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Dissecting the Effects of White Willow *Salix Alba* and Salicin on Yeast Longevity

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Abstract

Objective: Yeast cells are frequently used to model longevity and aging. Following the screening in yeast, chemical supplements and they are containing natural remedies could be pharmacologically assigned or repositioned as anti-cancerogenic and anti-aging substances. An extract from the white willow *Salix alba* is rich in salicin, a β-glucoside that contains D-glucose and salicylic acid. Salicin has a similar to aspirin anti-inflammatory effect. The extract also contains salicin-7-sulfate and physiologically active polyphenols.

Methods: Protein phosphorylation and protein expression analyses.

Results: The genetic yeast screen indicated that the anti-aging effect of *Salix alba* is linked to the PKH1/PKH2-dependent regulation of the Sch9 kinase. However, the phosphorylation of most PKH1/PKH2 targets, including YPK1/YPK2, was not affected in presence of *S. alba*. TORC1-dependent activation of Sch9 was inhibited, suggesting that similarly to aspirin or rapamycin, salicin acts to block TORC1 signaling either above or at the TOR1 level, respectively. Present in the *S. alba* extract salicin-7-sulfate could additionally decrease phosphorylation of Sch9 by blocking serine and threonine residues on the protein surface. Despite the decreased phosphorylation of Sch9, the phosphorylation of some other proteins was increased in the *S. alba*-treated cells. This occurred due to a kinase-swapping effect when the insufficient function of one kinase is compensated by other kinases with overlapping substrate specificity. Not only the phosphorylation but also the expression of numerous proteins was increased. Changes in the protein expression profiling were indicative of mild oxidative stress and activated UPR (Unfolded Protein Response).

Conclusions: Our findings suggest that *S. alba* has a complex effect on yeast cells: salicin-7-sulfate decreases protein phosphorylation, while salicin acts as an inhibitor of TORC1 signaling.

Keywords

Longevity; white willow; *Salix alba*; lipids, Sch9; inhibitor; rapamycin

Introduction

Several aging pathways are evolutionarily conserved between yeast and human, therefore yeast research became increasingly involved in studying aging [1,2]. Numerous chemical libraries were tested in *S. cerevisiae* in order to discover an ultimate "fountain of youth"-a unique chemical compound that inhibits pro-aging processes and signaling pathways increases longevity and renders organisms immortal [3].

The so-called Forward Chemical Genetics (FCG) screen yielded a handful of chemical compounds, often with previously unknown properties, that improved longevity phenotype in various experimental models [4]. The already known compounds and substances were tested in the scope of Reversed Chemical Genetics (RCG) in order to determine whether or not, in addition to already known physiological effects, they exhibit age-delaying properties [5]. The best-known example of such a compound is rapamycin, a naturally existing macrolide with immunosuppressing functions in humans, which inhibits pro-aging TORC1 signaling in several species and delays aging [6,7]. Other known anti-aging chemicals include resveratrol, spermidine, aspirin, caffeine, etc. [6,8,9].

In chemical screens, the compounds are often tested in combination with one another [6]. Known as convoluted testing, such an approach is applicable to exceptionally large libraries or when assayed compounds have synergistic effects [10-12].

Yeast aging is regulated by several pathways, including TORC1, cAMP-PKA, SNF1/AMPK pathways, and the ATG1 autophagy pathway [13-17]. These pathways regulate, *via* protein phosphorylation, the variety of longevity-defining processes, including gluconeogenesis, glyoxylate cycle, glycogen synthesis and degradation, mitochondrial respiration, maintenance of nuclear and mitochondrial genomes, mitochondrial lipid biosynthesis, and regulation of the Fkh1/Fkh2 (yeast FOXO) gene expression [18-20].

The TORC1 signaling could be inhibited by rapamycin, and most aging pathways could be regulated by nutrients, including glucose and nitrogen, and also genetically, by introducing gene deletions or mutations in genes of interest [6,14,15]. Polyphenol resveratrol, for example, is known to act as an anti-oxidant that decreases apoptosis [8]. The polyphenol-rich extract of cocoa also acts as an anti-oxidant, and it increases longevity in yeast and mammalian models [21,22].

A great emphasis was placed on testing the natural substances with already known physiological effects. We previously described six plant extracts that influence age-related yeast signaling pathways [12]. These extracts include *C. racemosa* (black cohosh), *V. officinalis* (valerian), *P. incarnata* (passionflower), *G. biloba* (maidenhair tree), *A. graveolens* (celery), and *S. alba* (white willow), which are part of the TCM (Traditional Chinese Medicine) network pharmacology supply [23,24]. Each extract acts as a geroprotector which delays the onset of chronological aging and decreases its rate. The implicated mechanisms involve the hormetic stress response, reduced concentrations of Reactive Oxygen Species (ROS), decreased oxidative damage to cellular proteins and lipids, enhanced resistance to oxidative stress; decreased accumulation of lipid droplets, and decreased levels of free fatty acids [12,25].

