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Research Article

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Yeast-Longevity Surrogates Obtained in Response to Steroid Lithocholic Acid

Tania (Tatiana) Iouk*, Anastasia Glebov

Abstract

Objective: The theories of programmed aging suggest the existence of genetic mechanisms that actively restrict organismal lifespan. We challenged yeast cells with a longevity steroid compound (lithocholic acid, LCA) to break the aging barrier and to also determine whether steroids elicit hormetic effect in absence of receptors.

Methods: Longevity assay, assaying GTPase activity, assaying telomeres, assaying mutations, assaying changes in gene expression using reverse transcriptase and PCR analysis.

Results: The quasi-immortal mutants were developed that were linked to recombinant long telomeres. Several anti-aging mutations and changes in gene expression were detected in some of these mutants.

Conclusions: Steroid compounds influence GTPase activity in the context of mitochondrial dynamics and they increase the length of telomeres. The obtained in response to a steroid LCA long-lived cells bear longevity mutations and they also exhibit changes in gene expression, suggesting that steroid-dependent signal transduction doesn't need soluble receptors to act.

Keywords

Longevity; Telomeres; Dynamin-Related Gtpase; Inhibition; Steroid; Gene Expression

Introduction

The theories of programmed aging imply the existence of the evolutionary forces that actively restrict organismal lifespan [1,2]. Such a restriction accelerates the death of an individual in order to benefit kin, i.e. to ensure that the fate of progeny is not jeopardized because of spontaneous errors, free radicals, glycation, and other detrimental factors accumulated by the parental organism.

An aging organism is often a threat to populations and ecosystems; therefore, mechanisms of programmed aging have developed that target both RLS (Replicative Life Span) and CLS (Chronological Replicative Lifespan) of a species [2,3]. In yeast, such a mechanism exists primarily in a form of TORC1 (Target of a Rapamycin Complex 1) signalling pathway, which functions in coordination with other nutrient-sensitive pathways, including RAS/cAMP-dependent protein kinase A and AMP-dependent protein kinase (Snf1) pathways [4-8].

Within the TORC1 signalling, a nutrient-regulated Tor1 kinase complex phosphorylates Sch9 (the yeast homolog of mammalian S6K). TORC1 accelerates an entropy-based cell death due to the: accumulation of ROS (reactive oxygen species), changes in subcellular redox homeostasis, the release of extrachromosomal rDNA circles, changes in the vacuole dynamics, protein aggregation, etc. [9-14]. TORC1 also decreases the RLS: Sch9-dependent phosphorylation of Rim15 kinase blocks the release of PP2ACdc55 phosphatase required for the cell-cycle re-entry and consequently, cells enter a G0 growth arrest [15-16].

If Sch9 and its upstream activator TOR1-kinase gene are responsible for programmed aging, the non-programmed chronological aging depends on a variety of genes, including the mitochondrial-protein genes, regulators of redox homeostasis, genes that regulate alkalinization of the vacuole, and protein sorting to the endosome, tRNA methylation genes, etc. [17-19]. The gene deletions were identified that increase RLS but not CLS. Among others, the implicated knockouts include those of retrograde-transport genes, which communicate mitochondrial malfunction to the nucleus, and some histone-deacetylase knockouts [20,21].

Yeast longevity is often co-incident with a decreased growth rate, and the long-lived mutants are frequently characterized by a decreased fitness [1,22,23]. The decreased fitness means the inability of yeast cells to grow on a specific carbon source, a decreased fecundity, or the decreased resistance to stress [24]. The competitive fitness, which is also often decreased in the long-lived cells, is determined based on the ability of a mutant to survive in a mixed yeast culture along with the wild-type strain or other mutant [22,24].

The longevity could be studied not only in the designated knockouts but also in natural isolates, chemically-challenged random yeast mutants [22,25,26]. In this study, we characterize in detail the subset of long-lived mutants obtained using a steroid Lithocholic Acid (LCA). LCA increases mitochondrial fusion while decreasing this organelle fission [26-29]. It increases chronological longevity in the liquid yeast cultures [27,28]. In addition, continuous exposure to this drug results in the appearance of long-lived mutants which have extended recombinant telomeres, mutations, and exhibit changes in gene expression [24,26].

