Chapter 3

Do senescence markers correlate in vitro and in situ within individual human donors?

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Abstract

Cellular senescence can be detected by several markers both *in vitro* and *in situ*, but little is known on how well senescence markers correlate within individual donors. By using data from highly standardized experiments, correlations between the same *in vitro* senescence markers were studied in duplicate short-term experiments, and between short-term and long-term experiments. In addition, different *in vitro* senescence markers measured within the short-term and long-term experiments were tested amongst each other for correlation. The different *in vitro* senescence markers were also tested for correlations with *in situ* p16INK4a cell positivity. From a total of 100 donors (aged 20-91 years), cultured dermal fibroblasts were assessed for reactive oxygen species (ROS), telomere-associated foci (TAF), p16INK4a and senescence-associated β-gal (SAβ-gal), both in non-stressed conditions and after supplementing the medium with 0.6 μM rotenone for 3 days (short-term experiment). In cultured fibroblast from 40 of the donors, telomere shortening, levels of ROS and SAβ-gal were additionally assessed, with or without 20 nM rotenone for 7 weeks (long-term experiment). In skin tissue from 52 of the donors, the number of p16INK4a positive dermal cells was assessed *in situ*. More than half of the correlations of the same senescence markers *in vitro* between duplicate experiments and between short-term versus long-term experiments were significant (with an average coefficient of 0.498). Half of the different senescence marker correlations were significant (average coefficient of 0.349) within the short-term experiments and within the long-term experiments. Within middle-aged donors, the different senescence markers *in vitro* were not significantly correlated intra-individually with *in situ* p16INK4a positivity. In conclusion, caution is warranted in comparing results obtained using different senescence markers and in extrapolating *in vitro* to *in vivo* findings.

Introduction

Five decades ago, Hayflick and Moorhead first described the phenomenon of limited replicative capacity of cultured primary cells, termed cellular replicative senescence [1;2]. It was postulated that this *in vitro* phenomenon of stable cell cycle arrest might be related to aging of the whole organisms *in vivo*. Since then many studies have focussed on cellular senescence *in vitro*, and have identified several triggers inducing senescence as well as pathways leading to senescence (reviewed in [3]). Considerable interest has also been given to the possible *in vivo* implications of senescence; by studying relevant functions, including embryonic development and attenuating liver fibrosis as well as consequences of senescence in animal models, notably age-related diseases, and tumorigenesis in neighboring cells [4-8]. In the last few decades [9] human tissues have been studied to detect cellular senescence *in situ*, providing knowledge on the prevalence of senescent cells in humans at older ages or with disease.

Apart from growth arrest, several other markers of cellular senescence have been studied (reviewed in [10]). A frequently used marker is senescence-associated β- galactosidase (SAβ-gal) activity, which is upregulated in, but not essential for senescence[9;11]. Other markers are based on triggers of senescence such as DNA damage foci or reactive oxygen species (ROS), expression of genes involved in cell cycle arrest or factors that are secreted by senescent cells[3;10;12]. Most of these markers have been established by detecting senescence *in vitro*, but some can also be used *in situ* [13]. However the number of studies in fibroblasts reporting on senescence *in situ* compared to *in vitro* is disproportionally small [14], and there is a lack of knowledge concerning the correlation of senescence markers between these conditions. In addition, only few attempts have been made to study the correlation between different senescence markers.

Our aim is therefore to study the correlations between (A) the same senescence markers and (B) different senescence markers within individual donors, using an unique dataset of highly standardized experiments including (1) *in vitro* short-term experiments; (2) *in vitro* long-term experiments, and (3) *in situ* within skin biopsies. Correlations were tested between the same senescence markers: (1A) *in vitro* between duplicate experiments and (2A) *in vitro* between short-term and long-term experiments. In addition, correlations between different senescence markers were tested: (1B) between *in vitro* markers within the same short-term experiments; (2B) between *in vitro* markers within the same long-term experiments; and (3B) intra-individually between *in vitro* markers and *in situ* p16INK4a positivity.

