===== REVIEW =====

Importance and Meaning of TERRA Sequences for Aging Mechanisms

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Abstract—Any theory suggesting an adaptive meaning for aging implicitly postulates the existence of specific mechanisms, genetically determined and modulated, causing progressive decline of an organism. According to the subtelomere–telomere theory, each telomere is covered by a hood formed in the first cell of an organism having a size preserved at each subsequent duplication. Telomere shortening, which is quantitatively different for each cell type according to the telomerase regulation, causes the hood to slide on the subtelomere repressing it by the telomeric position effect. At this point, the theory postulates existence of subtelomeric regulatory sequences, whose progressive transcriptional repression by the hood should cause cellular alterations that would be the likely determinant of aging manifestations. However, sequences with characteristics of these hypothetical sequences have already been described and documented. They are the [sub]TElomeric Repeat-containing RNA (TERRA) sequences. The repression of TERRA sequences causes progressively: (i) down- or up-regulation of many other regulatory sequences; (ii) increase in the probability of activation of cell senescence program (blockage of the ability to replicate and very significant alterations of the cellular functions). When cell senescence program has not been triggered and the repression is partial, there is a partial alteration of the cellular functions that is easily reversible by telomerase activation. Location of the extremely important sequences in chromosomal parts that are most vulnerable to repression by the telomeric hood is evolutionarily unjustifiable if aging is not considered adaptive: this location must be necessarily adaptive with the specific function of determining aging of the cell and consequently of the whole organism.

INTRODUCTION

There are two completely different paradigms to explain aging, which is here precisely defined as "increasing mortality with increasing chronological age in populations in the wild" [1]:

1) In the first paradigm ("non-programmed or non-adaptive aging paradigm"), aging is the progressive overall result of many degenerative phenomena that natural selection cannot oppose [2].

Many theories are belonging to the first paradigm. The main difference between them is the assumed single or main factor responsible for aging origin. A large group of these theories, which can be defined as "damage accu-

mulation hypotheses", hypothesizes that the cause of aging includes a very heterogeneous range of factors, limited only by the lively imagination of the proposers, e.g.: "wear and tear" of the cells and/or of the whole organism, inherent changes in the specified tissues (nervous, endocrine, vascular, connective, etc.), mechano-chemical deterioration of cell colloids, toxic products of intestinal bacteria, accumulation of metabolites or of "metaplasm", effect of cosmic rays, action of gravity, accumulation of heavy water, effect of the Aristotelian "entelechy", attainment of a critical volume-surface relationship, depletion of resources linking senescence to reproduction (see [3], p. 10).

Many theories of this group were proposed in the nineteenth century or the early twentieth century, but the persisting popularity of their basic concepts is demon-

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strated by the repeated re-propositions of analogous theories, in the following years continuing still today in renewed forms in which the cause of aging is attributed to: progressive and inexorable increase in entropy [4]; accumulation of chemical damage due to DNA transcription errors [5]; deleterious effects of oxidation [6]; oxidative effects of free radicals on the whole body [7]/on the mitochondria [8]/on the DNA [5]; inflammatory phenomena ("inflamm-aging") and immunological alterations related to age [9]; etc.

A different group of theories attributable to the first paradigm considers the origin of aging as the continuity of senescence along with morphogenesis or, on the contrary, cessation of the somatic growth (see [3], p. 10).

Despite their great variety, all these theories share the fact that natural selection is ignored or disregarded in the proposed mechanisms. Therefore, natural selection is implicitly excluded as a factor capable of countering the hypothesized causes of aging.

Another group of theories also belonging to the non-adaptive aging paradigm explicitly pretends to consider the mechanisms of natural selection. In these theories, natural selection is unable to completely counter aging because it is: (i) ineffective against mutations acting late in life (*mutation accumulation hypothesis*); (ii) weakened against genes with pleiotropic effects that are favorable in the early stages of life and subsequently harmful (*antagonistic pleiotropy hypothesis*); or (iii) limited by conflicting evolutionary necessities, e.g., sharing of limited resources between reproduction and organism renewal/maintenance needs (*disposable soma hypothesis)* [10].

Within the diversified theories of non-adaptive aging paradigm, there is an idea that unites them. Aging is always and only considered as an exclusively negative phenomenon and therefore without any characteristic for which it could be favored by natural selection or other factors. Consequently, with or without explicit consideration of natural selection, the existence of aging is due to insufficiency of the factors (natural selection or others) that could counter it, while the possibility that aging is anyhow favored by something is totally disregarded.

All this could be summarized in the statement that for the first paradigm, aging is a big failure of evolution!
2) For the second paradigm ("programmed or adaptive aging paradigm"), aging, although clearly harmful to the aging individual, is advantageous, under certain conditions, in terms of supra-individual selection. This allows us to hypothesize that aging may be the result of an adaptive process, i.e., a phenomenon genetically determined and modulated with its specific physiology, phylogenesis, and pathology [11, 12].

The idea of aging as a phenomenon favored by natural selection was perceived, but without any subsequent study, by Alfred Wallace (Wallace, 1865-1870 in [13], vol. I, 1889]) and next by August Weissmann [13], vol. I, 1891]. In 1961, Aldo Carl Leopold, a botanist, first proposed, among other things, that: "...in plants senescence is a catalyst for evolutionary adaptability" [14]; "We can safely assume that there are some internal biological mechanisms which bring about decline in viability and increase in vulnerability in such populations." [14]. Hence the suggested ideas imply that, at least in plants, aging is: (i) somewhat advantageous; and (ii) necessarily determined by specific "internal biological mechanisms."