Materials and Methods

Media, Growth Conditions, and Chronological Lifespan

Yeast were grown in the synthetic medium in presence of 0.2% glucose, as described [24,26,27,30]. For the CLS assay, cells were plated on the YEPD plates, and the colonies were scored at specific time points (Ibid.). Mitochondria were stained with a MitoTracker Green FM.

GT Pase activity

As described [31], using the NADH oxidation assay. MFN2 was from H. McBride [32]. Other GST-fused GTPases were described [33]. mdivi-1 compound was used at 2.5 μM concentration while optimal LCA concentration for the assay was 1.0 μM.

***Corresponding author:** Dr. Tania (Tatiana) Iouk, Department of Biology, Concordia University, Montreal, Quebec H4B 1R6, Canada, E-mail: tatiouk@gmail.com

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Telomeres assay

As described [34]. A pCT300 probe was used to detect telomeric fragments. DIG DNA labeling and detection kit were from Roche.

cDNA synthesis, PCR, and sequence analysis

RNA was synthesized using a hot-phenol method. The cDNA synthesis kit was from Thermo Fisher Scientific. The primers for PCR analysis and sequencing services were from Bio Basic Canada.

Results

LCA inhibits the GTPase activity

Lithocholic Acid (LCA) is a steroid compound that modifies the lipid composition of mitochondrial membranes to promote the fusion of this organelle (Figure 1A) [28,29]. Specifically, LCA increases the amount of cone-shaped phosphatidic acid in the mitochondrial membranes, thereby increasing their curvature at the contact points, while also promoting biogenesis of the Ugo1 protein which facilitates the fusion (Figure 1B) [29]. Mitochondrial fusion and fission (i.e. the division of mitochondria) are also regulated by the dynamin-related GTPase proteins (DRPs), which synchronously push or pull the outer and inner membranes of two mitochondria to trigger the fission or fusion, respectively [31]. Various chemical inhibitors are known to block the GTPase activity of DRPs, and we sought to determine whether or not LCA has an effect on the DRP-related GTPase activity [35] (Figure 1B).

The fluorescence microscopy revealed that 25μM LCA decreases the requirement for MGM1 and MDM30 genes that have a role in mitochondrial fusion (Figure 1C). Mgm1 controls mitochondrial inner membrane fusion and has several other functions, while Mdm30 controls the fusion at the level of ubiquitination of Ugo1.

We used the continuous coupled regenerative assay to measure the mitochondrial DRP GTPase [31]. As a positive control, we chose the inhibition of Dnm1 by an allosteric mdivi-1 inhibitor [35] (Figure 1D). LCA was tested on the MFN2 mammalian protein (orthologue of yeast Fzo1) that could regulate both fusion and fission of mitochondria, and on Dnm1 (Figure 1E). We determined that LCA inhibits the MFN2 when the substrate concentration is low (<40μM), and it stabilizes activity at a basal level (<200 AU) when the substrate concentration is high (Figure 1E). The "stabilization" likely occurs because of the dual nucleotide-binding and nucleotide-hydrolyzing nature of MFN2 when a portion of available enzyme forms a complex with LCA, while other, free from LCA portion, is hydrolyzing GTP [32]. As shown in Figure 1E (bottom), LCA had a weak inhibitory effect on Dnm1-a more potent, in comparison with MFN2, DRP-GTPase. If depending on the inhibitor and substrate (GTP) concentration, mdivi-1 provides anywhere between the 7- and 600-fold inhibition of the GTPase activity (sufficient to prevent yeast apoptosis [35]), LCA provides only the 2-60 fold inhibition (Figure 1F).

As shown in Figure 2A, the appreciable inhibitory effect of LCA was restricted to the mammalian MFN2, a relatively weak GTPase, while many other GTPases, including GEF (guanine-nucleotide exchange factor)-regulated proteins were unaffected (Figures 2A and 2B). The GTPase activity of another protein was decreased in presence of LCA, and that is of the Est3 subunit of yeast telomerase holoenzyme, which rebuilds chromosomal ends following each round of cell division. Est3 is the Oligonucleotide-Binding (OB) protein, which assists the assembly, binding, and function of telomere-holoenzyme at the ends of chromosomes [34]. In addition to the OB function, Est3 also contains nucleolytic activity (both ATPase and GTPase).