Methods

Study design

The Leiden 85-plus Study is a prospective population-based study[15] of inhabitants of Leiden (the Netherlands). Participants aged 90 years and young controls aged 18-25 years donated skin biopsies of the upper inner arm to establish fibroblast cultures[16]. As previously described [17], in the Leiden Longevity Study factors contributing to familial longevity are studied. Skin biopsies for *in situ* staining and fibroblast cultures were obtained from middle-aged to old (mean 63 years) offspring of nonagenarian sibling and their partners [16]. All participants in these studies have given written informed consent, and both studies were approved by the Medical Ethical Committee of the Leiden University Medical Center.

In vitro senescence markers

Detailed methods have been described previously [18-20]. In short, fibroblast strains from 10 young donors (passage 14), from 80 middle-aged donors (40 offspring of long-lived families and 40 partners – passage 10), and from 10 old donors (passage 14) were randomly selected for subsequent experiments. Fibroblasts were cultured for 3 days with or without 0.6 μM rotenone added to the medium (short-term experiments). The following senescence markers

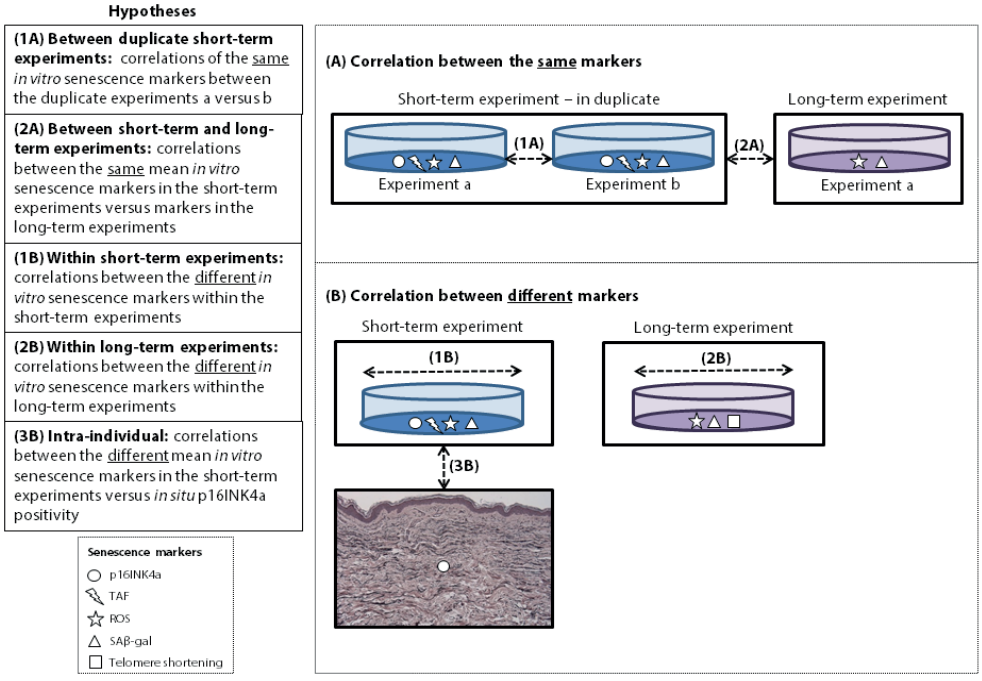

Figure 1. Explanation of hypotheses tested.