In 1988, aging was proposed as an adaptive phenomenon that was favored by natural supra-individual selection, in terms of kin selection, under particular ecological conditions (spatially structured populations and K-selection) [1]. Sixteen years later, in 2004 [15], and in subsequent years [16, 17], similar ideas were proposed by other authors who highlighted how aging could be beneficial in spatially structured populations (for the precise and detailed information on these adaptive hypotheses of aging, in order to avoid unnecessary repetitions, it is advisable to consult the mentioned works).

Furthermore, it was highlighted that aging, described as a phenomenon harmful to the individual to the point of death but advantageous in terms of supra-individual selection, is by no means something that is unique or a rare oddity. On the contrary, with the definition of the concept of phenoptosis as "programmed death of an individual" [18] and extension of the definition of this concept [19], it was pointed out that aging is one of the countless phenoptotic phenomena [18, 19], well known for some time [20] and which, among other things, shape the life tables of the species [21].

Although the non-adaptive aging thesis is still the most accepted and widespread paradigm [22-24], there are various theoretical arguments and strong evidence in support of the adaptive explanation of aging and against the opposite interpretation [10, 25]. However, it is not the subject of this work to reiterate or deepen the arguments and evidence against or in support of each of the two theses. This paper aims to focus attention on some findings that should allow clear and objective choice between the two paradigms.

As a matter of fact, fundamental distinction between the two paradigms is direct and logical consequence of their opposite ways of interpreting aging [11].

For the non-adaptive theory, since aging cannot have any physiological adaptive significance and is explained as a random cumulative effect of degenerative phenomena, it cannot be originated by hypothetical specific mechanisms shaped by natural selection and genetically determined and regulated. So, the possible existence of such mechanisms would be in clear and irremediable contrast with the non-adaptive hypothesis and therefore would make it completely untenable.

On the contrary, for the adaptive theory, the existence of specific mechanisms that cause aging, shaped by natural selection and genetically determined and regulated, is a necessary and essential prediction. Furthermore,

the existence of such mechanisms is an indispensable condition for the tenability of the thesis.

It is therefore clear that critical and decisive discrimination between the two theories based on empirical findings would be the existence or non-existence of such mechanisms. All this without excluding the possibility of future evidence for other theories, e.g., regarding the pleiotropic genes predicted by the antagonistic pleiotropy theory and of which there is currently a lack of evidence. This could be an explanation for the aforesaid mechanisms, giving new strength to the theories that do not assume an adaptive nature of aging.

AGING MECHANISMS ACCORDING TO THE SUBTELOMERE–TELOMERE THEORY

Within the programmed aging paradigm, the possible mechanism that appears much better supported by scientific studies is the one described by Fossel in 2004 [26] (in particular, chapter 3), and afterward deepened and defined as "subtelomere–telomere theory" [27]. In this paper, possible alternative interpretations of aging mechanisms in the context of the adaptive aging paradigm (see [28]) will not be considered because they seem to be much less justified by empirical findings. The subtelomere–telomere theory is explained in detail in other works [11, 26, 27, 29, 30] and here only a summary will be presented.

Hayflick and Moorhead, showed that normal somatic cells have limited duplication capacity contrary to the previous long-term beliefs [31]. Regarding the causes of this limit ("Hayflick limit"), in 1971 Olovnikov observed that the enzyme able to duplicate the DNA molecule (DNA polymerase) did not replicate a small terminal part of the molecule, the "telomere". This incomplete duplication should cause progressive shortening of the terminal part of the DNA molecule and could explain the Hayflick limit [32].

Two years later, Olovnikov himself observed that incomplete duplication was compensated in some cases by a specific enzyme, which explained the unlimited or greater duplication capacity of germline cells and stem cells, respectively [33]. This enzyme (telomerase) was found in 1985 [34] and its activity turned out to be under genetic regulation [35].

During the same period, the telomere was shown to be composed of a fixed sequence repeated several times [36], and that this motif was TTAGGG for humans [37] and for many other species [38]. Furthermore, it was discovered that the sequence is constant in each species and strongly conserved in the course of evolution. For example, the TTAGGG motif is present in all vertebrates and in many phylogenetically distant invertebrate species and a slightly different motif (TTAGG) is present in many other species (Telomerase Database, telomerase.asu.edu/sequences_telomere.html, accessed February 14, 2020).

A simple hypothesis based on these findings seemed plausible. In the absence of telomerase activity, telomere is shortened at each duplication, and when it reaches a critical length, the duplication capacity is exhausted, and, as a side effect, cellular functions are altered. Furthermore, the full or partial activity of telomerase allows unlimited or greater duplication capacity of the germline cells and stem cells, respectively. This hypothesis was, however, contradicted by the fact that the synchronized cell cultures (i.e., cells with an equal number of duplications) showed a progressive reduction of growth potential starting from the first duplications. This implied that even for cells with very little reduced length telomeres, there was a stochastic transition from a "cycling state" (duplication possible) to a "noncycling state" (duplication impossible) [39, 40]. To solve this contradiction, a model that could explain the experimental results was proposed by Blackburn [41]. According to this model, the telomere randomly oscillates between two states: (i) "capped", when it is protected by a particular nucleoproteinic hood (not better defined); and (ii) "uncapped", when this hood does not protect the telomere. Furthermore, Blackburn hypothesized that the balance between the capped/uncapped state is affected by the length of the telomere, i.e., the more telomere is shortened the more telomere is in the uncapped state, which makes it vulnerable to the transition to the "noncycling state".