LCA and telomeres

Previously, we described a lithocholic-acid-driven selection of the long-lived yeast mutants, [24,26]. In some cases, longevity was linked to recombinant telomeres (Figure 2C). After prolonged exposure to LCA, 17 individual long-lived survivor colonies of the ScBY4743 cells were selected and their genomic DNA was isolated and it was digested with the XhoI enzyme that produces a particular digestion pattern [34]. Using a DNA probe that detects 286 bp of telomeric repeats, we determined-using a Southern blot-the presence of recombinant products in many mutants. If a typical digestion pattern contained a terminal fragment of 1.2 kb (and a specific subset of larger bands (Figure 2C, a "wt" lane, the arrow is pointing to a terminal 1.2 kb fragment), the recombinant telomeres produced additional bands and

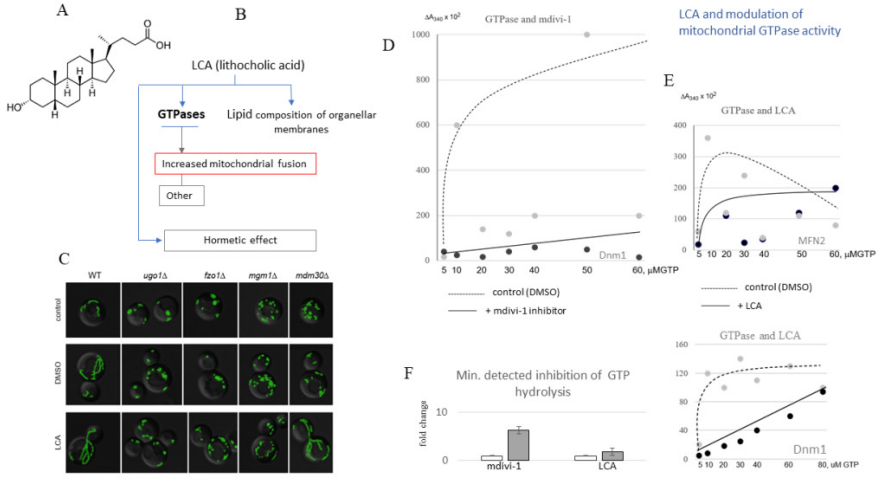


Figure 1: (A): The structure of Lithocholic Acid (LCA); (B): Known effects of LCA on yeast cells; (C): LCA improves mitochondrial fusion; (D,E): Assaying the effect of LCA on GTPase. The Dnm1 GTPase curve with the mdivi-1 compound is a positive control for allosteric inhibition; (F): LCA is a weak inhibitor of GTPase activity.

they exhibited an aberrant digestion pattern, as in the control rad50Δ cells (Figure 3A "telom. mutant" lane) in which the recombination RAD51 pathway is active [36,37].

LCA is predicted to have a dual effect on telomers (Figure 2D). Firstly, the drug decreases the activity of the Est3 telomerase subunit [38]. Secondly, it could modify telomere attachment to the nuclear envelope, hence previous studies showed that a relative content of several lipids is modified in presence of LCA [34,39]. Finally, LCA was shown to modify oxygen consumption in yeast (first very low then high), and it could trigger the oxygen-regulated activation of RAD51 pathway and recombination [40,41].

Changes in gene expression and mutations

Three mutants (3,5 and 12) showed a strong increase of mean CLS, suggesting that in addition to long telomeres, they contain mutations or changes in gene expression (Figure 3A) [24,26]. Unlike the other 14 mutants, mutants #3, #5 and #12 also had difficulty pinpointing phenotypes. They were sporulated, and at least four sporulating cells of each mutant were used to obtain tetrads #3(T1-T4), #5(T1-T4), and #12(T1-T4). Up to16 segregants were obtained for each mutant. Among these, at least 5 segregants of the #3 mutant remained exceptionally long-lived (3T1A, 3T1C, 3T2C, 3T3B, and 3T3D), 4 segregants of the #5 mutant (5T1D, 5T2C, 5T2D, and 5T3C), and 3 segregants of the #12 mutant (12T1B, 12T2B, and 12T2D) were also long-lived. For working purposes, the individual segregants were assigned back to the original mutant number: for example, the properties of 3T1A segregant were assigned to the original mutant #3, etc.

The SCH9 cDNA from all the mutants was sequenced to find a STOP codon in place of Asp186 in the number 5 mutant. The elimination of TORC1 signaling in the #5 mutant was confirmed by ACS1 (acetyl-CoA synthetase) expression (d.n.s). The #3 mutant also contained mutations in the SCH9 gene, however, these mutations weren't deleterious, i.e. they were characterized by the ≤ 1.0 SIFT (Sorting Intolerant From Tolerant) score, suggesting that the arising amino amino-acid substitutions do not significantly influence the protein function (Figure 3C)[42].