were assessed in fibroblast cultures in non-stressed and in rotenone-stressed conditions: median fluorescence intensity value of β galactosidase (SAβ-gal) and mean fluorescence intensity value of reactive oxygen species (ROS) were measured using flow cytometry, and the percentage of immunocytochemically stained p16INK4a positive fibroblasts was counted. The number of telomere-associated foci (TAF) was determined using immunofluorescence and PNA telomeric probe (53BP1 positive foci located at telomeres). 100 randomly selected nuclei were automatically scored for TAF. TAF are presented as the percentage of nuclei with ≥1 TAF per nucleus. These experiments were conducted in duplicate (experiments a and b) [18;20] (i.e. in parallel conducted repeated experiments for each strain). Furthermore alongside the above mentioned experiments, 10 fibroblasts strains from young, 20 from middle-aged (10 offspring, 10 partners) and 10 from old donors were randomly selected and cultured for 7 weeks, with or without 20 nM rotenone to generate chronic stress (long-term experiments). The median fluorescence intensity values of β galactosidase (SAβ-gal) and reactive oxygen species (ROS) were measured using flow cytometry. Telomere length was assessed with a flow-FISH kit and was expressed as the percentage compared to the reference cell line. The telomere shortening rate was further determined by comparing these measurements to telomere length at baseline and dividing the difference by the number of cumulative population doublings [19].

In situ senescence marker

As detailed previously [21], in order to detect p16INK4a in the formalin fixed paraffin embedded skin tissue, immunohistochemistry staining was used. Dermal p16INK4a cell counts were restricted to morphologically determined fibroblasts and normalized for the area of the dermis the cells were counted in. Dermal p16INK4a positivity is given as the number of p16INK4a positive cells per 1 mm^2.

Statistics

All analyses were performed using IBM SPSS Statistics 20. Not all data was normally distributed, these variables were naturally log transformed before evaluating the correlations by calculating the Pearson partial correlation coefficient, adjusted for experiment batch. The studied correlations are explained in Figure 1. First, correlations of the same senescence markers were analyzed using data of (1A) the short-term experiments a and b (duplicate experiments); (2A) the mean results of duplicates in the short-term experiments and the single measurements of the long-term experiments (as this experiment was performed once). Secondly, correlations between different senescence markers were analyzed using (1B) the mean results of duplicates within the short-term experiments; (2B) the single measurements within the long-term experiment; and (3B) the mean of the *in vitro* markers in the short-term experiments (mean results of duplicates) and *in situ* p16INK4a positivity. All *in vitro* markers were measured in a non-stressed and (rotenone) stressed condition. For data visualization

the percentage of fibroblasts staining positive for p16INK4a *in vitro* was plotted against the number of p16INK4a positive dermal cells *in situ*.

Results

Table 1 supplies the anthropometric and medical characteristics of the donors from whom the skin biopsies were obtained based on age (young, mean 23 years; middle-aged, mean 63 years; old, mean 90 years).

First, we studied correlations between the same markers, both in non-stressed and stressed conditions. The correlation of duplicates of each senescence marker (p16INK4a, TAF, ROS and SAβ-gal) were tested between experiment a and b of the short term experiments (Table 2). Most markers were significantly associated between experiments a and b (coefficients > 0.400), except for ROS which showed low, non-significant correlation coefficients.

Table 3 shows the correlations between ROS and SAβ-gal in the short-term versus the long-term experiments. ROS measures in the short-term experiment were significantly correlated to ROS in the long-term experiment. SAβ-gal was not significantly correlated between the short-term and long-term experiments.

Table 1. Characteristics of donors.

	Young (N=10)	Middle-aged (N=80)	Old (N=10)
Female, no.(%)	7 (70.0)	40 (50.0)	6 (60.0)
Age, years	22.8 (1.5)	63.2 (7.3)	90.2 (0.5)
Member of long-lived family	n/a	40 (50.0)	n/a
Body mass index, kg/m²	22.2 (1.8)[a]	26.2 (4.1)[b]	25.4 (3.8)
Co-morbidities			
Cerebrovascular accident	0/10 (0.0)	3/76 (3.9)	2/10 (20.0)
Chronic obstructive pulmonary disease	0/10 (0.0)	3/75 (4.0)	1/10 (10.0)
Diabetes mellitus	0/10 (0.0)	7/74 (9.5)	2/10 (20.0)
Hypertension	0/10 (0.0)	17/76 (22.4)	5/10 (50.0)
Malignancies	0/10 (0.0)	3/72 (4.2)	1/10 (10.0)
Myocardial infarction	0/10 (0.0)	0/75 (0.0)	3/10 (30.0)
Rheumatoid arthritis	0/10 (0.0)	0/76 (0.0)	3/10 (30.0)
Smoking, current	0/10 (0.0)	10/76 (13.2)	1/10 (10.0)