Another phenomenon needing explanation was the so-called "telomeric position effect", i.e., in yeast (*Saccharomyces cerevisiae*), the repressed nucleotide sequences associated with telomere shortening are located in the immediate proximity of the telomere [42] (a phenomenon afterward reported for other species [43], humans included [44], and confirmed to be related to telomere shortening [45]).

Furthermore, another interesting phenomena similar to the "telomeric position effect" were found in yeast. In this single-celled species, telomerase is always fully active and, therefore, telomeres do not shorten at each duplication [46]. In yeast, the mother cell divides into two slightly different cells: (i) the first, defined as a "mother", shows presence of extrachromosomal ribosomal DNA circles (ERCs) in the immediate proximity of the telomere; and (ii) the second, defined as a "daughter" (or "budding") cell, shows no presence of ERCs.

As duplications continue, the daughter cells show unlimited duplication capacity [47], while in the cells of the mother lineage there is a progressive accumulation of ERCs [48], progressive decline in the ability to withstand stress with growing vulnerability to apoptosis [49, 50], as well as these cells exhibit limited number of possible duplications (25-35 duplications [51]). Regarding the decline of the mother lineage cells, it has been reported that "several lines of evidence suggest that accumulation of ERCs is one determinant of life span" (i.e., the greatest number of duplications), and increasing metabolic

alterations occur proportionally to the number of duplications [52].

Moreover, in the specific yeast strains with defective telomerase (*tlc1Δ* mutants), cells of both mother and daughter lineages show telomere shortening at each duplication. Individuals of daughter cell lineage, although they have no ERC accumulation as wild-type strains, have a transcriptome similar to that of the normal individuals of the mother lineage with the same number of previous duplications [52].

This appeared to indicate that while in the wild-type strains ERCs repressed subtelomeric DNA, in the *tlc1Δ* mutants' repression of the subtelomeric DNA was caused by the sliding of the telomere heterochromatin hood related to telomere shortening.

An interpretation of these phenomena was proposed by Fossel: "... a heterochromatin "hood" ... covers the telomere and a variable length of the subtelomeric chromosome... As the telomere shortens, the hood slides further down the chromosome (the heterochromatin hood remains invariant in size and simply moves with the shortening terminus) ... the result is an alteration of transcription from portions of the chromosome immediately adjacent to the telomeric complex, usually causing transcriptional silencing, although the control is doubtless more complex than merely telomere effect through propinquity... These silenced genes may in turn modulate other, more distant genes (or sets of genes). There is some direct evidence for such modulation in the subtelomere..." [26], p. 50.

The progressive alteration of cellular functions in relation to telomere shortening, "telomeric position effect" in a certain sense, was defined in a closer examination of what Fossel proposed, perhaps more precisely for its consequences, as "gradual cell senescence" [10].

In the same years in which Fossel suggested his hypothesis, Blackburn's transition to "noncycling" state [41] was described as a specific cellular "program" that determined the condition defined as "cell senescence" [53], characterized by the block of duplication capabilities and profound changes in cellular functions ("dramatic changes in chromatin structure and gene expression") [53], and also by "apoptosis resistance, and frequently acquisition of a pro-inflammatory, tissue-destructive senescence-associated secretory phenotype (SASP)" [54].

In vertebrates, many cell types show a continuous turnover with distinct rhythms [55], due to a balance between various types of programmed cell death (e.g.: (i) keratinization and detachment of cells from epidermis and hair; (ii) detachment of cells from mucosae of body cavities; (iii) apoptosis and duplication of the specific types of stem cells. While in younger individuals the balance appears to be optimal, for older subjects' substitution of the lost cells becomes progressively slower and incomplete. This declining cell turnover together with the increasing number of cells with altered functions, due to
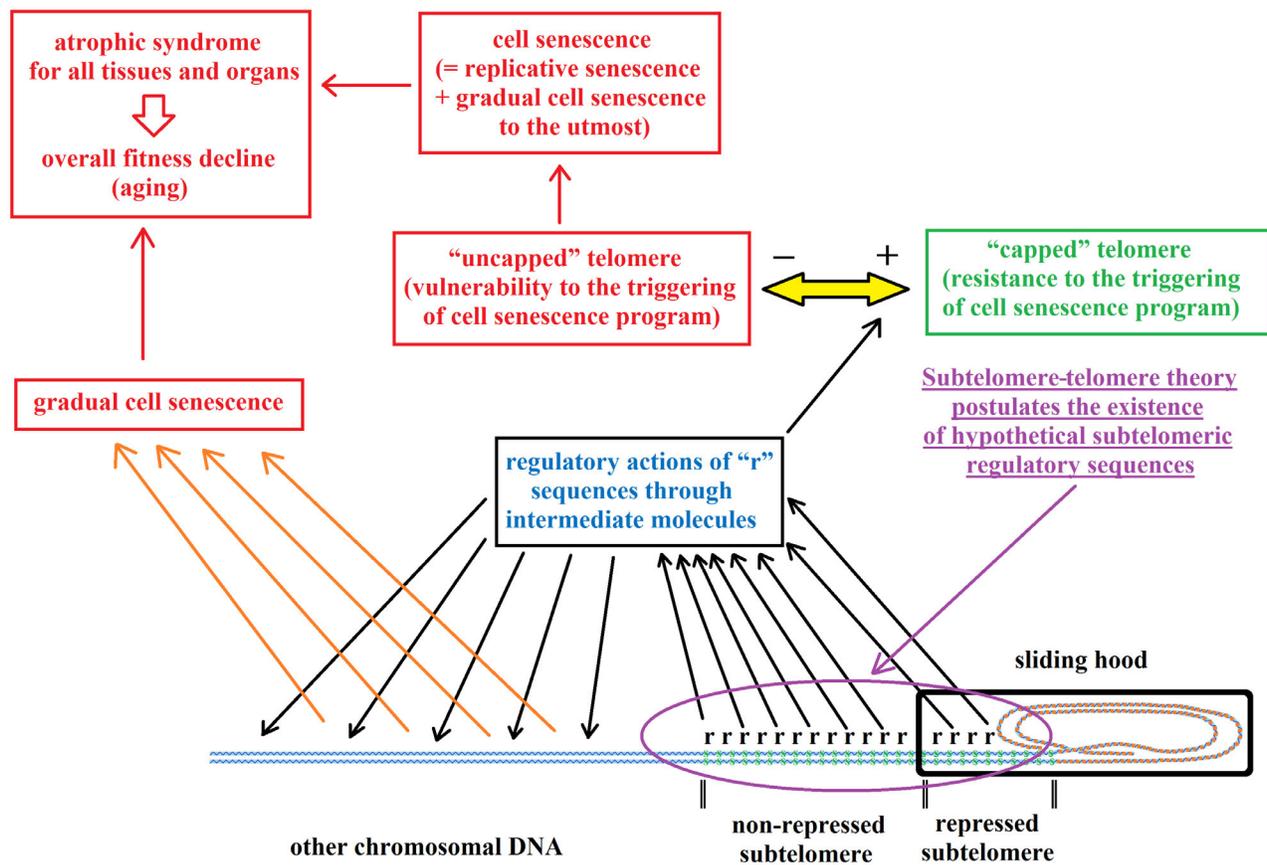
cell senescence or gradual cell senescence, determines a condition defined as "atrophic syndrome" [11] with:

"1) reduced mean cell duplication capacity and slackened cell turnover;

2) reduced number of cells (atrophy);

3) substitution of the missing specific cells with non-specific cells;

4) hypertrophy of the remaining specific cells;

5) altered functions of cells with shortened telomeres (gradual cell senescence) or complete transformation into noncycling state (cell senescence);

6) alterations of the surrounding milieu and of the cells depending on functionality of the senescent or missing cells;

7) vulnerability to cancer because of dysfunctional telomere-induced instability..." [29].

A possible objection for this general explanation of aging manifestations could be the seemingly inadequate explanation of aging for tissues or organs in which the main cells are perennial, i.e., without a turnover, including the central nervous system, retina, organ of Corti, and crystalline lens. For this question, the answer, which is intrinsic to point (6) in the above-reported description of the atrophic syndrome, is that aging of perennial cells is a consequence of the decline of the respective satellite or trophic cells. This topic has been discussed in detail in other works [11, 29, 56, 57] and for brevity will not be repeated here.

As for Fossel's hypothesis regarding the fixed size of the "hood" that protects the telomere ("... the heterochromatin hood remains invariant in size and simply moves with the shortening terminus..." [26], p. 50]), it is more precisely described by saying that, in the first cell of an organism, for each telomere, a hood with a size proportional to telomere length is formed, and that the size of each hood remains unchanged in all subsequent cellular duplications. As a consequence of the fixed size of the hood, the phenomena of subtelomere repression are related not to the initial length of the telomere and of the relative hood, but to the telomere shortening and the consequent relative sliding of the hood on to the subtelomeric region. The hypothesis of the fixed length of the hood for each telomere appears indispensable to explain many following phenomena (note that the points <a>, <b>, and <c> already have been discussed in detail elsewhere [30]):

a) Absence of correlation between the telomere length and life span when different species are compared [58];

b) Similar absence of correlation between individuals of the same species (e.g., (i) between donor and cloned animal [59]; and (ii) between two mouse strains with different telomere length [26], p. 60);

c) Subtelomeric repression in cells of daughter lineage of yeast *tlc1Δ* mutants (see before);

d) Considering that in each cell there are many telomeres (the number of telomeres, *n*, is equal to the

Fig. 1. Diagram of subtelomere−telomere theory. Figure inspired by Fig. 3 in [56]. Progressive repression of the hypothetical "r" sequences is suggested to cause gradual cell senescence and increasing probability of cell senescence. (Colored versions of Figs. 1-4 are available in online version of the article and can be accessed at: https://www.springer.com/journal/10541)

number of DNA molecules − two for each chromosome − multiplied by two − the number of ends of a DNA molecule − and so, in a human cell, $n = 23 \times 2 \times 2 = 92$), and that telomere length varies from chromosome to chromosome and also from end to end of the same DNA molecule [60], the fixed size of the hood, proportional to the initial telomere length, would avoid differences in the degree of telomere repression and the possibility that the aging of the entire cell could be conditioned by the length of the shortest telomere;

e) When telomerase restores telomere length, it is indispensable for each telomere to have a precise marker of the previous length: the hood, which is hypothesized to have a size that is fixed and proportional to the initial telomere length, could be this precise marker.

These concepts, which briefly describe the subtelomere−telomere theory, are represented in Fig. 1. The main point, which must be highlighted for the subsequent discussion, is that according to this theory the hood that covers the telomere slides on the subtelomeric sequence as a result of telomere shortening and inhibits hypothetical sequences ("r" in the figure), which should produce a general regulatory effect on the cellular functions. Existence of these postulated regulatory subtelomeric

sequences is essential for tenability of the theory but no direct empirical proof of their existence was proposed and has been reported yet.