The TOR1 cDNA, which encodes the upstream regulator of Sch9, was also analysed. Several mutations were potentially detected, however, all of them produced a high SIFT score. Two mutations were found in the UBP6 ubiquitin-peptidase gene, and the RPL8 and RPS26B genes also contained mutations.

The mutant number 12 had no mutations directly related to the TORC1 signalling. The initial sequencing analysis suggested the presence of a STOP codon in the DBP3 rRNA processing gene, i.e. a dbp3Δ mutation which could potentially increase replicative longevity [26,43]. However, the dbp3Δ mutation was not confirmed, and further analysis revealed possible mutations in the RPP2(POP7) gene, which encodes a subunit of the nuclear MRP RNase and P RNase that also regulates the function of telomerase [44,45]. The detected in the POP7 gene mutations (32T>K and 43S>R) increase the binding to nucleic acid, thereby decreasing the release of the substrate and a nucleolytic activity. Remarkably, because of a decreased rRNA processing, this mutant's cells were smaller in size than the control ScBy4742 cells (4.5+/-1.4μ versus 5.5μ for the ScBy4742 control), and they had a slightly decreased early-growth rate.

We sought to examine the expression of specific genes in long-lived mutants. Previously, LCA was shown to trigger the changes in the mitochondrial and metabolic-protein (TCA cycle enzymes)-expression [46]. mRNA was isolated from mutants to synthesize cDNAs. The specific primers were used to PCR-amplify individual cDNAs and to compare changes in the expression. The primers were designed so that a representative 1kb fragment of each cDNA

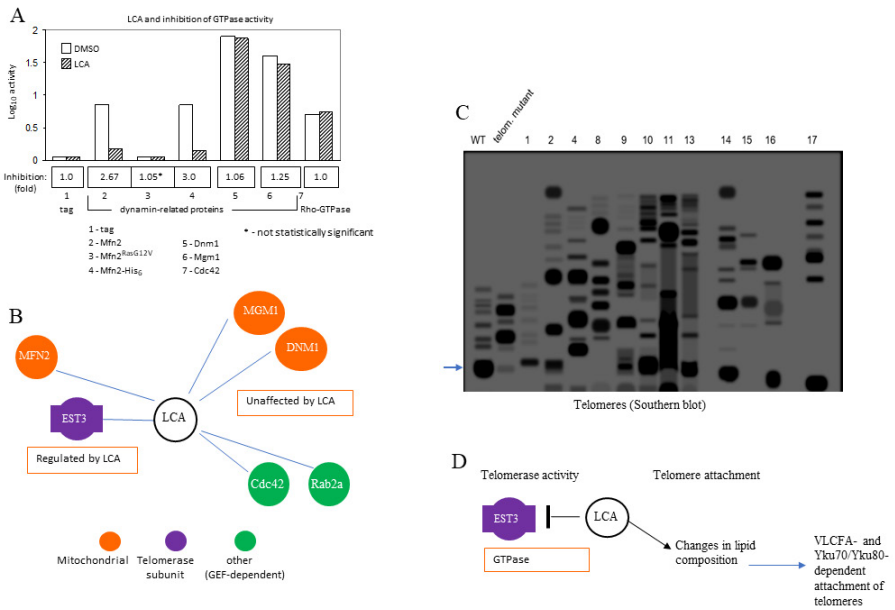
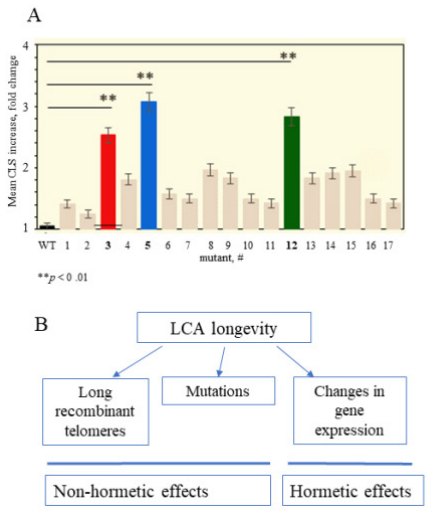


Figure 2: (A): The effect of LCA on GTPases; (B): The LCA-inhibited GTPases include the MFN2 (mitofusin 2) and the oligonucleotide-binding Est3 subunit of telomerase holoenzyme; (C): longevity was linked to recombinant telomeres; (D): LCA is predicted to have a dual effect on telomers.