SD: standard deviation. a: N=8, b: N=77. N/a: not applicable. Data are depicted as either mean (SD) or number (%). Diseases and intoxications are given as no./total known (%).

Secondly, we studied correlations between different senescence markers. In the Supplementary Material, correlations between different senescence markers within the short-term

Table 2. Senescence markers and their correlations between duplicate short-term experiments (1A).

	Distribution of markers		Correlation coefficient	P-value
	Experiment a	Experiment b		
Non-stressed				
p16INK4a, %	0.90 (0.45; 1.65)	1.61 (0.76; 2.71)	0.702	<0.001
TAF, %/nucleus	24.2 (16.9; 31.0)	24.4 (18.5; 32.1)	0.418	<0.001
ROS, FI	1477 (1280; 1706)	1455 (1295; 1762)	-0.111	0.354
SAβ-gal, FI	2959 (2389; 3813)	2987 (2187; 3951)	0.527	<0.001
Stressed				
p16INK4a, %	2.17 (1.10; 4.17)	4.70 (2.33; 6.48)	0.623	<0.001
TAF, %/nucleus	20.6 (14.8; 27.9)	21.9 (16.0; 26.7)	0.414	<0.001
ROS, FI	2003 (1734; 2376)	1972 (1653; 2366)	0.139	0.244
SAβ-gal, FI	4251 (3405; 5345)	4044 (3180; 5233)	0.452	<0.001

N=100. Marker distribution is given as median (IQR). Correlations are Pearson's partial correlation coefficient, adjusted for batch. All markers in experiment a were correlated with the same markers in experiment b. FI: fluorescence intensity. PD: population doublings. P16INK4a: percentage of p16INK4a positive cells; TAF (telomere associated foci): percentage of nuclei with ≥1 TAF/nucleus; ROS: mean fluorescence intensity peak reactive oxygen species; SAβ-gal: median fluorescence intensity peak senescence-associated β galactosidase.

Table 3. Senescence markers and their correlations between short-term versus long-term experiments (2A).

	Distribution of markers		Correlation coefficient	P-value
	Short-term experiment	Long-term experiment		
Non-stressed				
ROS, FI	1559 (1356; 1734)	1500 (1366; 2205)	0.419	0.010
SAβ-gal, FI	2973 (2445; 3732)	3452 (2905; 4660)	-0.009	0.959
Stressed				
ROS, FI	2095 (1753; 2324)	1835 (1553; 2205)	0.426	0.009
SAβ-gal, FI	4171 (3530; 5231)	4090 (3417; 5205)	-0.006	0.972

N=40. Correlations are Pearson's partial correlation coefficient, adjusted for batch. All mean markers of short-term experiments A and B were correlated with the same markers in the long-term experiment. FI: fluorescence intensity. ROS: mean fluorescence intensity peak reactive oxygen species; SAβ-gal: median fluorescence intensity peak senescence-associated β galactosidase.