We are therefore faced with a case that is frequent in scientific research. On the basis of empirical evidence, a theory is constructed which hypothesizes phenomena that must be confirmed or disapproved by the appropriate research. Experimental confirmation of the theory validates and reinforces it, while lack of confirmation invalidates the theory or at least makes it unsustainable in the proposed form.

TERRA SEQUENCES

Although the extent of subtelomeric regions is not precisely defined in the works cited below, signs of the great importance of these parts of DNA molecules for cellular functions and data compatible with the possible existence of the hypothesized "r" sequences has been known for some years:

− In humans, subtelomeric regions show a mosaic of multiple common sequences (more than 40 types), with various open reading frames [61-63].

— Subtelomeres show an "...unusual structure: patchworks of blocks that are duplicated..." [64], with "...long arrays of tandemly repeated satellite sequences" [65]. These characteristics have been observed in numerous animals and plants [66].

— "Human subtelomeres are polymorphic patchworks of inter-chromosomal segmental duplications at the ends of chromosomes... Cytogenetics and sequence analyses reveal that pieces of the subtelomeric patchwork changed location and copy number during primate evolution with unprecedented frequency. Half of known subtelomeric sequence formed recently through-human-specific sequence transfers and duplications. Subtelomeric dynamics result in a gene-duplication rate significantly higher than the genome average and could have both advantageous and pathological consequences in human biology" [67].

— In human subtelomeres, "of the 20.66 Mb of subtelomeric DNA analyzed, 3.01 Mb are subtelomeric repeat sequences (Srpt), and an additional 2.11 Mb are segmental duplications" [68].

— "The highly variable subtelomeric repeat regions are filled with recently shuffled genomic segments, many of which contain sequences matching transcripts and transcript fragments; the rapid duplication and combinatorial evolution of these regions has generated an extremely diverse set of subtelomeric alleles in the human species, the complexity and potential significance of which is only beginning to be understood" [61].

— Subtelomeric regions have a low gene density and the total of single-copy subtelomeric genes, in humans, is not more than three hundred [62, 68].

— Subtelomeric sequences are important for pivotal cellular activities, such as interactions with processes of ion transportation and uptake of nutrients [69-72], and cell cycle regulation [61, 73].

— "All this taken together underlines the important role of subtelomere, able to function as a factor, which optimizes the work of different cellular systems." [74].

However, all these data by no means represent a proof of the existence of the postulated "r" sequences, while other empirical findings, which are in part quite recent, show subtelomeric sequences that have all the characteristics required for their identification as "r" sequences:

— "The pro-terminal DNA sequences associated with the long-arm telomeres of human chromosomes X/Y (Xq/Yq) and 10 (10q) were isolated nearly 20 years ago and named TelBam3.4 and TelSau2.0, respectively (Brown et al., 1990). The two sequences share a conserved repetitive region that extends for about 1.6 kb (nucleotides 2110-3117) and about 1.3 kb (nucleotides 408-1789) until about 280 nucleotides (nt) upstream of the terminal array in TelBam3.4 and TelSau2.0, respectively... This conserved region contains three different repetitive DNA tracts: the most centromere-proximal tract comprises tandemly repeated 61-base-pair (bp) units (five repeats in TelBam3.4 versus six repeats in TelSau2.0); a second, more distal tract comprises 29-bp tandem repeats (nine repeats versus 18 repeats); a third tract comprises five tandemly repeated 37-bp DNA units in both sequences... We refer to the tandem repeat-containing region as "61-29-37 repeats" and to the about 280 nt located between the last 37-bp repeat and the telomeric hexamers as *pre-tel*" [75].

— The first report about the transcription of subtelomeric/telomeric sequences was published in 1989 for *Trypanosoma brucei* [76] and in 1994 for birds' lampbrush [77]. These sequences, defined as TElomeric Repeat-containing RNA (TERRA), have been described in humans [78, 79], mouse [79], Zebrafish [79], plants [80], and yeast [81, 82]. It has been highlighted that TERRA sequences are evolutionarily conserved in vertebrates [83].

— "The first human subtelomeric promoters that were identified comprise CpG dinucleotide-rich DNA islands shared among multiple chromosomes ends [75]. These CpG islands are characterized by the presence of the so-called 61-29-37 repeats located directly upstream of TERRA Transcription Start Site (TSS) and at ~1 kb from the telomeric tract...".

— In humans, a TERRA transcript originates from a specific subtelomeric sequence, which is adjacent to the repeated motif of the telomere, and from some of these motifs [84] (TTAGGG in human DNA, which become UUAGGG in the transcripts, as T is substituted by U in any transcripted RNA). The length of TERRA transcripts depends on the length of the subtelomeric TERRA sequences [85, 86]. Since TERRA sequences are mainly composed of subtelomeric sequences, for their definition it would be more accurate to say subTElomeric Repeat-containing RNA.

— In mammals, TERRA transcripts are observed only in nuclear cellular fractions, show telomeric 5′-UUAGGG-3′ RNA repeats, and have a length that may go from about 100 bases up to more than 9 kilobases [78, 79]. Production of the TERRA transcripts is mediated by the action of RNA polymerase II, which starts from the subtelomeric DNA and proceeds toward the telomeric repeated motif, including some of them in the transcription [78, 79].

— TERRA sequences, which are long noncoding DNA sequences, constitute a general feature of eukaryotic cells and "are emerging as new key players in several important biological processes" [84]. TERRA transcripts originate from the subtelomeric promoters located on at least two-thirds of chromosome ends [75, 87-90].