C

	LLS (mut)				
Gene	#	aa	nucleotide	gene function	SIFT score
SCH9	mut3	75 I > F	223 A > T	effector kinase of TORC1	1
		209 T > S	626 C > G		1
	mut5	186 E > Stop	556 G > T		0
UBP6	mut5	57 E > K	169 G >A	ubiquitin release	1
		155 E > K	463 G > A		1
RPL8	mut 5	54 D >Y	165 T > C	ribosomal protein	n/d
		60 R > T	177 G > C		n/d
RPS26B	mut 5	30 I > V	88 A > G	ribosomal protein	1
FKH1	mut5	3'-UTR *		transcription factor	n/a
XBP1	mut12	3'-UTR *		transcription factor	n/a

Figure 3: (A): Mean CLS increase, in comparison with wild-type; (B): Three exceptionally long-lived mutants (a.k.a. LLS - long-lived survivors) are additionally predicted to contain mutations or changes in gene expression: (C): The summary of relevant mutations detected.

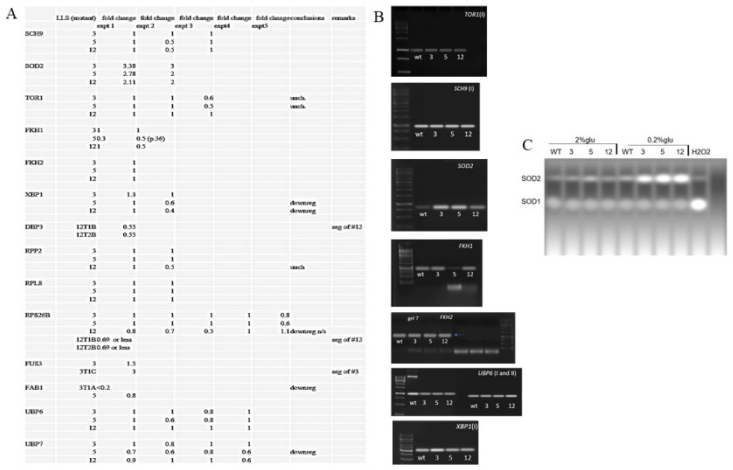


Figure 4: (A): The summary of changes in gene expression; (B): Reverse-transcriptase PCR was used to detect changes in gene expression. The 1kb fragments of individual cDNAs were amplified simultaneously; (C): The SOD2 activity.

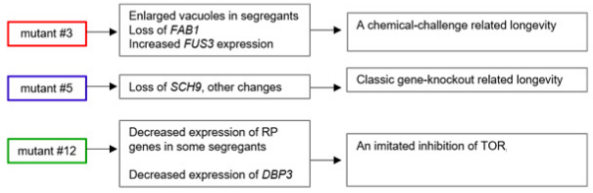


Figure 5: The summary of three long-lived mutants.

sequence was amplified, therefore numerous sequences were synthesized simultaneously using the 1 min elongation time (at 72˚C). The DNAs were run on the agarose gel, and the amounts were quantitated. Overall, >50 sequences were analyzed, and the changes for some genes are presented in Figure 4A.

The expression of SCH9 and TOR1 mRNAs wasn't affected in any of the mutants. The expression of the mitochondrial SOD2 superoxide dismutase (Mn^{2+}) gene was increased in the number 3 and 5 mutants (Figure 4B) [47,48]. However, when examined using a nitro blue tetrazolium, the SOD2 activity wasn't increased under normal growth conditions (2% glucose). It was increased only under calorie-restriction conditions (0.2% glucose), however low glucose is known to increase the expression of many mitochondrial proteins, including SOD2 (Figure 4C).

There were no changes in the expression of metabolic genes, including those regulating the gluconeogenesis (FBP1), accumulation of storage carbohydrates (TPS1, GSY2), TCA cycle (ACO1, IDH1, IDH2, KGD1), and genes linked to the biosynthesis of amino-acids and mitochondrial-genome maintenance (ILV5, ILV6, LYS12). The expression of a mitochondrial chaperon HSP60 gene was increased in all three mutants.