We specifically became interested in the effect of the white willow *Salix alba* extract, (also known as PE21-plant extract 21) on yeast longevity. *Salicaceae* family is characterized by the presence of phenolic glycosides, including salicin and salicin-7-sulfate, and it also contains polyphenols [25,26] (Figure 1).

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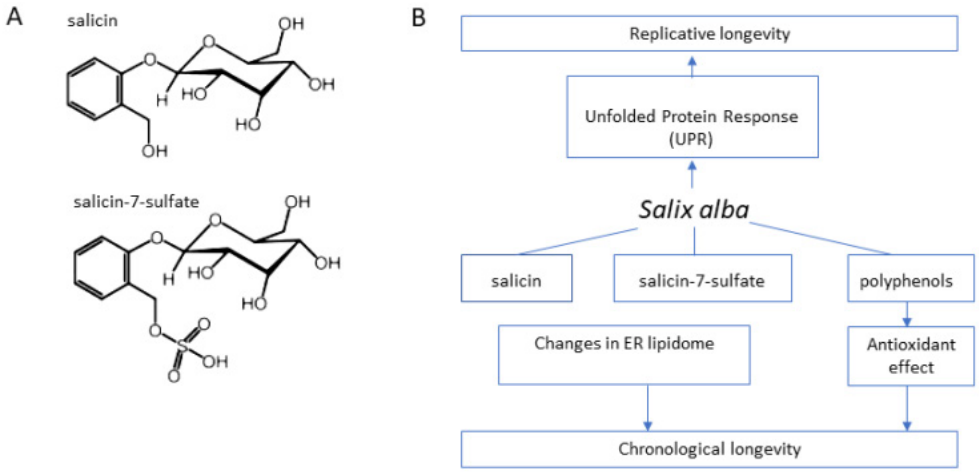


Figure 1: (A): Plant extract of *Salix alba* contains salicin and salicin-7-sulfate; (B): Previously elucidated effects of *S. alba* on yeast longevity [25].

Salicin is a β-glucoside composed of D-glucose and salicylic acid (Figure 1A). This is a naturally occurring prototype of aspirin, primarily known for its anti-inflammatory effect [27]. Aspirin increases longevity in some multicellular models, however, it has no striking effect on yeast longevity [6]. Moreover, aspirin could elicit a negative effect on yeast physiology since it decreases the production of 3-OH oxylipins, the products of incomplete β-oxidation of fatty acids required for yeast cell adhesion and reproduction [28].

In mammalian models, aspirin and salicin are known to inhibit the NF-κB protein complex, which transcriptionally controls cytokine production and which is known to be a part of the pro-inflammatory ROS-activated ERK pathway: aspirin prevents degradation of the NF-κB inhibitor IκB, therefore NF-κB is unable to enter nucleus [29,30]. In addition, aspirin and salicin appear to directly block the NF-κB dependent transcription from the Igκ enhancer. The binding of salicin to NF-κB-interacting and other proteins is stronger than that of aspirin. Salicin is known to bind to various enzymes, including the mammalian cyclooxygenase-2 [31].

After being endocytosed by yeast cells, salicin travels to the Endoplasmic Reticulum (ER), where it introduces changes in lipid composition of the ER membrane [25] (Figure 1B). Specifically, it increases the synthesis of glycerophospholipids at the expense of decreased amounts of triacylglycerol. Salicin also causes activation of the Unfolded Protein Response (UPR), a pancellular stress response that occurs following the accumulation of unfolded or misfolded proteins in the ER [25,32]. The activation of UPR in response to salicin was predicted based on the overexpression of various proteins with a role in the protein folding [25]. The upregulation of these proteins could occur due to hypoacetylation when lysine residues of the ER-localized proteins fail to become acetylated, and the proteins could no longer be transported into Golgi [33]. Salicin could potentially trigger protein hypoacetylation inside the ER. The implicated mechanism is likely to involve the glucosidase-dependent reaction, which transforms salicin into a salicylic acid, which in turn scavenges acetyl groups inside the ER lumen [34]. Noteworthy, either decreased acetylation of the ER proteins or salicin-dependent changes in the ER lipidome could contribute to the detected ER stress and UPR [25].

While inducing stress, UPR is known to decelerate age-related decline in protein homeostasis and increase replicative longevity [25,35]. However, various chemicals that induce ER stress and activate the UPR in tissue or yeast culture system, including tunicamycin, thapsigargin, Brefeldin A, dithiothreitol (DTT), and MG132, are not considered true anti-aging compounds despite their positive effect on replicative longevity [35,36].

