DROSOPHILA MELANOGASTER CONTROL MECHANISMS OF TELOMERE LENGTH MAINTENANCE AND IME4 PUTATIVE ROLE IN THE RETROTRANSPOSONS REPLICATION AND TELOMERE ELONGATION

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Summary: Senescence occurs in every living organism. Revolutionary studies of McClintock, Blackburn and Szostak led to the discovery of telomeres, telomerase enzyme complex and telomere shortening-related aging processes. Current scientific knowledge brings the scientists closer to the recognition of the basic courses and processes behind organisms' senescence, although this advance seems to be more regressive than progressive in modern days when multiple studies discover new possibilities and bring even more questions than answers. *Drosophila melanogaster* is used as a good model of organism that represents genomic, proteomic and epigenetics behaviors very close to the higher organisms, including primates and humans. Though the telomere structure in *D. melanogaster* differs distinctly from other organisms (Arthropods' telomeres stay as an exception to the repeated, palindromic DNA telomeric sequences found in other eukaryotes), conserved nature of *IME4* and similar cellular involvement in the telomere maintenance may answer the question whether mRNA methyltransferases (human's homolog of *IME4* is MTA-70) can actively participate in telomere elongation. This review focuses on genetic aspects of *D. melanogaster* telomere maintenance and presents hypothetical approaches that could be utilized in the fruit flies telomeres experiments, and also indicates conjectural application of the knowledge in the human aspect of gerontology.

Key words: Drosophila, *dm IME4*, telomere length maintenance, ageing

INTRODUCTION

In multicellular world of animals cells decide about everything what happens in an organism as a whole structure. In 1912, French surgeon and biologist Alexis Carrel, established and grew a culture of heart fibroblasts of a chicken in his

own laboratory; the cells were growing incessantly for the following 34 years. This gave the scientific world a bright insight into cell immortality, and Carrel described that aging is "an attribute of the multicellular body as a whole" [68]. It is generally shared knowledge that each eukaryotic cell has repeated, in tandem or dispersed form, sequences not being expressed, but acting as a specific protection cap [6]. The term *telomere* owes its name to Hermann Muller and Barbara McClintock who described thoroughly the ends of human chromosomes (*telos* from the Greek means "end" and *meros* stands for "part"). The true revolution in telomeres genetics began with studies by Elizabeth H. Blackburn, Jack W. Szostak, and Carol W. Greider, awarded the Nobel Prize in 2009 by their contribution in newly emerging branch of biomolecular science which today is known as gerontology. Blackburn and Szostak mapped the repetitive sequences of the ends of protozoan organism' cells (*Tetrahymena thermophile*) and presented to the world the sequence being a hexametric scheme: CCCCAA/GGGGTT [2]. In the following decades Hayflick, Greider, Blackburn and de Lange's researches led to the discovery of one of the most important enzymes (enzyme complexes) in the living world – telomerase [15, 7, 69]. Telomerase is a reverse transcriptase that extends and maintains the length of a telomere and thereby increases the number of divisions of one cell. Maintenance of telomeres is important because of their role in genome stability and replication problem [20, 25]. These are participation in the last stage of replication, preservation from homologous recombination and non-homologous replication, protection from inexpedient activity of exonucleases, and, what was the highlighted issue of the past few years, telomeric looping effects on proximal gene expression patterns [71, 17, 58]. Scientists have no doubts – telomeres are important part of genome homeostasis in eukaryotic cells.

However, not all *Animalia* possess telomeres in the context of short repeated nucleotide sequences. According to the definition, telomeres are short tandem repeated sequences of DNA found at the ends of linear eukaryotic chromosomes [50]. Telomeres shortening occurs in many phyla of *Animalia,* including *Taeniopygia guttata, Tachycineta bicolor, Pygoscelis adeliae, Oceanodroma leucorhoa*, and even Echinodea [65, 32]. Generally speaking, with some exception of Nematodes and Arthropods, $(TTAGGG)_n$ sequence is conserved in most Metazoa [70]. What is even more, some plants exhibit specific telomere sequences which also undergo shortening processes during cell cycle M phase [30]. It is not well known why some Arthropods and Nematodes lack TRFs (Telomere Restriction Fragments) at the end of their chromosomes, but the structure of some of them are nowadays well recognized and systemized. In this article, *Drosophila melanogaster* in considered as one of the examples of eukaryotic organisms deprived from telomere tandem repeats and here are presented some assumptions focusing on epitranscriptomics aspects of telomere maintenance in *D. melanogaster* and the control of the biomolecular mechanisms.