The mutant number 5 showed the strongly decreased expression of FKH1, one of the two FOXO transcription factors in yeast (Figures 4A and 4B). In mammals, the Forkhead-box O (FOXO) transcription factors suppress tumor growth and they control cell differentiation [49,50]. The FOXO transcription is known to maintain cellular homeostasis during aging in the (*C. elegans*) nematode, however, it could also cause age-related disease [51,52]. In yeast, the overexpression of FKH1 was linked to a modified sensitivity to mild oxidative stress, including nutrient-related oxidative stress [53,54]. The expression of the RPS26B mRNA was increased in the mutant #5 cells.

Notably, the mutant number 5 is highly resistant to stress, including oxidative, high-temperature, and salt-inducible stress [24]. In part, the resistance to stress was linked to the decreased expression of the FKH1 transcription factor [53-56].

Mutant #3 exhibited the increased FUS3 expression in some experiments, and it was characterized by enlarged vacuoles. There were several lines of evidence suggesting that #3 is a chemical-challenge product, rather than a genuine longevity mutant (Figure 5) [25].

Mutant #12 lacked the expression of the XBP1 transcriptional-repressor gene, DBP3 and RPP2 rRNA processing genes, and of the RPS26B ribosomal-protein gene. XBP is known to repress yeast genes as cells enter quiescence. The down-regulation of XBP1 expression could indicate the mutation in the 3'-UTR of this gene in the #12 mutant, which was missed since only a subset of cDNAs was sequencing-analyzed suggests that regulation of programmed aging is compromised in the #12 mutant. Together, the down-regulation of XBP1 in the #12 and down-regulation of the FKH1 transcription factor in the #5 mutant suggest that the LCA-related hormetic effect is linked to these two TFs.

Discussion and Conclusion

The steroid LCA (Figure 1A) influences lipid metabolism and mitochondrial function, and it also has a hormetic effect on yeast cells that manifests in increased longevity [24,26]. In contrast to receptor-mediated signalling, which exists in *C. elegans*, *D. melanogaster*, and higher eukaryotes, the hormetic effects of hydrophobic compounds in yeast involve direct sensing by transcription factors that are followed by the regulation of gene expression [57-61]. Unlike ergosterol and fatty acid, which reconfigure metabolism without increasing lifespan, the exogenously added LCA increases longevity. Initially, the LCA-dependent longevity appeared restricted only to optimizing yeast metabolism, decreasing the levels of ROS (reactive oxygen species), and protein oxidation levels [27]. However, following exposure to LCA, the long-lived yeast isolates were identified (also known as LLS, long-lived survivors) that had increased lifespan, often however at expense of competitive fitness [24,26]. This finding suggested that LCA elicits a long-lasting effect, similar to that of estrogen, corticosteroids, or AAS (Anabolic-Androgenic Steroid), when the effect of steroid compound remains evident after the compound itself is withdrawn.

When added to yeast cells, LCA could induce oxidative stress, however, the mitochondrial function becomes restored in less than 24 hours, and it remains strongly enhanced during aging [28,40]. Either early oxidative stress or subsequent hyperoxia could induce the Rad52/Rad59 or RAD51-dependent recombination pathways respectively to recombination-lengthen the GC1-3-repeat-rich chromosomal ends [36,41].

The recombination at telomeres involves the decreased activity of telomerase, a reverse-transcriptase enzyme that post-mitotically replicates telomeres [34,36]. Telomerase consists of several subunits, including the Est3 protein that has a GTPase activity [38,62,63]. Lithocholic acid inhibits some GTPases, including the MFN2 mitofusin and Est3 protein (Figures 1D-1F, Figures 2A and 2B) [32,63]. The LCA is unable to inhibit GEF-chargeable GTPases or super-powerful GTPases such as Dnm1 (Figure 1E and Figure 2A).

It should be noted that est 3Δ mutation does not block the function of telomerase, however, chemical inhibition of the Est3 could have the blocking effect sufficient to decrease the function of telomerase holoenzyme in presence of LCA [38, 62]. We hypothesize that LCA-dependent inhibition of GTPase- or OB- the activity of Est3 had triggered activation of recombination within GC (1-3)- rich telomeric-repeat regions (Figure 2C).

The LCA-dependent changes in the ER lipid composition, which decrease the availability of DAG while increasing the ceramide-to-DAG ratio, are also beneficial to telomeres because the VLCFA-containing ceramide mediates a protein-dependent attachment of telomeres (Figure 3) [27]. Notwithstanding, this attachment, which also involves Yku70/Yku80 proteins, would primarily benefit the telomerase-dependent chromosomal-end maintenance and not the recombination [34].