— Binding sites of the TERRA transcripts are found outside of subtelomeres and telomeres (mostly in intronic and intergenic sections of the genome). TERRA transcripts appear to have a pivotal role in the regulation of gene expression. Depletion of the TERRA transcripts in

the mouse embryonic stem cells was shown to be associated with the reduced telomere protection [91, 92]. "TERRA read coverage was high within subtelomeric regions of nearly all chromosomes (Chr), most prominently Chr. 2, 9, 13, 18, and the sex chromosomes, with targets being as much as tens of kilobases away from the telomeric repeat... TERRA also bound within internal chromosomal regions and within genes, where it favored introns... TERRA binds chromatin targets throughout the genome. ...TERRA binds both in *cis* at telomeres and in trans within or near genes." [91]. There is evidence of "...significant changes in expression of TERRA targets relative to non-targets after TERRA depletion..., indicating that TERRA target genes were more likely to be affected by TERRA depletion... Interestingly, subtelomeric target genes were consistently downregulated... Internal target genes could either be up- or down-regulated... In the mouse ES [embryonic stem] cell genome, we identified thousands of cis and trans chromatin binding sites" [91]. "TERRA binds to many genomic loci outside telomeres where the noncoding DNA appears to play important regulatory functions related to gene expression..." [84] "The vast majority of TERRA-binding sites were found outside of telomeres, mostly in distal intergenic and intronic regions of the genome, where TERRA regulates gene expression. Importantly however, TERRA depletion in ES (embryonic stem) cells was also associated with telomere deprotection, suggesting that TERRA is nevertheless important for mouse telomeric integrity" [84].

— Cycling endurance exercise increased the levels of TERRA transcripts in skeletal muscle biopsies obtained from healthy young volunteers. This supports the idea that exercise is an anti-aging factor [89].

These data show that TERRA sequences:

(i) are widespread among eukaryotic species and evolutionarily conserved;

(ii) are documented in the form of two sequences (TelBam3.4 and TelSau2.0), although other sequences are likely existing;

(iii) are placed in the area of DNA molecule subject to repression in correlation with telomere shortening (telomeric position effect);

(iv) originate transcripts that have regulatory actions on sequences placed in the adjacent segment of the DNA molecule (up-regulation) and on sequences placed in other parts of the DNA (up- or down-regulations)

(v) perform actions described in the previous point (iv) on all cellular DNA molecules and not only on the one in which each of them is present;

(vi) determine greater stability of the telomere–heterochromatin hood complexes and therefore lower probability of triggering cell senescence program;

(vii) when repressed, cause general alteration of cellular functions.

Therefore, TERRA sequences characteristics perfectly coincide with those of the hypothesized "r"

sequences. Furthermore, the reported data allow to distinguish and define three distinct segments in the terminal DNA parts, which are (starting from the end of the molecule):

— telomere proper (consisting of the monotonous repetition of a motif);

— regulatory subtelomere ("subtelomere R"), where TERRA sequences with their general regulatory functions are present and can be gradually repressed in correlation with telomere shortening;

— amplifier subtelomere ("subtelomere A"), where there are a series of sequences with multiple regulatory functions, up-regulated by the action of the TERRA sequences and therefore with lower activity when TERRA sequences are repressed. These sequences amplify and alter the regulatory actions of TERRA sequences.

Sequences that are in other parts of the chromosome and are up- or down-regulated by the TERRA transcripts are not part of the above-said segments.

These concepts are summarized in Fig. 2, which represents a re-designed version of Fig. 1. The main difference between the two figures is substitution of the hypothetical "r" sequences with the real TERRA sequences.

Some characteristics of the above described mechanisms deserve some brief clarification:

Transcriptional repression of TERRA sequences.

— In mice, telomere shortening affects epigenetic status, i.e., methylation pattern of subtelomeric DNA, and determines modifications of histones for both subtelomeres and telomeres [93]. "Furthermore, the abrogation of master epigenetic regulators, such as histone methyltransferases and DNA methyltransferases, correlates with loss of telomere-length control, and telomere shortening to a critical length affects the epigenetic status of telomeres and subtelomeres" [93].

— In human leukocytes, "...shorter telomeres are associated with decreased methylation levels of multiple cytosine sites located within 4 Mb of telomeres... significant enrichment of positively associated methylated CpG sites in subtelomeric loci [was observed] (within 4 Mb of the telomere) ($p < 0.01$)" [94]. This causes modifications in the gene expression profile and increase of the risk of age-related diseases [94].

— In Terc($-/-$) mice, the methylation of subtelomeric DNA appeared decreasing in relation to telomere shortening [95].

— "Both healthy controls and sarcoidosis patients showed that long telomeres (>9.4 kb) decrease and short telomeres (<4.4 kb) increase with aging, accompanying relative increases of long telomeres with subtelomeric hypermethylation and short telomeres with subtelomeric hypomethylation. This suggested that the aging-related telomere shortening is associated with the surrounding subtelomeric hypomethylation" [96].

These empirical findings appear to indicate that: (i) subtelomere is subject to methylation and demethylation

activities by specific enzymes; (ii) longer telomeres are associated with greater levels of methylation, and vice versa; and, therefore, somehow (iii) the hood interferes with subtelomere methylation thus repressing TERRA sequences.

Structure of the heterochromatin hood. Structure of the hood that covers and protects telomere is unknown. However, it is possible to suggest a working hypothesis, starting from the fact that the telomere is protected by groups of proteins, defined as shelterin complexes [97] (Fig. 3).