In this study, we show that salicin acts as an inhibitor of TORC1 activity, which decreases activation of pro-aging Sch9 kinase. TORC1-dependent activation of Sch9 kinase (the yeast homolog of mammalian S6K) is a centerpiece of yeast aging [13]. The activation itself is a two-step process. The PKH1/PKH2 kinases phosphorylate T570 residue within the activation loop of Sch9, while TORC1 phosphorylates five additional residues in the Sch9 C-terminus [13]. TORC1-dependent phosphorylation of Sch9 activates series of phosphorylation events downstream of Sch9 that increase protein synthesis and aggregation while decreasing autophagy, the function of the vacuole, and the ability to re-enter the cell cycle [37-40].

The deletion of the SCH9 gene (sch9Δ) strongly increases chronological lifespan (CLS) [14,41]. In contrast to sch9Δ, the deletion of TOR1 (tor1Δ), an upstream activator of Sch9, has a relatively modest effect on yeast longevity. It appears that sch9Δ is associated not only with the loss of specific kinase activity but also with strong expression of SOD2 (Mn^{2+}- dependent mitochondrial superoxide dismutase) and other components of mitochondrial electron transport chain that sequester free oxygen radicals and decrease oxidative damage to yeast cell [41,42]. Therefore, a not actual loss of the Sch9 kinase activity, but associated with this loss changes in gene expression appear to define sch9Δ longevity phenotype [41-43].

Sch9 kinase is also crucial for regulating Replicative Lifespan (RLS). Sch9-dependent phosphorylation of Rim15 kinase inhibits the release of a Cdc55 phosphatase from its inhibitory protein complex, and consequently, it blocks cell-cycle re-entry in aging yeast cells [40]. Several other kinases, including PKA and Pho80-Pho85, are also responsible for the phosphorylation of Rim15 and G0 entry, however, they primarily function in young yeast in the context of meiosis [44]. In contrast, TORC1-dependent phosphorylation of Sch9 functions mostly to terminate age-related G0 arrest.

In addition to salicin, *S. alba* contains salicin-7-sulfate [26] (Figure 1A). This chemical derivative is predicted to block serine and threonine residues on the protein surface to inhibit their phosphorylation [26,37]. We reasoned that either alone or in combination with salicin, salicin-7-sulfate influences protein phosphorylation inside the yeast cell so that distinct phosphorylation pathways become affected.

Previous studies showed that prolonged exposure to *Salix alba* could lift TORC1-dependent inhibition of SNF1-AMPK signaling, and to also enable the activation of the PKA (cAMP-protein kinase A) pathway [12,15]. The Snf1 kinase (yeast prototype of mammalian AMPK) enables the transcription of glucose-repressed genes, thereby altering the metabolic outlook of yeast cells towards the increased lipid catabolism, including the increased β-oxidation of fatty acids; while increasing peroxisomal and mitochondrial function [45,46]. The glucose-sensitive PKA pathway, which regulates longevity, may lead to filamentous growth and autophagy [14,15]. In addition, it manifests in increased phosphorylation and overexpression of several Heat-Shock Proteins (HSPs) that were detected in the *S. alba* treated cells [25].

In this study, we show that extract of *S. alba*, which contains salicin and salicin-7-sulfate, decreases the phosphorylation and activation of Sch9. Salicin acts either upstream TORC1 or at the TORC1 level, as a weak inhibitor of this kinase complex and it indirectly decreases Sch9 phosphorylation. Salicin-7-sulfate, on the other hand, is predicted to directly inhibit Sch9 phosphorylation by blocking phosphorylatable residues on the protein surface. Both salicin and salicin-7-sulfate appear to increase the in vivo requirement for kinase activity, thereby driving up protein phosphorylation and expression. Our findings suggest that the longevity effect of *Salix alba* involves negative regulation of TORC1 signaling, however, changes in protein phosphorylation appear to coincide with the UPR and oxidative stress.

Material and Methods

Yeast growth

S. cerevisiae strains in the *BY4742* backgrounds were grown in a synthetic medium with 2% glucose. An extract from the bark of *Salix alba* (PE21) was added to the final concentration of 0.1% [12].

Protein phosphorylation

Protein phosphorylation was detected by SDS-PAGE and immunoblotting. The Sch9 C-terminal fragment was obtained as described [13]. Full-length Sch9 was detected using polyclonal antibodies, while C-terminal fragment was detected using a 12CA5 antibody. Other antibodies used are indicated in figure legends. YPK1 was a Myc-tagged protein. Inhibition of protein phosphorylation was determined following a 6-hour long incubation of yeast cells with the components indicated.

DNA microarray

DNA microarray was performed as described [41], using GeneChip Yeast Genome 2.0 Array (Thermo Fisher Scientific). Protein quantitation using mass-spectrometry was as described [25].