The lithocholic acid has no known mutagenic effects. It increases the longevity of both glucose-limited and non-glucose-limited yeast cells, by modifying yeast lipidome and decreasing GTPase-dependent mitochondrial fission [27, 29]. The GTPase-regulating activity of LCA is thought to be secondary to its lipidome-related function, which manifests in decreasing the DAG levels in ER (endoplasmic reticulum) and increasing the levels of phosphatidic-acid lipid in mitochondrial membranes [28]. The yields of several other lipids including cardiolipin and the cardiolipin- biosynthesis intermediates, which define the curvature of mitochondrial inner membrane are also increased in response to LCA, however, this drug does not have a direct effect on cristae formation [28]. The grown in presence of LCA aged yeast cells contain giant mitochondria that no longer possess a tubular structure [28]. It, therefore, appears that in aging cells LCA does not support mitochondria and that it perhaps fully redirects its influence towards telomeres.

Chemical inhibition of dynamin-related GTPase activity and Est3, both of which are archaic GTPases (of bacterial origin), does not impact other GTPase-driven processes inside the yeast cell [35]. Lithocholic acid does not inhibit polarized cell growth and endocytic pathway the regulation of which involves Rho-type and Rab-family GTPases [64, data not shown].

In this study, we characterized the subset of long-lived yeast mutants [24,26]. The three mutants were exceptionally long-lived, while other mutants demonstrated a modest increase in longevity [24,26] (Figure 3A). Among those mutants, an average increase of mean CLS was 1.64 ± 0.4 fold (i.e. approximately 21 days as opposed to 13 days for the control cells). A modest increase was detected for some mutants (as low as 1.25 ± 0.06 fold), however, the typical increase of mean CLS was 1.88 ± 0.07 fold.

It should be noted that in liquid culture, long telomeres have a modest effect on yeast longevity. By comparison, plated on solid agar cells with long telomeres would increase replicative longevity from

approximately 80 to 150 and more generations, i.e. more than 2 fold [34,36]. In the classical RLS assay, which follows reproductivity of a single mother cell, we would see the increase from 30-40 to up to 70 daughters. It should also be noted that spontaneous telomeric recombination is exceptionally rare in the wild-type laboratory strain(s). Lithocholic acid increases the frequency of telomeric long-lived survivors up to 3×10^{-8} cells [24]. By comparison, the activation of Rad51 pathway (in tlc1Δ cells) is associated with a much higher frequency of telomeric mutants of up to the 1×10^{-6} cells level [34,36].

In this study, we primarily focused on the exceptionally long-lived 3, 5, and 12 mutants and their segregants. The maximal CLS was 47 days or more in liquid cultures, which is very long for ScBY4741-4743 cells that normally remain viable in the flasks for no more than 3 weeks [65-66]. The industrially relevant budding-yeast species could live in liquid culture for 90 days, however, these species lack programmed aging and they accumulate mutations and chromosomal alteration events while experiencing loss of heterozygosity [67]. These cells can't be used to model cell cycle and study numerous other processes.

Mutant # 3 and its segregants exhibited an upregulated mitochondrial function and large vacuoles. The expression of the FAB1 gene (1-phosphatidylinositol 3-phosphate 5-kinase that regulates synthesis and turnover of phosphatidylinositol 3,5-bisphosphate PtdIns (3,5) P2 and has a role in functioning the lysosome-like vacuole [64]) was decreased in these cells, while the FUS3 expression was increased (Figure 4A). The cell-wall integrity was compromised in the segregants in a manner resembling a "mating-induced cell death" that depends on the MID2 cell wall sensor and also on YPK2 [68,69]. The expression of UBP7, an ubiquitin-specific protease gene that cleaves ubiquitin-protein fusions, was increased in these cells. At least one segregant of the long-lived mutant #3 resembled the obtained by genome shuffling chemically-resistant long-lived strain [25]. The defect-related phenotypes are sometimes cured when the segregant is mated back to a wild-type strain; notwithstanding, a competitive fitness of a resulting diploid could still be decreased for example when cells are grown on 0.1% ethanol [26].