RETROTRANSPOSONS IN THE FLIES' TELOMERES

Drosophila melanogaster telomeres are unusual because of three different retrotransposons arrays comprising the fly's end of chromosomes and protecting them before deleterious harmful agents and cellular processes [39, 4, 46, 45]. These are HeT-A, TART (divided into three groups TART A, TART B, TART C) and newly discovered TAHRE [66]. All three families of the retrotransposons belong to non – LTR retrotransposons. In general, there are described two types of transposable elements: class I (or I TE) functioning through reverse transcriptase and class II (or II TE), called also DNA transposons, which use transposase (the cut-and-paste transposition). I TE is consisted of two subgroups, i.e. LTR retrotransposons and non-LTR retrotransposons. LTR retrotransposons (Long Terminal Repeats retrotransposons) with long repeats flanking internal coding region, and further divided into Ty3-*gypsy*-like, Ty1-*copia*-like, and BEL-Pao-like groups. In contrast, non-LTR retrotransposons do not possess repeated sequences at their coding end, are much longer than LTR retrotransposons and usually contain one or two open reading frames (ORFs) [24].

All three retrotransposable elements of *D. melanogaster* share common features and sequences, which suggests that all are conserved and come from the same ancestor. All three are found in the end of telomeres, but not centromeric and other euchromatin regions. Also, all three have ability to replicate by reverse transcriptase system and are influenced by telomere-associated sequences (TASs), sources of telomere position effect (TPE) [13,34,21]. What is even more, all three retrotransposons have very long 3' untranslated regions (UTRs) that sometimes encompass half the elements (having in mind that retrotransposons like *HeT-A* may be as long as 12 kb) [4]. It is believed that 3'-UTRs serve as stabilizing factor for *Drosophila* in the similar manner as G-quadruplex structures, formed of shelterin proteins [16], in other animals act [4]. TAHRE element has not been yet thoroughly examined, but HeT-A and TART retrotransposons are composed of tandem head-to-tail arrays with their truncated 5' ends oriented towards the terminus [39]. What is even more interesting, around 600 bp away from 3'UTR from HTT (HeT-A, TART, TAHRE sequences assay) is found promoter that carries unidirectional transcription from 3'end to 5' end, initiating with 3' oligo(A) tails needed for target-primed reverse transcription [21]. Using 3' terminus to prime reverse transcription seems reasonably, because if transcription started at 5' end of the transcript, these telomere elements would not be able to form a junction at the 5'end resulting in a loss of the part of chromosome distal to an internal insertion. The process of transcription is mainly found in HeT-A regions of telomeres, and HeT-A transcripts are in abundance in comparison to TART and TAHRE elements[51]; nowadays it is established that HeT-A/TART arrays form around 75,943 bp on the end of 4R and 19,199 on XL, with complete constitu-

tion of HeT-A retrotransposable elements of 61.4% (four and possibly five complete HeT-A elements distributed throughout 4R array and one in XL)[48]. Considering the fact of HeT-A mRNA transcripts abundance in nucleus and cytoplasm, its truncation at 5'end due to invariant orientation and 3' promoter localized very close to 5'end TAS region, there may be drawn a conclusion that this genomic position is intentionally developed by natural processes of evolution, where the loss of ~75 nt after each new cell cycle in *Drosophila* leads to activation of looping processes and epigenetics changes at histones and DNA methylation affecting directly the promoters functioning and telomeres elongation.