Results

Initial FCG analysis of more than 70 plant extracts revealed that

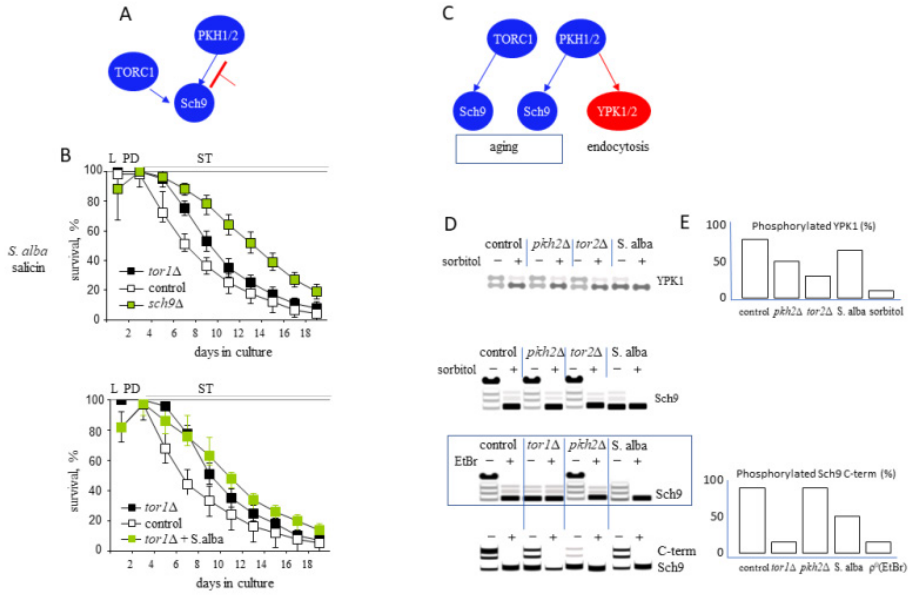


Figure 2: (A): Activation of Sch9 is a two-step process, which involves PKH1/PKH2- and TORC1- dependent phosphorylation; (B): Longevity curves. L- logarithmic phase of growth, PD- post-diauxic, and S is stationary phase of growth; (C): In addition to their role in the Sch9 activation, PKH1/PKH2 are responsible for the phosphorylation of YPK1 and YPK2 kinases at plasma membrane and endocytosis; (D): top: Western blot depicts the phosphorylation of YPK1 under various experimental conditions. "Sorbitol +" are the negative-control samples: adding 1M sorbitol to cell cultures triggers osmotic stress that abolishes phosphorylation of YPK1/YPK2. Salicin does not significantly influence the phosphorylation of YPK1. Salicin was added to cultures at the 0.5% concentration. tor2Δ mutation eliminates the principal regulator of the YPK/YPK2. Bottom: Western blot using anti-Sch9 antibody shows that phosphorylation of Sch9 is decreased when salicin is added to cells. "EtBr +" is a negative control for protein phosphorylation: EtBr renders yeast cells ρ0, i.e. mtDNA- deficient, and causes dephosphorylation of numerous proteins; (E): Quantitation of chemiluminescence.

several of them, including salicin-containing white-willow bark extract, increase the chronological longevity of TOR1Δ cells. TOR1Δ gene knockout lacks the principal kinase component of the TORC1 complex. An additional branch of the regulation involves Pkh1/Pkh2 serine-threonine kinases which act upstream TORC1 (Figure 2A).

Culture aliquots were withdrawn daily, and after 10^3-10^4 fold dilution, cells were plated on the YPD plates to determine their ability to form colonies (% survival) (Figure 2B). As shown previously, tor1Δ mutation, which decreases phosphorylation of pro-aging Sch9 kinase, causes a moderate (20%) increase in mean CLS. The salicin-rich extract prepared from white-willow bark (*Salix alba*) was increasing the mean survival interval by at least 50%.

The activation of Sch9 depends on the PKH1/PKH2 kinases and TORC1 kinase complex which phosphorylate one residue within the Sch9 activation loop and five or more residues in the C-terminus, respectively (Figure 2C)[13]. PKH1/PKH2 also have a role in endocytosis, in which they activate YPK1/YPK2 kinases. As shown in Figure 2D, the phosphorylation of YPK1 was not impaired in presence of salicin, suggesting that it has little effect on the PKH1/PKH2 kinase activity overall, and YPK1/YPK2-dependent endocytosis was not visibly affected. Conversely, the phosphorylation of Sch9 was decreased in presence of the *S. alba* extract (Figure 2D), suggesting that it could inhibit TORC1-dependent phosphorylation of Sch9.

The experimental proxy of salicin-7-sulfate heparin decreases phosphorylation of Sch9

The comparative analysis of salicin and rapamycin chemical structures suggests that salicin is unlikely to act as a potent inhibitor of TORC1 activity (Figure 3A). The additional inhibition could occur due to the presence of salicin-7-sulfate, which was also detected in the *S. alba* extract and which is predicted to bind to specific amino-acid residues on the protein surface, thereby decreasing protein phosphorylation [26,37].

