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Tanshinones extend chronological lifespan in budding yeast *Saccharomyces cerevisiae*

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Abstract Natural products with anti-aging property have drawn great attention recently but examples of such compounds are exceedingly scarce. By applying a high-throughput assay based on yeast chronological lifespan measurement, we screened the anti-aging activity of 144 botanical materials and found that dried roots of *Salvia miltiorrhiza* Bunge have significant anti-aging activity. Tanshinones isolated from the plant including cryptotanshione, tanshinone I, and tanshinone IIa, are the active components. Among them, cryptotanshinone can greatly extend the budding yeast *Saccharomyces cerevisiae* chronological lifespan (up to 2.5 times) in a dose- and the-time-of-addition-dependent manner at nanomolar concentrations without disruption of cell growth. We demonstrate that cryptotanshinone prolong chronological lifespan via a nutrient-dependent regime, especially essential amino acid sensing, and three conserved protein kinases Tor1, Sch9, and Gcn2 are required for cryptotanshinone-induced lifespan extension. In addition, cryptotanshinone significantly increases the lifespan of *SOD2*-deleted mutants. Altogether, those data suggest that cryptotanshinone might be involved in the regulation of, Tor1, Sch9, Gcn2, and Sod2, these highly conserved longevity proteins modulated by nutrients from yeast to humans.

Keywords Chronological lifespan · Cryptotanshinone · Amino acid · Tor1/Sch9/Gcn2/Sod2 · Nutrient-sensing pathway · Conserved longevity mechanism

Introduction

Natural products with anti-aging capacity have been receiving great attention in the academic community. Compared with compounds that target a single age-related disease, compounds with anti-aging activity could increase the overall quality of life by extending the healthy lifespan while delaying the onset of aging-associated diseases, such as cardiovascular disease, cancer, osteoporosis, diabetes, hypertension, and Alzheimer's disease (Fontana et al. 2010). So far, a few natural products such as resveratrol (Howitz et al. 2003; Baur and Sinclair 2006) and rapamycin (Harrison et al. 2009) have targeted conserved longevity mechanisms and have been proposed to act as dietary restriction mimetics to slow aging in multiple model organisms (Steinkraus et al. 2008). Longevity effects of resveratrol and rapamycin, as well as the other promising longevity factors (e.g. Sir2, Tor1, Sch9, Ras2), were first discovered in a yeast-aging model (Fabrizio et al. 2001; Howitz et al. 2003; Powers et al. 2006). The budding yeast *Saccharomyces cerevisiae* has served as a leading model organism for studying evolutionarily conserved mechanisms relevant to human aging and age-related diseases (Kaeberlein 2010; Longo et al. 2012).

We established a high-throughput assay based on measuring the yeast chronological lifespan in order to rapidly identify the anti-aging compounds from natural products (Wu et al. 2011). By applying this assay, we have screened 144 plant materials, including traditional Chinese herbs and legumes (see supporting materials for details), and found that the roots of *Salvia miltiorrhiza* Bunge (Danshen in Chinese) have high activity. Herein, we reported the in-depth study on this plant.

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S. miltiorrhiza Bunge is a commonly used traditional Chinese medicine (TCM) for the treatment of coronary heart disease, hyperlipidemia, cerebrovascular diseases, angina pectoris, and acute ischemic stroke (Zhou et al. 2005). Recently, a drug based on the root extract of *S. miltiorrhiza* Bunge has passed Food and Drug Administration (FDA) phase II clinical trials for treatment of cardiovascular disorders (Xu 2011). Previous investigations have shown that cryptotanshinone, a major tanshinone in *S. miltiorrhiza* Bunge, possesses multiple biological activities relevant to chronic diseases, such as stroke (Yu et al. 2007), Alzheimer's disease (Yu et al. 2007), atherosclerosis (Zhou et al. 2005), cancer (Chen et al. 2010; Chen et al. 2012), and type 2 diabetes (Kim et al. 2007). Moreover, studies on the mechanism of action indicate that cryptotanshinone involves mediation of several signaling pathways that are highly conserved in multiple species, such as the mammalian target of rapamycin (mTOR) pathway (Chen et al. 2010; Johnson et al. 2013), AMP-activated protein kinase (AMPK) pathway (Kim et al. 2007; Chen et al. 2012), and phosphatidylinositol 3-kinase (PI3K) pathway (Don et al. 2007). Recent studies have suggested that these pathways are involved in the regulation of aging among different species (Steelman et al. 2011; Salminen and Kaarniranta 2012). Thus, it is plausible that cryptotanshinone could act as an anti-aging compound by targeting these conserved longevity pathways. To address whether cryptotanshinone induces longevity, here, we used a high-throughput assay in the yeast chronological aging model and demonstrated that cryptotanshinone extends chronological lifespan (CLS) significantly. In addition, our data indicate that a few evolutionarily conserved protein kinases may mediate the CLS extension by cryptotanshinone in budding yeast *S. cerevisiae*.