HeT-A AND *TART* SIMILARITIES AND DIFFERENCES

HeT-A and TART transcripts comprise one domain of telomeric region of the flies' telomeres (HTT arrays) alongside with TAS domain. Though, they are different non-LTR retrotransposons they share many similar features and genomic characteristics, undistinguishable between them two. First of all, both are non-LTR retrotransposable elements with transposing capability to chromosome ends due to conserved sequence of their *gag* protein domains. Furthermore, they form one-comprised-of-two arrays sometimes written as HeT-A/TART found only in TRFs. Nevertheless, they share two characteristics that distinguish them from other retrotransposons in *D. melanogaster*; first, they bind only to the ends of chromosomes, and second, each one contains long sequence of 3' UTR.

However, besides these similarities, these two elements feature functional and structural differences that cannot be interpreted as instance. *HeT-A* is remarkable because its DNA sequence does not encode reverse transcriptase protein, but only *gag* protein domain. This is why *HeT-A* has only one ORF. Besides that, *HeT-A* is the most abundant retrotransposon found in *D. melanogaster'*s Northern mRNA transcripts pool [51]. *HeT-A* and *TART* are dependent on Gag protein targeting the chromosome end. Once transported to nucleoplasm Gag proteins form specific aggregates (Het dots) at the end of a chromosome intermingling among themselves (between *HeT-A* and *TART*'s expressed proteins) [51]. Generally *gag* domain in telomeric retrotransposons is required for RT to reach the end of a chromosome and insert newly formed DNA sequence to the 3'end. Exact mechanism behind the process is not well understood. Ultimately, with reference to 3'-UTRs of *HeT-A*, both 3'-UTRs and 5'end of the retrotransposon contain sequences working as sense promoters; and what is even more startling, *TAHRE* region preserves the same structural functionality [37]. *HTT* arrays are organized in tandem head – to – tail arrays always in the same direction, and therefore 3' of one element is followed by the 5'-UTR of the downstream element.

TART possesses two ORFs: ORF1, which encodes for *gag*, and ORF2, which encodes for *pol*, reverse transcriptase (RT). ORF1 and ORF2 are separated by a short spacer [66,59], and ORF1 has a cluster of three CCHC-type zinc knuckles – conserved domain in non-LTR retrotransposons [59,35]. Interestingly, despite the fact of highly conserved 3'-UTR sequence in all three retrotransposons, the length and sequences of three subfamilies of *TART* elements have diverged. The experiments before brought evidence that there is a strict correlation between the level of *TART* and *HeT-A* transcripts [48], which suggests that *HeT-A* increased expression of mRNA transcripts goes along with reduced number of *TART*'s transcripts, but containing *pol* domain, absent in *HeT-A* sequence.

Another striking feature of *TART* is that it produces both sense and antisense strands. In the article of Elena Casacuberta and Mary-Lou Pardue [47], the authors managed to explore phylogenetic distribution of specific telomeres structure themes among four different species of *Drosophila* genus. They discovered that *D. virillis* and *D. americana* produced both the sense and antisense strands of *TART* transcript, and they noticed that antisense RNA was in excess. Later on Northern hybridization to RNA showed that *D, virillis* and *D. Americana* had significantly less amounts of sense and antisense strands found than in *D. melanogaster* or *D. yakuba*. There are other studies proving that both strands are expressed [22]. So far, it is not acknowledged why both strands are produced and what factors stay behind that. However, there may be a simple explanation provided basing upon the previous experiments given. Antisense strands are produced in excess, which might indicate epigenetics incorporation in the antisense expression; for example, H3K36me3 methylation or H3K27 deacytlation, or other epigenetical changes may influence transcription mechanism to express antisense strand even more than sense strand. This might be seen as a preventive mechanism before exaggerated production of *pol* transcripts capable to elongate fly's telomeres. *HeT-A* transcripts comprise more than 60% of the whole pool of retrotransposons in *Drosophila*, although without RT motif protein they are redundant. In this point, *TART* expression seems to be a trigger that highly pulled, it is capable to initiate telomere elongation in accordance with *HeT-A* transcripts. However, it is worth remembering that *TAHRE*, though still not completely explored and with one to three copies a chromosome, is *TART* alike and may play a role in the process of telomere length maintenance.