The analysis of chemical structure suggests that salicin-7-sulfate could have a similar to heparin effect on protein modification and protein binding. Heparin is a naturally occurring dimerized glycosaminoglycan that contains sulfates.

There are two consensus sequences for heparin-protein recognition [-X-B-B-X-B-X] and [-X-B-B-B-X-X-B-X-], where X are hydropathic (Ala, Gly, Ile, Leu) or phosphorylatable polar residues (Ser, Thr, Tyr), and B are basic residues (Lys, Arg, and sometimes His). It was suggested that N-acetylated glucosamine (GlcNAc) and not sulfite groups are involved in the binding to Lys, Arg, and His. Consequently, it is not heparin but the GlcNAc-containing heparan-sulfate has a prominent role in extracellular signaling [47-49]. Notwithstanding, heparin is frequently used as an "experimental proxy" to study the effects of heparan-sulfate on protein binding, regulation of signaling cascades, etc.

We determined that heparin decreases phosphorylation of Sch9 (Figure 3B). The inhibition was >5.0 fold when heparin was present at 10,000 U/ml concentration at which it is known to inhibit metabolic activity and the biofilm formation by yeast cells (in *C. albicans*) [50]. Partial inhibition of phosphorylation was detected when >2,000 U/ml of heparin were present in cell culture. Salicin, on the other hand, was not inhibiting activation of Sch9.

The phosphorylation of histone H1 was not decreased in presence of heparin. Moreover, it was likely increased, hence Hho1 is phosphorylated by several kinases, including PKA, and the phosphorylated form appeared enriched in heparin-treated cells, suggesting probable activation of the PKA pathway (Figure 3B, lower panel).

We then sought to also assay the phosphorylation of Snf1 to determine that this AMPK could also be phosphorylated when cells

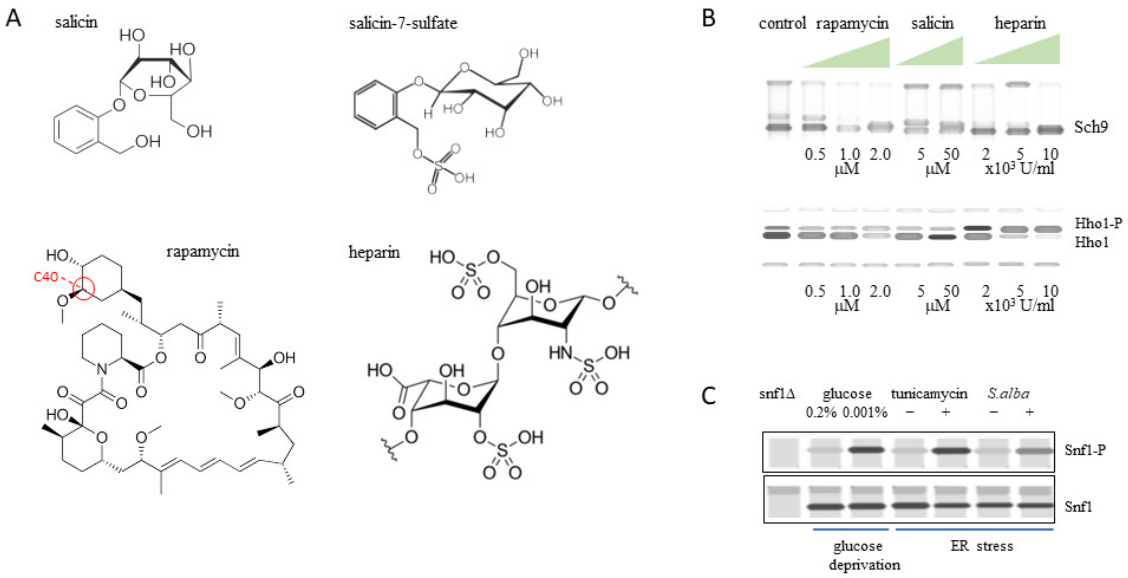


Figure 3: (A): The structure of salicin, a principal component of *S. alba* extract, is different from that of rapamycin, the TORC1 inhibitor. The extract also contains salicin-7-sulfate, which could be compared to heparin; (B): Inhibition of the Sch9 phosphorylation. Sulfate-containing heparin was used in place of salicin-7-sulfate. The phosphorylation of histone H1 is a positive control; the histone was detected using rabbit anti-Hho1 antibodies; (C): Snf1 is phosphorylated in presence of *S. alba*. Snf1 was detected using anti-phospho-AMPK and anti-Snf1 antibodies. 2.5μm/ml tunicamycin was applied for 3.5 h.

were exposed to *S. alba* plant extract (Figure 3C). This finding is consistent with the previously suggested activation of UPR in cells that were cultured in presence of *S. alba* [19,25]. The 3.5 h-long incubation with tunicamycin was a positive control for UPR activation.