Materials and methods

Materials

The wild-type strain *S. cerevisiae* BY4742 (*MATα his3*Δ1 *leu2*Δ0 *lys2*Δ0 *ura3*Δ0) and single-gene deletion mutant strains in the BY4742 genetic background were obtained from Thermo Scientific Open Biosystems (Huntsville, AL, USA). The culture of each yeast reference strain was aliquoted into 10 μL and stored at –80 °C. All L-amino acids were from GL Biochem (Shanghai, China), and yeast nitrogen base (YNB), peptone, agar, and yeast extract were from Amresco (Solon, OH, USA). YPD Broth, tanshinone IIA, tanshinone I, cryptotanshinone, and other chemicals were from Sigma-Aldrich Chemical Company (Singapore). HPLC grade acetone, acetonitrile, and methanol were obtained from Tedia Company (Fairfield, OH, USA). The 96-well polystyrene microplates with flat bottom were purchased from Fisher Scientific (Nunc, Rochester, NY, USA). Other solvents were of HPLC grade from commercial sources.

Lifespan and yeast cell growth assay

The determination of chronological lifespan (CLS) of yeast was carried out according to the method described previously (Wu et al. 2011). In brief, the yeast cells were prepared by transferring a streaked strain from frozen stocks onto YPD (1 % yeast extract/2 % peptone/2 % dextrose) agar plates. After incubating the cells at 30 °C for 2 days, a single colony was picked and inoculated into a 1.0-mL YPD liquid medium (Sigma YPD Broth, Louis, MO, USA) in a 4-mL glass sample vial and cultured at 30 °C for 2 days in a flat incubator at 200 rpm. The 2-day YPD culture was diluted with autoclaved 18 mΩ Milli-Q grade water (1:10) and stored in a refrigerator at 4 °C for 2 days. After 2-day incubation at 4 °C, 5 μL (≈1×10^4 cells) of the diluted culture was transferred to 1.0 mL of different aging media and maintained at 30 °C, 200 rpm for the entire experiment. Tanshinones in methanol or dimethyl sulfoxide (DMSO) with several concentrations (2.0 μL) were added at initial inoculation (0 h), exponential phase (12 h), and stationary phase (24 h). Each experiment was performed at least in triplicate. Cell cultures were incubated at 30 °C without replacing the aging medium throughout the experiment. After 2 days of culture in aging media, the cells reached stationary phase and the first age-point was then taken. Subsequent age-points were taken every 2–4 days. For each age-point, 5.0 μL of the mixed culture was pipetted into each well of 96-well microplate (Nunc, Rochester, NY, USA). One hundred microliter YPD medium was then added to each well. The cell population was monitored with a PowerWave XS microplate reader (BioTek, Winooski, VT, USA) by recording OD660 every 5 min during 12–24 h.

Intracellular ROS quantification of yeast cells

To quantify intracellular reactive oxygen species (ROS) level of yeast cells, 2 μL H_2DCFDA (Invitrogen, Eugene, OR, USA) from a fresh 5-mM stock solution in DMSO was added into 1.0 mL yeast aging culture at 30 °C for 1 h. The culture was then washed twice in sterile distilled water and suspended in 1.0 mL of 50 mM Tris/Cl buffer (pH 7.5). Twenty microliter of chloroform and 10 μL of 0.1 % (*w*/*v*) sodium dodecyl sulfate (SDS) were added, and the cells were incubated at 200 rpm for 30 min to allow the dye to diffuse into the buffer. The culture was centrifuged at 5,000 rpm for 5 min, and the fluorescence of the supernatant was measured using a Synergy HT microplate reader (Biotek, Winooski, VT, USA) with excitation at 480 nm and emission at 520 nm.

Data analysis

The raw data from the microplate reader were exported to Excel (Microsoft, San Leandro, CA, USA). From the growth curves, the viability of the yeast can be obtained according to our previous report (Wu et al. 2011). Survival integral (SI) of each aging culture was defined as the area under the survival curves (AUC). The analysis of variance for each set of biological replicates was carried out with the SAS statistical program (version 9.2, SAS Institute Inc, Cary, NC, USA), and differences between the means of SI for treatments were determined by Duncan's multiple range test at $P<0.05$.

Results

Tanshinones, particularly cryptotanshinone, extend yeast lifespan in a concentration and the-time-of-addition dependent manner

Taking advantage of the yeast chronological aging model, high-throughput assays were employed by us and by another laboratory for rapid quantification of the CLS (Murakami et al. 2008; Wu et al. 2011). Subsequently, we applied this assay to screen 144 plant materials for their anti-aging activity (Supplementary Table S1). From these results, we singled out the root extract of *S. miltiorrhiza* Bunge, which showed the highest activity (Supplementary Fig. S1). We further fractionated the crude extract (using solvents) and, using the HTS as a guide, pinpointed the active compound such as tanshinones (i.e., cryptotanshinone, tanshinone I, and tanshinone IIA), which turned out to be commercially available (Supplementary Fig. S2). Therefore, to investigate the mechanisms, we purchased these compounds from a commercial supplier. We determined the longevity efficacy of tanshinones in a range of doses and at different addition times. As shown in Fig. 1, there was a dose-response relationship between the yeast CLS and the tanshinones concentrations, while higher or lower concentrations either reduced or could not enhance cell survival.