However, the article form 2009, written by Kalmykova, Rozovsky, Kwon, and Shpiz presents that *HeT-A* transcripts also produce antisense strands of mRNA in the manner, which has not yet been identified precisely, but read-through transcription from an adjacent external promoter (like the one found in the *Caenorhabditis elegans* in transposon Tc1 [60]. The antisense strands contain many introns inserted in the mRNA sequence. The authors document a repeat – associated short

interfering rasi – RNA – mediated system is crucial in bidirectional transcription. Moreover, it is emphasized that both strands, sense and antisense, are affected by rasi – RNA machinery, suggesting that rasi – RNA – mediated interplay between sense and antisense transcripts in nucleus.

TAS AND TPE EFFECT ON RETROTRANSPOSONS EXPRESSION

There are found two domains in *D. melanogaster* telomeres: terminal restriction region consisted of retrotransposons arrays (HTT) and subterminal repetitive telomere-associated sequence (TAS). TAS domain exhibits telomere position effect (TPE) on expression of retrotransposon arrays in telomeric region, although this functioning is limited only to telomere region and does not surpass the distance of the telomeres (TPE – OLD). In the article of James M. Mason & Radmila Capkova Frydrychova et al. [21], the authors reported that deletion of the 2L TAS array leads to dominant suppression of TPE and compared the results with the the expression level of *w* transgene inserted between TAS and HTT arrays, and the experiment showed that flies with a TAS deficiency (with different lengths) displayed a noticeable increase in the level of *w* mRNA transcripts by Northern hybridization.

There are two factors affecting expression efficiency of DNA transcripts. *Trans* participants are considered as all the proteins: transcriptional factors, methyltransferases, spliceosome enzyme complexes, and others; proteins that are directly involved in the change of expression patterns. *Cis* elements are the sequences of DNA affected by *trans* elements. Reciprocal interactions between these two groups is the key to understand the processes behind expression schemes and transcripts levels.

In the experiment by Mason & Frydrychova et al., 2L TAS deficiency did not result in increased transcripts levels of *HeT-A*, which indicates that TAS has influence only on proximal telomeric regions, which is in contrast to TPE effects in mammals [58]. This downregulation of retrotransposons expression is limited to some extent, but it has not yet been determined how many bases away from TAS domain may be localized in the range of this specific TPE. Moreover, it has been determined that TAS has the ability to up- or downregulate the read-through transcription of *HeT-A* because of distal – to – proximal transcription of *HeT-A* starting at a promoter in the 3'-UTR of an upstream of the retrotransposon. Stimulation of TAS on $P\{w^{var}\}$ expression because of TAS deficiency presented the data correlating with the hypothesis that read-through expression occurs in *cis* position upstream and downstream, but in the proximity of a promoter. What is even more,

the authors observed the suppression of TPE on non-homologous chromosomes due to deficiency of the 2L TAS: suppression of silencing the *w* transgene in 2L, 2R, and 3R telomeres. They suggest that Polycomb group proteins, like *Mcp*, may bind to TAS region and mediate long-range interactions. Another study [54] reports putative RNAi role in the telomere length and intertelomeric effects. There is still little known about TAS functioning in *Drosophila* telomere elongation process, but current knowledge clearly indicates heterochromatin modifications of TAS in TPE downregulation changes of proximal to retrotransposons promoters with read – to – through transcription accessibility. Telomere "capping" structure, functionally alike to human shelterin complex, may be seen as a missing link between TPE, epigenetical modifications and retrotransposons (telomere elongation) expression levels.

ELONGATION CONTROL MECHANISMS AND *DROSOPHILA* TELOMERES CAPPING STRUCTURE

McClintock, in 1930's and 1940's, found in her studies a broken chromosome end in some maize tissues that might have induced a cycle of chromosome fusion, anaphase bridges and new chromosomal breaks; something which today is known as DSB and NHEJ [41, 42]. The article from 1997 [38] was titled *Chromsome Ends: All the Same Under Their Caps*, and the authors captured the essential importance in the title. They stated that subtelomeric region of chromosomes is conserved from yeasts to humans and spotted that two domains exist in telomere structure: one with tandem repeated sequences and the second with centromere – proximal longer blocks of contiguous homology [19]. They also wrote about the case of Arthropods, who lack telomerase and specific short microsatellite sequence of nucleotides in telomeres; they suggested an evolution theory saying that initially recombination was about to maintain the telomeres length by utilizing the satellite arrays and retrotransposons, and then retrotransposons either became active or their control became regulated by unknown mechanisms. Nowadays, we know a little bit more about the mechanisms.