Protein phosphorylation is increased in the *S. alba*-treated cells

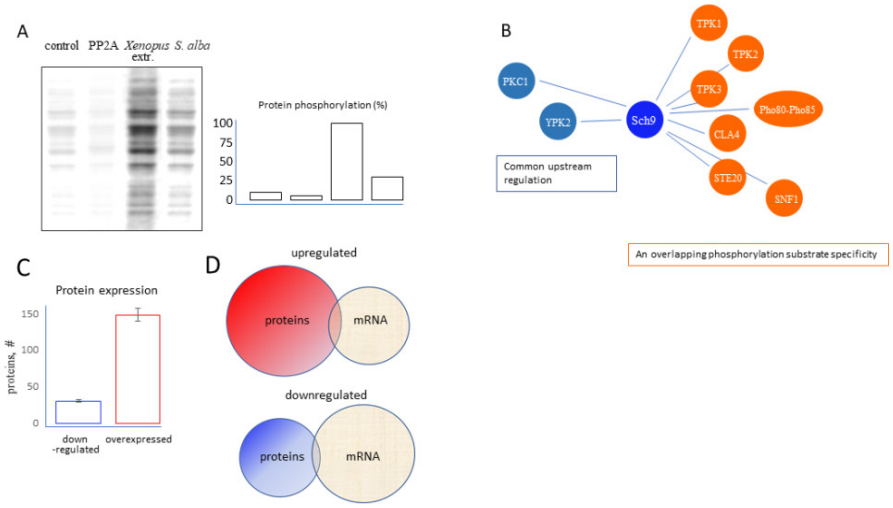
Despite the predicted inhibitory effect of salicin-7-sulfate on protein phosphorylation, an overall phospho-modification of yeast proteins was not decreased in yeast cells, and it was possibly increased instead. The increased protein phosphorylation could be achieved under various experimental conditions (Figure 4A), including kinase overexpression (HRR25), supplying the recombinant kinase activity (along with ATP and $MgCl_2$) or the naturally obtained kinase activity, for example in a form of Xenopus oocyte extract [51].

Protein phosphorylation is also increased in cells with a deregulated kinase activity (Figure 4B). The decreased activation of Sch9 triggers a feedback response that increases protein phosphorylation inside the yeast cell. Previously, we showed that deletion of a small regulatory peptide within Ste20 kinase (Ste20Δ269-289), which has an overlapping with Sch9 phosphorylation substrate specificity, increases phosphorylation of numerous proteins, thereby creating a swapped-kinase effect [52] (Figure 4B). We hypothesize that a decreased activation of Sch9 kinase in presence of the *S.alba* plant extract creates such an effect. The kinases whose function is increased are likely to include AGC kinases (PKC1, YPK1) with a similar to Sch9 mechanism of activation, PKA (protein kinase A), and several other functionally unrelated serine-threonine kinases which possess nevertheless and overlapping with Sch9 phosphorylation substrate specificity (Figure 4B). The subset of such kinases was elucidated previously [53,54].

Protein overexpression in the *S. alba*-treated cells

The expression of many proteins was increased in exposed to *S. alba*-extract yeast cells [25] (Figure 4C-D). The increased levels of some proteins were linked to the activation of UPR, while overexpression of some heat-shock proteins and other proteins was potentially coupled to the increased protein phosphorylation, which frequently coincides with protein overexpression.

However, as shown in figure 4D, the mRNA and protein overexpression profiles were poorly matched, suggesting that protein



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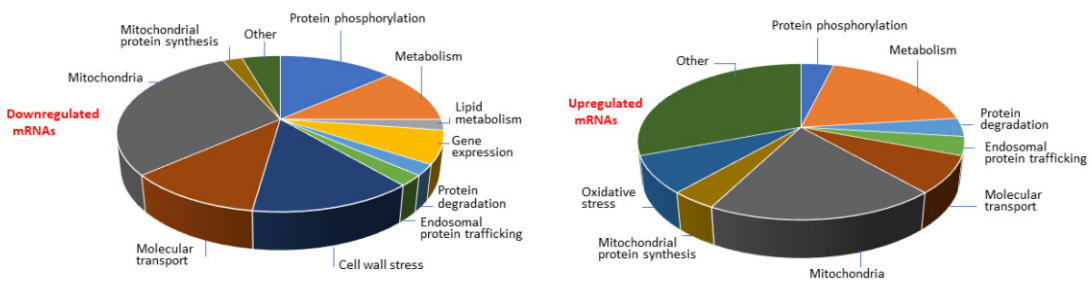

Figure 5: Typical changes in the mRNA expression in cells with a swapped Sch9-like kinase activity.

overexpression is not linked to transcription and that increase in protein levels occurs at the post-transcriptional or post-translational level. The typical mRNA expression profile of cells with the strongly increased kinase activity is shown in Figure 5. Mitochondrial-function genes were equally likely to be up-regulated or down-regulated, while oxidative-stress genes were up-regulated, suggesting oxidative stress. Protein-phosphorylation genes were repressed, thereby indicating excess kinase activity inside the cell. The overexpression of some oxidative-stress and mitochondrial-protein synthesis genes ("upregulated mRNAs", Figure 5, right) could be interpreted as part of UPR. The same appears to be the case for the upregulated protein-degradation genes.