(Supplementary Table 1) and long-term experiments (Supplementary Table 2) are given. In the short-term experiment each marker was tested against the 3 other markers, both in non-stressed (6 combinations) and stressed condition (6 combinations). Of these 12 senescence marker combinations, 6 were significantly correlated (in non-stressed and stressed conditions 3 each). P16INK4a showed the highest correlations with other markers. In the long-term experiment a total of 6 marker combinations were tested in both non-stressed and stressed conditions of which 3 senescence marker combinations were significantly correlated, mainly with ROS (2 in the non-stressed condition, 1 in the stressed condition).
In vitro senescence markers (both in non-stressed and stressed conditions) were tested for correlation with *in situ* p16INK4a positivity of dermal fibroblasts (Table 4). No significant correlations were observed between *in situ* p16INK4a positivity and any of the *in vitro* senescence markers (ROS, TAF, SAβ-gal or p16INK4a). In Figure 2, *in vitro* p16INK4a positivity in non-stressed and stressed conditions are plotted against *in situ* p16INK4a positivity of dermal fibroblasts, further showing this lack of intra-individual correlation.

Table 4. Intra-individual correlations: in vitro senescence markers versus in situ p16INK4a positive human fibroblasts (3B).

	Coefficient	P-value
Non-stressed		
p16INK4a	0.064	0.655
TAF	-0.030	0.835
ROS	-0.097	0.498
SAβ-gal	-0.042	0.772
Stressed		
p16INK4a	0.091	0.527
TAF	0.014	0.922
ROS	-0.095	0.506
SAβ-gal	0.023	0.871

Values are depicted as Pearson's partial correlation coefficient, adjusted for batch. Data for *in situ* and *in vitro* senescence markers were available for N=52 donors. P16INK4a positive dermal fibroblasts: number of positive cells per 1mm2 dermis. All *in vitro* variables are the mean of short-term experiments. P16INK4a: % of p16 positive cells; ROS: mean fluorescence intensity peak; SAβ-gal: median fluorescence intensity peak; telomere-associated foci (TAF): % of nuclei with ≥1 53BP1 foci per nucleus, coinciding with telomeric DNA.

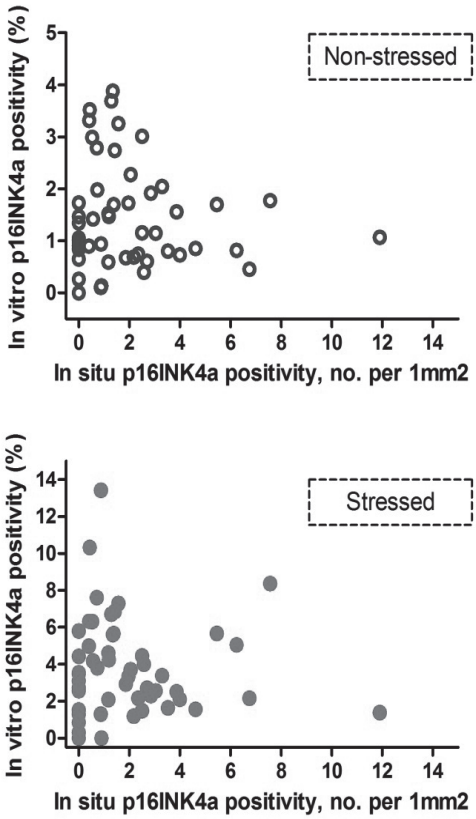

Figure 2. Intra-individual correlations: in vitro versus in situ p16INK4a positivity
Each dot represents an individual donor, N=52. *In vitro* p16INK4a positivity: percentage of p16INK4a positive cells - mean of experiments A and B. *In situ* p16INK4a positivity: number of p16INK4a positive cells per 1mm^2 dermis.

Discussion

In individual donors the same senescence markers more than half correlations were significantly correlated *in vitro* (1A) between duplicate experiments and (2A) between short-term versus long-term experiments, with high correlation coefficients. Within the experiments the different senescence markers were significantly correlated to each other, within both the short-term (1B) and long-term experiments (2B), in half of the correlations tested. On average correlation coefficients were lower than for the same markers correlations. Assessment of (3B) correlations between *in situ* p16INK4a positivity with different *in vitro*

senescence markers showed a lack of correlation, both with *in vitro* markers in non-stressed and stressed conditions.