HOAP (*cav*), HP1 (*Su*(*var*)205), and PROD are the substantial proteins involved in the *Drosophila* chromosome cap. HP1 binds directly DNA in the cap structure and interacts with histone H3 methylated at Lys9 (H3Me2K9) [49]. From string-db.org we may find that *Su*(*var)3-9* (suppressor of variegation) specifically trimethylates Lys9 of H3 representing a specific tag for epigenetic transcriptional repression by recruiting HP1. In Perrini et al., 2004, the authors showed that mutations in the gene encoding HP1 lead to a 100 fold increase in *HeT-A* transcripts [49]. In another experiment *Su(var)205* mutations led to more than 100

fold higher *HeT-A/TART* attachments compared to *Su(var)205*$^{+}$ flies [53]. These conditions led not only to higher retrotransposons transcripts levels, but also to greater telomere elongation – the process, which is not only affected by a number of retrotransposons levels in nucleoplasm, and this suggests that there must be other processes staying behind this profound mechanism.

HOAP is a DNA binding protein resembling HMG family (high mobility group proteins) because of amino-terminal part, but with three copies of a novel repeated sequence [3, 56, 29]. In the experiments by Kellum & Gatti [29], deficiency in *caravaggio* (*cav*) gene leads to both lethality and the telomere fusion phenotype of *cav* mutation homozygotes. It also has been established that HP1 is not necessary required for HOAP to bind DNA. At the same time is it has been showed that HOAP binds the ends of *Drosophila* chromosomes in a sequence – independent manner, but telomere – binding factor has not yet been identified. Again, in string-db.org one can find functional correlation between *cav* and *E(var)3-9*, but also with rad50 – crucial component of the MRN complex needed in DBS repair; this may make us think that amino-terminal part of resemblance with MRN proteins and direct association with histone methylation are indicators of HOAP being affected or affecting epigenetical changes. However, first of all HOAP is needed by *Drosophila* cells to maintain telomere capping structures.

PROD, along with HP1, is a protein that binds not a telomeric DNA, but also it localizes strongly to the centric heterochromatin of the second and third chromosomes and to >400 euchromatic sites [62, 63]. PROD binds to a region just upstream of the *HeT-A* promoter; heterozygous *prod* mutants display elevated levels of *HeT-A* transcripts in ovaries and this suggests that *prod* acts as a functional repressor in *Drosophila* telomere elongation [63]. Together with HP1, those both proteins are *Drosophila* repressors, helping in prevention of telomere elongation rather than preventing from telomere shortening. And this is the most outstanding difference between Arthropods and the rest of the *Animalia* kingdom: flies contain telomeres elongation control mechanisms, while humans' cells, for instance, evolved mechanisms protecting against too rapid telomere shortening. This just indicates that retrotransposon system of telomere replication must be controlled because of its simple in its nature process of functioning.

As it was mentioned above, epigenetical changes are substantial in telomere length maintenance in *Drosophila*. In general, there are four different histones 3 and 4 modifications considered when searching for DNA permissiveness and proteins expression in subtelomeric structure. Trimethylation at lysine 4 of histone 3 (H3Me3K4) is associated with transcriptionally active chromatin, while trimethylation at lysine 9 and 27 of histone 3 (H3MeK9 and H3MeK27, respectively), and acetylation at lysine 12 of histone 4 (H4AcK12) are assumed to be associated with inactive chromatin. Several other histone modifications concerning acetylation of lysine residues of histones H3 and H4 were detected and associated with

actively expressed regions of DNA. The four core histones can harbor a variety of post-translational modifications, and *per se* they state about the fact of the importance of such epigenetic marks not only for transcriptional factors, but also for capping proteins and elements serving for telomere elongation mechanisms.