The mutant number 5 was highly resistant to stress, including the hydrogen-peroxide inducible stress [24]. This resistance was combined with the inhibited TORC1 signalling and with the increased expression of SOD2 (Figures 4B and 4C). The sch9 186E>Stop mutation was detected in this mutant and the TORC1 inhibition was confirmed by the ACS1 expression. The FKH1 transcriptional-factor expression was decreased in this mutant. In the mammalian cells, the FOXO members are known to protect against oxidative stress, while in yeast Fkh1/Fkh2 transcription factors also protect against nutrient-related oxidative stress [51-53].

The mutations in UBP6, a ubiquitin-specific protease gene [70] were also detected in the #5 mutant, however, none of these mutations is predicted to influence this protein function (Figure 3C). The mutations in UBP genes were detected in the long-lived ScBy4742 cells previously that decrease function of proteasome while increasing amounts of ubiquitinated proteins [55, 70]. The decreased function of proteasome however is not consistent with longevity, and UBP-gene mutations, consistently detected in the exposed to chemicals long-lived mutants should not be considered a part of these mutants' longevity profiles [71].

In addition to the UBP genes, the chem-challenge related longevity involves mutations in the: SSA1 (the Hsp70-like regulator of protein homeostasis) gene, ARO1 gene that has a role in the synthesis of aromatic acids, trehalose-H+ symporter gene MAL1 gene, a regulator of redox homeostasis GSH1 gene, NADP(+)dependent glutamate dehydrogenase GDH1 gene, transcription-factor encoding NRG1 gene, and several others [25,72].

The results suggest that LCA-exposed mutants developed not only anti-aging changes (genetic and chromosomal), but also mutations that bring them closer to industrial yeasts. While the sch9 mutations, in general, are associated with longevity, the sch9C44S is predicted to decrease protein aggregation [73-74].

It was surprising that among the mutations detected in this study, several were sch9 (Figure 3C). The studies by others also showed that SCH9-gene mutations are quite frequent in the exposed to chemicals long-lived survivors ([25] and D. Pinel, personal communication). These mutations, however, often have a high SIFT score.

SCH9 gene is located on the left arm of the VIII chromosome, upstream of the SKN7 transcriptional-regulator gene that has a role in oxidative, osmotic, and cell wall stress. The skn7 and the neighboring-gene mutations were not reported as frequent ones, suggesting that a specified locus is not particularly susceptible to mutagenesis. It is surprising overall that a principal longevity barrier set by nature in a form of the SCH9 gene is so easy to challenge, and that yeast do not seem to struggle to protect it against mutations. Moreover, loss of Sch9 kinase function often goes unnoticed by cells since several other kinases have an overlapping with Sch9 phosphorylation substrate specificity [75-77].

Before we conclude, we would like to reflect again on the claim that LCA is a hormetic compound for yeast cells. In mammalian cells, the effects of steroids are mediated either by soluble receptors - to activate transcription, or by plasma-membrane receptors, such as G protein-coupled receptors (GPCRs) to induce mitogen-activated protein kinases, adenylyl cyclase, protein kinase C, and heterotrimeric GTP-binding proteins [78].

The steroid-induced GPCR-mediated signaling constitutes the non-classical receptor-mediated pathway. In yeast, LCA is unable to bind to GPCRs (Ste2 or Ste3), which recognize only the mating-pheromone peptides. Instead, this steroid becomes endocytosed and deposited on the inner surface of the Plasma Membrane (PM), coupled to the yeast sterol-binding receptor proteins [28]. From there, LCA becomes internalized by mitochondria which in yeast cells frequently form contacts with the PM. As mitochondria age, LCA is released in the cytoplasm to then join the ER compartment and reach the nuclear envelope [28]. The LCA-dependent regulation of GTPase activity at mitochondria and at the nuclear periphery (where it acts towards chromosomal telomeres) cannot be considered a hormetic action, however, LCA- dependent inactivation of TORC1 pathway and downregulated expression of XBP1 and FKH1 transcription factors constitute a hormetic effect (Figure 3B) [27].

Previously, we stated that polygenic longevity traits were developed in yeast long-lived mutants in response to a steroid LCA with some of these traits being dominant [26]. We also stated that LCA elicits a poorly understood hormetic effect on yeast cells [24,26]. We now gained additional evidence to support our ideas and to also suggest that a hormetic effect of an exogenously added steroid compound involves the regulation of gene expression in yeast cells [46].

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Author Affiliations

Top

Department of Biology, Concordia University, Montreal, Canada