6	24
DC8	NCI-156-1	<i>TINF2</i>	p.R282H (c.845 G-A)	Male	4	3.3	3
Con1	GM05757	/	/	Male	7	/	/
Con2	GM23973	/	/	Male	19	/	/
Con3	GM23976	/	/	Male	22	/	/
Con4	GM03349	/	/	Male	10	/	/

* Cells were grown in 3% v/v oxygen. For some DC patient-derived cells, their limited replicative capacity precluded detailed experimental analysis.

^ Lymphocyte telomere lengths measured by flow cytometry with fluorescence *in situ* hybridization at Repeat Diagnostics, Inc. (Vancouver, BC) and reported in kilobases (kb).

+ ND, not determined because the patient underwent hematopoietic cell transplantation prior to study enrollment.

Appendix Table S2. Concentrations of different cytokines in cell culture supernatant and cell lysate.

Unit (pg/ml)	DC1 (mean±SD)				DC2 (mean±SD)			
	Supernatant		Cell lysate		Supernatant		Cell lysate	
	Veh	NR	Veh	NR	Veh	NR	Veh	NR
FGF-2	44.82±7.05	47.85±1.34	1357.03±33.98	1371.6±30.52	39.21±10.09	37.45±8.70	1330.71±38.57	1310.29±89.82
TGF- α	2.76±0.12	3.14±0.15	1.68±0.36	2.49±0.05	1.00±0.18	0.81±0.16	0.14±0.06	0.16±0.02
G-CSF	1.77±0.74	0.64±0.81	3.5±1.89	2.77±0.20	1.71±0.30	0.54±0.43	1.59±1.46	2.25±0.91
Flt-3L	2.87±0.63	2.36±0.24	5.59±1.03	4.48±0.70	1.09±0.18	1.58±0.87	2.67±0.68	2.46±1.40
GM-CSF	6.67±0.75	6.42±0.62	5.63±0.31	5.28±0.50	5.42±0.53	4.94±1.11	5.15±0.56	5.10±0.36
Fractalkine	22.67±6.32	20.75±2.31	21.00±7.37	21.14±0.58	9.97±4.80	9.79±6.28	13.03±0.00	15.63±5.29
IFN α 2	3.72±1.02	3.36±0.79	5.48±1.32	5.28±0.53	2.72±0.39	2.93±0.35	3.00±0.28	3.68±0.55
IFN γ	3.29±0.24	2.87±0.20	2.98±0.98	3.37±0.77	3.44±1.60	2.72±0.54	3.01±0.27	2.88±0.48
GRO α	21.98±6.34	25.23±4.69	23.37±7.06	22.77±2.57	8.49±3.21	13.14±5.99	13.96±0.00	8.8±4.47
IL-10	0.80±0.26	0.72±0.38	0.65±0.25	0.69±0.16	0.61±0.05	0.41±0.21	0.51±0.04	0.45±0.08
MCP-3	12.15±1.59	15.08±0.83	11.58±4.08	10.31±1.15	8.77±2.03	7.56±2.43	5.82±0.93	5.47±2.74
IL-12P40	2.91±1.09	1.42±0.47	2.30±0.60	2.57±0.17	1.87±0.20	1.8±0.76	1.14±0.08	1.38±0.68
MDC	47.74±5.59	32.24±11.59	86.32±55.89	66.02±40.30	16.85±6.38	12.39±9.50	24.06±0.00	17.55±3.01
IL-12P70	1.36±0.39	1.14±0.45	0.88±0.87	0.91±0.13	0.22±0.21	0.73±0.13	0.60±0.32	0.46±0.25
PDGF-AA	0.43±0.02	0.46±0.06	1.53±0.24	1.32±0.07	6.46±0.43	6.85±0.54	2.45±0.08	3.36±0.06
PDGF-BB	1.14±0.65	1.98±0.91	5.62±2.11	4.12±1.96	1.94±2.70	0.63±0.34	12.43±1.85	7.95±2.63
IL-15	1.04±0.16	0.73±0.23	2.35±0.53	2.67±0.08	0.55±0.29	0.64±0.08	2.10±0.11	2.14±0.15
sCD40L	0.81±0.20	0.93±0.13	0.87±0.24	0.83±0.18	0.72±0.10	0.64±0.03	0.58±0.00	0.52±0.10
IL-1R α	6.70±1.69	6.65±0.23	109.34±6.50	73.99±3.23	5.17±0.58	4.83±1.08	11.62±5.09	6.01±0.96
IL-1 α	0.46±0.12	0.58±0.32	0.96±0.18	1.29±0.25	0.25±0.18	0.72±0.38	0.25±0.11	0.36±0.19
IL-6	4.49±0.27	3.23±0.34	5.87±0.49	2.44±0.04	2.84±0.26	2.25±0.18	2.89±0.15	2.26±0.18
IL-8	1.57±0.14	1.55±0.00	3.84±0.33	2.83±0.03	3.16±0.26	3.20±0.18	5.84±0.49	2.82±0.75
MCP-1	122.27±6.58	94.15±13.03	67.28±3.68	42.76±1.03	60.70±3.19	49.58±1.25	37.97±1.62	36.69±3.54
MIP-1 α	2.31±0.31	3.00±0.95	3.06±1.04	2.98±0.45	2.29±1.15	2.44±0.72	1.94±0.37	2.11±0.15
MIP-1 β	1.58±0.38	2.46±0.28	1.18±1.02	1.05±0.19	0.64±0.76	0.91±0.76	0.37±0.00	0.78±0.95
TNF α	0.29±0.08	0.35±0.07	0.24±0.08	0.26±0.02	0.26±0.04	0.31±0.01	0.25±0.01	0.25±0.04
TNF β	0.72±0.19	0.77±0.07	0.88±0.52	1.01±0.13	0.62±0.45	0.41±0.28	0.33±0.27	0.53±0.42
VEGF- α	7.25±0.49	7.43±1.03	9.78±1.28	7.80±0.25	29.26±2.05	38.24±1.76	33.27±0.52	53.60±5.67
IL-18	1.32±0.34	1.67±0.49	4.05±1.18	5.07±1.44	1.32±0.48	0.87±0.43	6.09±3.37	5.30±1.03

All values are mean $\pm$ standard deviation (SD) of triplicates.