ncRNA elements are most abundant family of RNA strands found in a cell and the fact may seem not so surprising that they are also involved in HTT regulation in *Drosophila melanogaster.* Repeat-associated small interfering RNA (rasi-RNA) is a control mechanism of cells downregulating all three retrotransposons transcripts. rasi-RNA requires the members of the Piwi subfamily of Argonaute proteins: PIWI, AUB and AGO3 that bind directly to small RNAs, piRNAs (piwi-interacting RNAs) using them through their identification, slicer, and cleavage activities [40, 2, 9]. However, it is not understood how exactly rasi-RNA control the levels of HTT transcripts in cells. Brennecke et al., 2007 [9] suggest that PIWI binds sense piRNAs from *HeT-A* and *TAHRE*, whereas the same proteins bind antisense rasiRNAs from *TART*. AUB is associated with antisense rasi-RNA of all three HTT retrotransposons, and AGO3 binds entirely sense strands of HTT arrays. The fact of AUB protein binding antisense strands is correlated with what was mentioned above in the article about antisense strands of *HeT-A*. Here, Brennecke et al., exhibit the data of *TAHRE* antisense rasi – RNA elements found in cells [9]. In the further experiments mutations in helicase genes *spn-E* and *armi*, genes playing a central role during spermatogenesis and oogenesis by repressing transposable elements and preventing their mobilization, caused elevation of the three HTT retrotransposons [52, 64, 18, 9]. s*pn-E* mutants in ovaries are accompanied by chromatin modifications of genomic *HeT-A* elements and other retrotransposons [31]: dimethylation at lysine 4 residue of H3, a decrease in the methylation marks of H3Me2K9 and H3Me3K9, and a reduction of HP1 proteins associated with *HeT-A* promoters. This depicts an interesting problem. Disturbances in rasi – RNA system (including pi – RNAs and Piwi proteins) leads to a decrease in methylation pattern of HTT genomic regions, and this in turn, as long as we can assume, precludes HP1 from binding telomere capping. rasi – RNA machinery may interact directly with retrotransposons transcripts and "destroy" them through cleavage process, but it cannot interfere directly with histones and their posttranslational changes; thereafter, it may be presumed that rasi-RNA must correlate with proteins that are involved in epigenetics and may modify the histones' residues.

Besides those proteins, there are other ones that were identified as potent elements corresponding to telomere elongation control mechanisms. Reduction in Ku70/80 concentrations, components of NHEJ repair pathway, leads to a significant upregulation of HTT transcripts [43]. Furthermore, PcG proteins are also considered as contributors in telomeric elongation controlling [1]. Two Polycom-group repressors have been immunolocalized to TAS – PC and E(Z). In *Drosophila*, PcG family maintains silenced state of genes expression by mis-

cellaneous manners, like methylation catalysis, demethylation, deacetylation or ubiquination. Finding PcG proteins in TAS is consistent with the data that TAS is heterochromatin silencing gene expression in its proximity by the activity of TPE. In sequence, *Telomere elongation* (*Tel*) and *Enhancer of terminal gene conversion* (*E(tc))* have recently been described as dominant genetic factors of telomere elongation in *Drosophila* [61, 44]; mutations or deficiencies resulted in telomere shortening in both somatic and polytene chromosomes. In conclusion, *Tel, E(tc), ku70/80 complex* may be involved in HTT transcripts elevation, whereas *PROD, JIL-1, Z4* or *E(Z)* and *HP1* lead to HTT arrays' expression suppression; we also could incorporate many other proteins like ATM, Rad50, or MRE11 – all involved in DNA damage and repair pathways. Interestingly,Török et al. observed one promising correlation [63]. The more copies of retrotransposons found in telomeric region, the more PROD proteins bind the genomic DNA and this results finally in the reduction of HTT (mainly *HeT-A*) arrays inserted in the end of chromosome. This and the knowledge of HP1, HOAP and other rasi – RNA proteins activities may guide us through still obscure field of *Drosophila* genetics.

SENESCENCE PROCESS IN *DROSOPHILA*

Senescence in *Drosophila melanogaster* is not linked to telomeres shortening like in humans. Aging in the flies is on the contradiction with observations in human cells with shorter and longer telomeres. Biessmann et al. demonstrated that *D. melanogaster* strains with longer telomeres exhibited lower fertility, fecundity (GIII females with short telomeres had laid significantly more eggs and produced more pupal profeny), but they did not find any evidence that *D. melanogaster'*s telomere length correlates with life span. In organisms that use telomerase the length of the telomeres is kept within limits, while in organisms like *D. melanogatser* elongation occurs spontaneously and must be, ironically, suppressed and specific regions of genome must be silenced.