The adapted picture of a telomere protected by shelterin complexes, where the POT1 protein locks onto the end-point of the telomere and appears tilting [98] (Fig. 4, upper part) suggests that the hood might be composed of a series of shelterin complexes hooked together by the POT1 protein (Fig. 4, lower part). This hypothesis is sup-
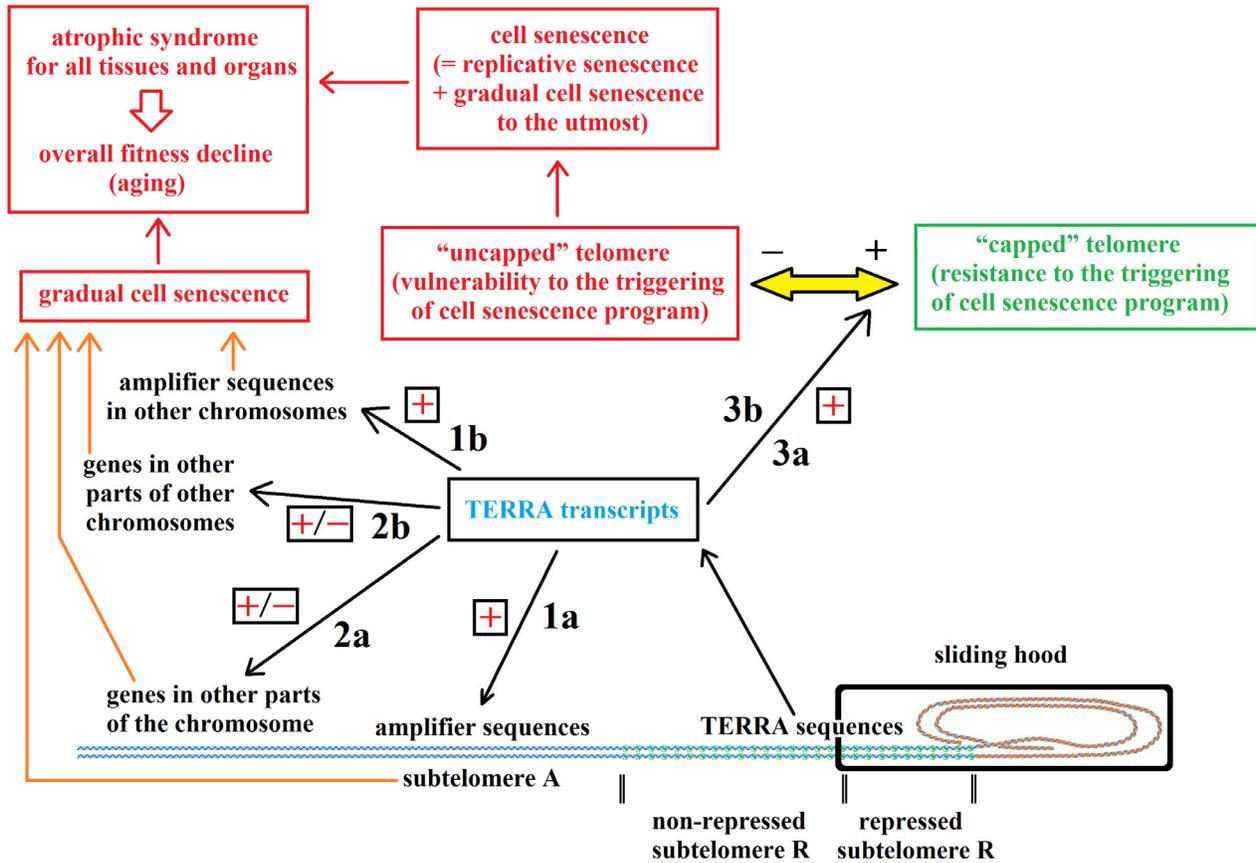
Fig. 2. Diagram of subtelomere–telomere theory (redesigned Fig. 1): 1) actions on near amplifier sequences; 2) actions on non-near amplifier sequences; 3) actions on the equilibrium capped/uncapped telomere. Actions are: a) on the same DNA molecule; and (b) on other DNA molecules of the same cell; and can be: (+) up-regulations; or (−) down-regulations. The progressive repression of TERRA sequences causes a reduced action of TERRA transcripts and, hence, progressive gradual cell senescence and increasing probability of triggering cell senescence program.
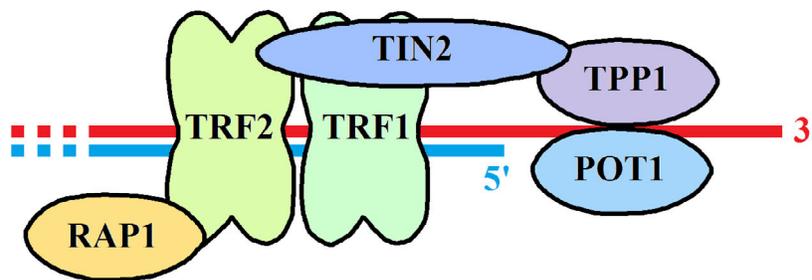
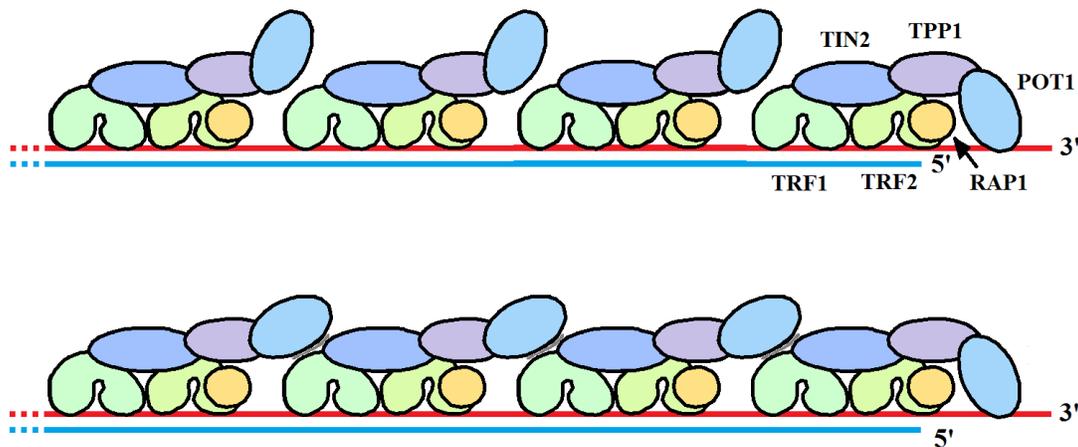**Fig. 3.** A diagram of shelterin complex (adapted from [97]).