Several lipid-metabolism genes were downregulated and some endosomal-trafficking and cell-wall stress genes were also repressed (1.5-2.0 fold, $p<0.05$) suggesting, that a PKH1/PKH2-dependent regulation of YPK1/YPK2 could be compromised in response to salicin.

Discussion

Previous studies show that a salicin-rich extract of *S. alba* increases longevity [12,25]. The underlying mechanisms are thought to involve changes in lipid metabolism, including the decreased accumulation of Free Fatty Acids (FFA), decreased liponecrosis, and probable activation of the UPR, which stimulates replicative longevity or RLS (Replicative Life Span) [2,25,40].

The CLS (Chronological Life Span) was also increased [12] (Figure 2B). In part, the increase was associated with a drop in the levels of FFA and decreased likelihood of liponecrosis [12,25,45]. By itself, however, the decrease in FFA levels does not warrant longevity, because neither extreme calorie restriction nor the absence of the FA-synthesizing, FA-transporting, and FFA-cleaving genes increases maximal CLS.

Yeast longevity is primarily linked to the inhibition of TORC1-dependent phosphorylation of Sch9 kinase (the yeast homolog of mammalian S6K) and to modulated mitochondrial function: in order to become long-lived, yeast cells must decrease oxygen consumption and oxidation during early growth, so that mitochondria remain functional at the late stationary phase [11,55].

The extract of the *Salix alba* plant was shown to trigger mild oxidative stress during the first 2 days of growth [25]. This temporary stress and a decreased mitochondrial membrane potential ($\Delta\Psi$) are consistent with a steroid-compound-induced longevity phenotype [11,55]. The stress then becomes eliminated due to the presence of polyphenols and also due to detected overexpression of the oxidative-stress proteins (e.g. Ctt1, Gpx2, Grx1, Grx2, Hsp31, Sod1, Trx1, and Trx2) [25]. The recovery was even more successful when white willow was used in combination with a black-cohosh root extract, which contains the stimulating mitochondrial dynamics and function steroid compound [12,21].

In this study, we show that in addition to the previously shown effects on lipid metabolism, UPR, and protein expression, the extract of *S. alba* appears to also influence protein phosphorylation. These two sets of findings are not in conflict, since the SCH9 kinase gene was previously linked to the regulation of lipid metabolism [43,54].

The TORC1-dependent activation of Sch9 appeared to decrease in response to *S. alba* (Figure 2D). The best-known TORC1 inhibitor is rapamycin (a.k.a. sirolimus) has several analogs including everolimus, temsirolimus, and ridaforolimus [6,56,57]. All these compounds share a central macrolide chemical structure and have the unique R groups at the C40 position (Figure 3A)[58]. In yeast, rapamycin binds to the evolutionary conserved FKBP12 domain of Tor1 to mimic nutrient deprivation, inhibit Sch9, and to also decrease ribosomal assembly and protein synthesis. Independently of Tor kinases, rapamycin is known to decrease yeast nucleotide excision repair [59,60].

There is no structural resemblance between salicin and rapamycin (Figure 3A), however similarly to aspirin, salicin could act on or upstream TORC1 with the inhibitory effect being additionally amplified by salicin-7-sulfate. In this study, a sulfate-containing heparin glycosaminoglycan was used to imitate the effect of salicin-7-sulfate. Unlike the monomeric salicin-7-sulfate, the heparin exists as a dimer which then forms oligomers, and has a greater than a salicin-7-sulfate number of sulfate groups on its surface [47-49]. At high concentration, heparin, which becomes endocytosed by yeast cells, is known to modify cell metabolism and it is also known to preserve the formation of translation preinitiation complex and ribosomes, thereby decreasing longevity. In mammals, the LMW (Low-Molecular-Weight) forms of heparin increase longevity in a manner that doesn't involve kinases. However, heparin was shown to inhibit the phosphorylation and autonomous activity of $Ca^{(2+)}$/calmodulin-dependent protein kinase II in vascular smooth muscle cells, and it also inhibits casein type II kinases (YCK1 and YCK2 in yeast) while stimulating the phosphorylation of some protein substrates by the catalytic subunit of cAMP-dependent Protein Kinase (PKA) [61,62].