Fly's maximum life – span is measured as 50 – 80 days, and reaching the further days of their life – span, flies exhibit many age-related functional deficits [23]. The symptoms are observed like decreased percentage of phototaxis [33], shorten duration free flight [33], reduced learning abilities [10], or decreased rest during night [57]. However, general life – span of flies is not disturbed because of telomeres shortening.

Walker et al. implied increased expression of inflammatory markers (antimicrobial peptides, AMPs) and metabolic defects, including impaired insulin/insulin – like growth factor signaling pathway (IIS) as factors contributing "effective" aging in *D. melanogaster* [67]. The authors findings are consistent with the "hyperfunction theory of aging" [8], which is very similar to Harman's theory of free

radicals in humans [can I refer to my previous article?]. Accumulation of AMPs in *D. melanogaster* leads to death. In accordance to ISS pathway, it was showed that *Drosophila* lacking *chico*, insulin receptor substrate, live longer than wild types [12]. Clements M. et al. [55] demonstrated similar finding in mice lacking IRS1 (insulin receptor substrate 1) that stayed long-lived with physical improvements. The same results were obtained by many other groups showing that IIS pathways is very important in cells aging, however, still this is dubious if IIS may be considered as one of the most significant factors contributing decreased life-span not only in flies, but only in higher invertebrates and vertebrates [26, 11].

IME-4 CONJECTURAL ROLE IN *DROSOPHILA* TELOMERE ELONGATION CONTROL SYSTEM

OVERVIEW

IME-4 (inducer of meiosis 4) is an N6-methyladonosine transferase in *Drosophila melanogaster.* Its role was showed, among others, in spermatogonial differentiation, meiosis, oogenesis, first stages of *Drosophila* development processes, and cell signaling pathways [28, 27, 36]. Its role was exhibited in interactions with many proteins, including DC1, WTAP, or XIST (mice homolog METTL3)[28], and Notch pathway proteins [27].

In the case of rasi – RNA elements, HP1, PROD and even histone methyltransferases, one could notice that there is a link missing between mechanisms of activation/repression and genes expression required for telomere elongation. It is obvious that there must be many proteins, identified or still unidentified, that share common contribution to these convoluted mechanisms, but a conserved nature of *IME-4* in its homologs in animals may suggest that if post-transcriptional changes seem substantial in proteins functionality and that so far thousands of proteins have been explored to interfere with *IME-4* (methods like RIP or iCLIP provide new and new insights about correlations among proteins), putative *IME-4* role in telomeric region maintenance in *Drosophila melanogaster* seems more than logical.

Five various manners of proteins opulence in organisms occur: alternative splicing process, epigenetical factors, epitranscriptomical factors, chromosomal looping effects on promoters, enhancers and silencers, mutations in DNA coding sequences, and a combination of all of them. Every element of this "web of correlations" is important and none must be omitted. Therefore, *IME-4* activity as a ubiquitous mRNA methyltransferase seems to be a good point where the studies upon the "web of correlations" of *Drosophila* telomere maintenance start. Its putative role in post – transcriptional modifications in some of the most significant factors of this complicated system must be evaluated in the future.

POSSIBLE APPROACHES

The first thing that must be tested is the nature of HTT transcripts in *D. melanogaster.* It is still not well recognized whether transposable elements are denoted as mRNA particles, at least in some cases. Alongside with ncRNAs, transposons are useful evolutionary tools of genomic changes and multicellularity development [64]. HTT transcripts contain poly(A) ending sequences, and as mentioned above, antisense transcripts of HTT, especially *TART*, contain multiple introns; although it does not give a visceral insight if telomeric retrotransposons might be found as true mRNA elements. Thus, in the perspective of protein-protein correlations in nucleoplasm it seems crucial to examine genomic nature of HTT transcripts, referring to the post-transcriptional modifications.