Fig. 4. Upper part: inspired by Fig. 1-B in [98]; lower part: possible structure of the hood.

ported by the fact that: "Telomere uncapping through either TRF2 shelterin protein knockdown or exposure to telomere G-strand DNA oligonucleotides significantly increases the transcription of TERRA..." [99].

Repression/non-repression by the hood of the adjacent subtelomere. It is unknown how the hood inhibits the adjacent subtelomere. However, it seems unlikely to suggest complete detachment of telomeres from their hoods as this would lead to problems for the subsequent exact re-pairings. The two ends of each hood may oscillate between the close contact with the DNA molecule and partial separation from it allowing subtelomere methylation in this second state on one end, and access of telomerase and of the protein-enzymatic complexes that determine DNA molecule duplication on the other end.

Formation of the hood and its replication with the same size at each duplication. These topics are unexplored. However, they obviously are critical for better understanding of the subtelomere−telomere-heterochromatin hood-telomerase machinery.

Activation of the cell senescence program. Triggering of the cell senescence program is correlated with the telomere shortening but how this happens is unknown. Activation of the cell senescence program could originate from one single hood−telomere complex, when complete detachment of the hood from the telomere occurs. Perhaps, somehow this leaves the telomere permanently uncovered and then stimulates the blockage of the other telomeres.

CONCLUSION

Subtelomere (or, more precisely, subtelomere R) has general regulatory sequences, the TERRA sequences, whose repression determines numerous cellular alterations that are manifestated by the macroscopic signs of aging. Due to their position, these sequences are subject to the telomere position effect, i.e., their repression when the telomere shortens. Such vulnerable position for extremely important sequences is difficult to explain without accepting the thesis that aging is adaptive.

The phenomena described within the subtelomere−telomere theory (limitations in telomerase activity, gradual cell senescence, cell senescence, TERRA sequences, etc.) do not appear at all justifiable as a general defence against cancer, as proposed by some supporters of the non-adaptive aging thesis, for the reasons already discussed in other works [11, 25, 30, 100]:

a) In a human population studied in the wild, many died because of the progressive age-related decline to resist various noxae (age-related fitness decline, aging), while no case of cancer was described (although some rare cases of death in elderly subjects might be due to cancer). Similarly, in animal populations in the wild, cancer cases are very rare while there is an age-related progressive mortality increase (aging), which contributes to the majority of deaths. If the mechanisms underlying the progressive increase in age-related mortality, whatever they are, were a defense against cancer, we would have the absurdity that a hypothetical defense against a rare cause of death kills most of the population.

b) "Young" and "old" individuals of species showing no detectable age-related fitness decline ("animals with negligible senescence"; e.g., rainbow trout and lobster) have in the wild the same telomerase activity without any age-related increased vulnerability to cancer, as it is shown by their constant mortality at any age.

c) When telomeres are shortened, this causes DNA instability and increases probabilities of the cancer onset.

d) Progressive decline of cellular functions related to telomere shortening (gradual senescence) is an implausible defense against neoplastic cell proliferation.

e) In mice, selective elimination of the senescent cells prevents several age-dependent changes, delays pro-

gression of the cancer diseases and increases longevity. Moreover, the secretory phenotype of senescent cells has oncogenic actions. This contradicts the suggestion that the senescent cells are a defense against cancer.

f) Gradual senescence and cell senescence progressively weaken immune system efficiency increasing vulnerability to cancer and cancer incidence.

g) In the "mother" lineage of eukaryotic unicellular species such as yeast, phenomena as replicative senescence, apoptosis, and overall decline of cellular functions related to the number of duplications (caused by ERC accumulation and not by telomere shortening), are known and defined as aging. They cannot be a defense against cancer, which is impossible in unicellular species.

According to the subtelomere–telomere theory, the repression of regulatory sequences of fundamental cellular importance is a pivotal part of the aging mechanisms and so TERRA sequences and their transcripts appear to constitute the core of aging mechanisms. Their existence and actions show clear evidence of the reality of genetically defined and modulated mechanisms that could determine aging.

However, it is essential to confirm the hypotheses proposed about the hood (first of all, its fixed length and how this length is preserved at each duplication) before we can consider the overall subtelomere–telomere theory as acceptable or corroborated. Furthermore, before this possible confirmation, it is important and necessary to carefully consider alternative hypotheses − in particular the antagonistic pleiotropy theory and the disposable soma theory − as potential explanations for the role of TERRA sequences, even if these theories also require evidence.

Ethics declarations. The authors declare that they have no conflict of interest in financial or any other sphere. This article does not contain any studies with human participants or animals performed by any of the authords.

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