In contrast to rapamycin, which directly inhibits TORC1 signaling, the salicin and salicin-7-sulfate are thought to decrease the Sch9 phosphorylation in part by redirecting the signaling towards PKA or AMPK longevity pathways (Figure 3, B and C)[2,14,15,19]. Sch9 (TORC1) and PKA pathways co-operate in the induction of specific anti-aging processes: the simultaneous inactivation of Sch9 and PKA is required for example to activate autophagy, a process by which yeast cell consumes the portions of its organelles to increase longevity [63].

Because the exact configuration of yeast metabolism and yeast longevity depends on glucose concentration, as many as four pathways are involved in glucose sensing, with the Ras2/Gpa2 and PKA pathways acting at the plasma membrane, the TORC1 signaling present in the cytosol and in the nucleus, and Snf1/AMPK pathway acting predominantly towards the nucleus to regulate histone acetylation and gene expression in the cultures with exceptionally low glucose [15,16,19,64,65].

As already mentioned, together with salicin-7-sulfate, salicin decreases the TORC1 signaling. While salicin-7-sulfate could, similarly to heparin, directly block the phosphorylation sites on the protein surface, the inhibition is thought to be indirect overall and it is thought to be linked to the ER stress response and the AMPK/Snf1 activation [25] (Figure 3C).

It is possible that in addition to Snf1, other kinases became activated since overall phosphorylation was increased in cells grown in presence of *Salix alba* extract (Figure 4A). The decreased activation of Sch9 is known to cause swapping the kinase activity in favor of such kinases as Ste20 and several others that have the RXRXKS or XXRRXS consensus sites [53,54] (Figure 4B). These kinases could have different from Sch9 mechanisms of activation. In the case of Ste20 for example, the upstream regulator is not TORC1 but the Cdk (Cdc28-Cln1,2), and in absence of the phosphorylation, Ste20 could be activated by binding Cdc42. The Sch9-related sharing of kinase activity and the

implicated kinases were studied in detail previously [53-54].

The inhibition of Sch9 phosphorylation in *S. alba*-treated cells and other changes in protein phosphorylation are regulated by: (i) the ER stress response, i.e. UPR [25]; (ii) blocking the phosphorylation sites in presence of salicin-7-sulfate (Figure 3B); (iii) swapping a kinase activity related to Sch9 inhibition [54].

Similarly to salicin, aspirin is known to act against TORC1: it decreases the phosphorylation of S6K in response to the TNF-α signaling [57]. One of the implicated mechanisms involves aspirin-dependent activation of AMPK, which inhibits mTORC1 activity either through phosphorylation of tumor suppressor TSC2 or through the inhibitory phosphorylation of RPTOR, the essential component of mTORC1 [56, 57]. Here, aspirin-dependent inhibition of TORC1 involves the cross-talk between signaling pathways and is not related to the ER stress response. Moreover, in most experimental models, the ER stress is known to activate and not inhibit the TOR pathway. In Drosophila, for example, activating transcription factor 6 (Atf6), a major stress-responsive ER transmembrane protein, is responsible for ER stress-induced TOR activation [66].

We, therefore, suggest that, despite the UPR-related activation of the AMPK pathway, which is predicted to inhibit TORC1, other mechanisms are engaged at the level of kinase inhibition and kinase-swapping activity that decrease activation of Sch9 following prolonged exposure to *Salix alba*.

The increased protein phosphorylation is often concurrent with the increased protein levels. In the *S. alba*-treated cells, protein overexpression was linked to the: (i) activated UPR, (ii) transcriptionally induced gene expression that is not related to UPR, and (iii) post-transcriptional overexpression. The transcriptionally induced protein expression included the oxidative-stress responsive genes and mitochondrial protein genes (including primarily the mitochondrial- ribosomal protein (mRP) genes). The activation of mRP genes could indicate the decreased activation of Tor1 and Tor2, which regulate the expression of cytoplasmic ribosomal-protein genes, thereby also confirming a decreased TORC1 signaling in the *S. alba*-treated cells.

Other affected proteins included the heat-shock and functionally related proteins, such as Sis1(Hsp40 co-chaperone), Ssa4, Sse2, Sti1 (Hsp90 co-chaperone) and some oxidative-stress inducible proteins (GPX2 and GRX2) [25].

The described in this study regulatory effects of salicin and *S. alba* are rather important. There is an ongoing search for novel molecules and natural remedies to substitute rapamycin. Known for its immunosuppressive and cancerogenic effects, rapamycin becomes an increasingly unwanted chemical today, when we are dealing with the consequences of the COVID-19 pandemic. Future studies will establish to which extent the plant extract of *S. alba* could substitute the anti-inflammatory and immune-suppressive chemicals that are currently in use. It will be a valuable find that will enable us to discriminate against rapamycin, metformin, and aspirin whose positive inhibitory effects are overshadowed by their negative impacts.

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