According to Pardue & DeBaryshe [46], *HeT-A* and *TART* are not affected by TPE nor methylated by methyltransferases. This lack of genomic control seems distressing, however, there are precise protein and RNA mechanisms staying behind telomere elongation mechanisms. These mechanisms are consisted of pre- and post-transcription phases that intertwine and result in fine genomic and proteomic control. The greatest emphasis must be put on post-transcriptional processes involving already transcribed HTT particles binding to telomere sites and being affected by control mechanisms factors: *HP1, cav,* rasi-RNA, etc. Thorough and careful methods must be applied to detect a putative role of *IME4* in the process of *Drosophila* telomere elongation. This is RIP (RNA-binding protein immunoprecipitation-microarray profiling) to investigate RNA-binding proteins (RBPs), in particular *IME4* as a gene expression pattern facilitation regulating factor, and methylation detecting method (e.g. meRIP-seq, miCLIP) in order to scan for methylation pattern of the studied HTT transcripts. The comparison in data between the potentially methylated mRNA sites and *IME4* binding sites would give thorough insight into the alleged role of *IME4* in telomere elongation. Furthermore, the results could be compared to the new data from *IME4* knock-downs.

Even more, *IME4* knock-downs seem useful tool to study the correlation between *IME4* and proteins actively engaged in telomere capping in *D. melanogaster.* This idea might be implemented to proteins like HP1, PROD, and HOAP. Hypothetically, this approach could be executed to every actively acting particle in the telomere elongation, including RNAs and crucial transcription factors.

Another significant exploration method is artificial telomere shortening. In Shay & Wright et al. [58], the researchers used CRISPR/Cas9 system to artificially shorten the telomeric and subtelomeric region of chromosome 5p. By infecting BJ (human fibroblasts) with Cas9 expressing lentivirus and gRNA, along with NHEJ inhibitor SCR7 (to prevent DNA repair system from DSBs repair), they fully succeeded having

obtained shortened chromosome 5p. Due to the fact that *D. melanogaster* telomere length is usually well maintained, detection of *IME4* in hypothetical supportive process of the telomere length maintenance causes proper timing troubles. By incorporating the idea of artificial telomere shortening, adequate and applicable short length of the telomeres could bring the discernment of specific proteins and particles being involved in the process of telomere elongation and maintenance. Simultaneously a couple of different factors' transcripts levels would have to be examined, i.e. HP1 (as a main telomere capping protein), *IME4*; histones methylation patterns could also be scrutinized, including H3MeK9 and H3MeK27 (inactive chromatin) and H3MeK4 (active chromatin). Significantly changed and varied levels of *IME4* may present very direct evidence of *IME4* involvement in the telomere maintenance process.

Besides the experiments presented, additionally the methylation patterns in telomeric and subtelomeric regions of the chromosomes might be studied before and after the *IME4* knock-down. In approval of this approach, one could suggest that *IME4* may be incorporated indirectly in the process of the telomere elongation in the fruit flies. Splicing machinery proteins, chromatin remodeling proteins, transcription factors and many other might be considered as potential effectors of *IME4*, and the changes in the crucial players in the telomere maintenance (splicing proteins in HTT post-transcriptional arrangements or in telomere capping telomeres, as an example). Methylation pattern is substantial in almost every genomic (cellular) process, and TAS active role upon *cis* transposable elements in subtelomeric region stays as a good indicator of that reasoning. Splicing process involves many proteins, including the proteins engaged in the telomere elongation control mechanisms. Finally, transcription factors, besides their huge number and diversification, could be potential targets for further *Drosophila* telomeres experiments.

CONCLUSION

Since the first discoveries of telomeres, tandem repeated sequences in yeasts, and exploration of telomerase complex in *C. elegans* brought the scientific world to the point where more questions than answers rise. Peculiar arrays of three different, but descending from one ancestor [37], retrotransposons in *D. melanogaster* seem to act highly differently from the protective systems we know. Even profound analysis of the "web of correlations" would not give an answer to the questions about telomere elongation, regulation mechanisms, and putative factors being in proximal distance to the telomeric regions and interconnecting with proteins from DNA repair pathways, transcription factors, ORFs, PcG, and intra- and intercellular signaling pathways. The answers to all these questions must be found.

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