The significant correlations of the same markers between duplicate experiments (in the short-term experiments) and between the short-term and long-term experiments indicate senescence markers are reasonably stable *in vitro* under standardized conditions. Most correlations between duplicate experiments show that the experiments were adequately reproducible, and the influence of technical issues is limited. However, ROS showed poor reproducibility between duplicates which hampers interpretation of other tested associations with ROS. Although the same markers were also correlated between the short-term and long-term experiments, this was less often the case than for the between duplicate experiment correlations. This finding is not surprising, as cell strains of an individual could respond to short-term and long-term stress differently. Indeed, in a previous study the relation between results of short-term and long-term experiments of senescence markers was also not always consistent: SAβ-gal in the stressed condition in the short-term experiment was negatively associated with the maximum replicative capacity of the strain (a long-term outcome), whereas a positive but nonsignificant trend was seen in the non-stressed condition [22].

Because senescence can be triggered in response to multiple factors and be induced through different pathways, it has been advised to use a marker of cell cycle arrest plus at minimum two senescence markers [23]. On individual cell level there is no hundred percent concordance of multiple different markers, as is the case for e.g. p16 and SAβ-gal [24], p16 and p21 [25], and γH2AX foci and p21 [26]. One of these studies also showed that SAβ-gal, senescence associated heterochromatin foci and the combination of Ki67 with γH2A.X foci were superior to other marker combinations in predicting growth curves of MRC5 fibroblast cultures [26]. A recent review [27] discussed the shortcomings of frequently used markers to assess *in vitro* senescence and particularly the difficulties of using these markers to detect *in vivo* senescence. We confirm the importance of this stance based on our results on correlations between different senescence markers. Only a half of the tested senescence marker combinations were significantly correlated within the experiment. The *in vitro* senescence marker that was most correlated to other *in vitro* senescence markers was p16INK4a. This was also the marker with the highest correlation coefficient between experiment a and b (between duplicate experiments). This stableness of duplicates could thus explain the observation that p16INK4a correlated most frequently to the other markers.

A recent review has shown that while some *in vitro* observations on fibroblast ageing have also been observed *in situ* in skin tissue, many observations have not been tested *in situ* yet [14]. To our knowledge, this is the first study in humans to directly correlate senescence markers *in*

vitro and *in situ* in cultured fibroblasts and biopsies from the same individual to assess whether both are reflective of a common (epi)genetic propensity to induce cellular senescence. In mice microRNA expression profiles were compared in cultured cells and aged mouse brains, which showed only very little similarities in expression [28]. The lack of correlation between *in vitro* and *in situ* senescence markers we have observed, was not altogether surprising. While experimental set-ups allow controlling of many variables, this also decreases the natural context of human cells. It has been observed that the process of establishing fibroblasts strains from skin biopsies itself can result in a selection of a subgroup of fibroblasts. Fibroblasts from subsequent outgrowths of single skin biopsies were shown to differ in their proliferation capacities [29]. Outgrowth from different dermal layers results in higher culture survival time in fibroblasts from the papillary dermis compared to reticular dermis [30;31]. Also, different culturing conditions were shown to have effects on replicative lifespan [32], and the process of cell culture itself has been suggested to drive some of the senescence findings *in vitro* [33;34]. We used atmospheric oxygen culture conditions which in itself is thought to be a stressor [35]. This can be seen in our scatterplot showing some individuals with high p16INK4a positivity *in situ* and low p16INK4a positivity *in vitro*, which might have resulted from selection of senescence resistant fibroblasts during expansion. Therefore, *in vitro* experimental data from cells derived from one individual might not be representative for the cell populations in their tissues under *in vivo* conditions. Due to this lack of intra-individual correlation we demonstrated here, problems might arise in extrapolating observations from *in vitro* experiments to *in vivo* implications. On the other hand, perhaps the *in vitro* characteristics of the selected subpopulation of primary cells could still reflect *in vivo* cellular capacities in specific situations, such as disease or in the presence of environmental stressors.

This study uses unique data on multiple senescence markers *in vitro* established from 100 individual fibroblast strains. We regard the high number of fibroblast strains as a strong point of this study. All culturing procedures and experiments were conducted under highly standardized conditions. A limitation of the study is that we did not include a marker for proliferation such as Ki-67. The association between *in vitro* and *in situ* p16INK4a positivity could only be evaluated in 52 subjects that had both measurements, and in a middle-aged age range. While this limited the power to detect significant associations, we could also not discern any trend for correlation. Another limitation of the present study is at the same time a limitation of many human studies in general: we have detected p16INK4a *in situ*, but cannot (yet) study cellular senescence *in vivo* in humans. Studies aiming to detect cellular senescence *in vivo* in animal models have shown that inter-individual variability is high, especially at older ages [36;37]. Inter-individual variation of senescence might also be influenced by genetic polymorphisms. In human peripheral blood T-cells, one atherosclerotic disease-related SNP was shown to associate with decreased expression

of INK4/ARF transcripts [38]. Further analysis of intra-individual correlation between *in vitro* and *in vivo* senescence associated markers within animal models could help to better explore this lack of correlation. Another limitation of our study is that for *in situ* measurements we only have data of one senescence marker, p16INK4a, whilst consensus is lacking on which (panel of) markers should be used to appropriately detect senescent cells *in situ*.

In conclusion this study shows unique data on senescence markers in human fibroblasts *in vitro* and *in situ* in human skin tissue, which can help in interpreting *in vitro* senescence results. On an individual donor level, *in vitro* markers of senescence correlated within and between experiments, but *in vitro* senescence markers and *in situ* fibroblast p16INK4a positivity were not correlated. Therefore, while *in vitro* studies on cellular senescence can provide us with valuable mechanistic insights, the validity of its use as a model to study the natural variation in human aging should be questioned and further tested. Caution is warranted when extrapolating results from *in vitro* studies towards *in vivo* implications.

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Supplementary table 1. Correlations between the different in vitro markers within the short-term experiment (1B).

	p16INK4a	TAF	ROS	SAβ-gal
Non-stressed				
p16INK4a	n/a	-	-	-
TAF	**0.253 (0.011)**	n/a	-	-
ROS	**0.321 (0.001)**	-0.048 (0.636)	n/a	-
SAβ-gal	0.151 (0.134)	0.054 (0.598)	**0.232 (0.021)**	n/a
Stressed				
p16INK4a	n/a	-	-	-
TAF	**0.227 (0.024)**	n/a	-	-
ROS	**0.299 (0.003)**	0.038 (0.706)	n/a	-
SAβ-gal	0.162 (0.109)	**0.215 (0.032)**	0.115 (0.255)	n/a

N=100. Values are depicted as Pearson's correlation coefficient (P-value), partial correlation with adjustment for batch. n/a: not applicable. P16INK4a: % of p16 positive cells; Telomere-associated foci (TAF): % of nuclei with ≥1 53BP1 foci coinciding with telomeric DNA per nucleus; ROS: mean fluorescence intensity peak; SAβ-gal: median fluorescence intensity peak. All *in vitro* variables are the mean of duplicate experiments.

Supplementary table 2. Correlations between the different in vitro markers within the long-term experiment (2B).

	ROS	SAβ-gal	Telomere shortening
Non-stressed			
ROS	n/a	-	-
SAβ-gal	**0.341 (0.042)**	n/a	-
Telomere shortening	**-0.466 (0.004)**	-0.011 (0.949)	n/a
Stressed			
ROS	n/a	-	-
SAβ-gal	**0.436 (0.008)**	n/a	-
Telomere shortening	-0.220 (0.198)	-0.030 (0.862)	n/a

N=40. Values are depicted as Pearson's correlation coefficient (P-value), partial correlation with adjustment for batch. n/a: not applicable. ROS: mean fluorescence intensity peak; SAβ-gal: median fluorescence intensity peak; Telomere shortening: percentage of shortening per population doubling

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