The phase of cell growth seems to be a critical factor for modulating CLS influenced by nutrient signaling. Consistent with this hypothesis, our data showed that tanshinones extend CLS only when it is applied to cells that are entering stationary phase, not when applied to cells that are already in stationary phase (Fig. 1). It is similar to the effect reported for caffeine (Wanke et al. 2008), rapamycin (Powers et al. 2006; Pan et al. 2011), spermidine (Eisenberg et al. 2009), and lithocholic acid (Goldberg et al. 2010; Burstein et al. 2012) in the promotion of longevity of yeast chronological aging. These compounds as well as calorie restriction (CR) and other nutrients with anti-aging property are added before yeast cell entering stationary phase (day 2). In laboratory mice and rats, CR and other

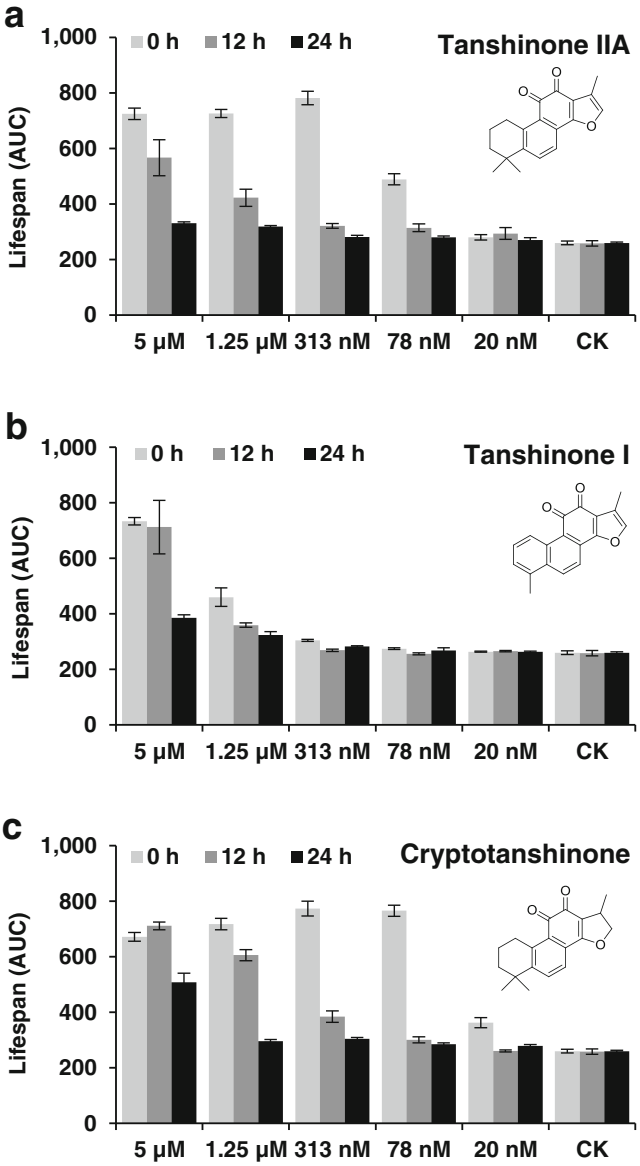

Fig. 1 Cryptotanshinone extends yeast lifespan in response to nutrients. **a**–**c** Cryptotanshinone-extended yeast chronological lifespan (CLS) in a concentration- and the-time-of-addition-dependant manner. Tanshinones (tanshinone IIA (**a**), tanshinone I (**b**), and cryptotanshinone (**c**)) in methanol or DMSO with several concentrations (2.0 μL) were added into 1.0 mL synthetic defined (SD) medium (≈1×10^4 cells) at initial inoculation (0 h) or 12 and 24 h after inoculation. Area under the survival curve (AUC) represents the survival integral for lifespan comparison and *error bars* represent standard error of the mean (SEM) within four replicates

nutrients can achieve maximal benefit for longevity only if they are applied during the rapid growth period (Weindruch and Walford 1982; Yu et al. 1985). Previous studies indicated that they prolonged lifespan via nutrient signaling pathways (Fontana et al. 2010). In fact, compounds targeting nutrient signaling pathways are effective in regulating longevity of an organism, since cells require nutrients in response to compound-induced physiological change in the organism (Howitz and Sinclair 2008; Fontana et al. 2010). Overall,

our results may implicate that the tanshinones prolong CLS via a nutrient-dependent regime.

Cryptotanshinone induced CLS extension is prevented by amino acid restriction

To determine which nutrient factors alter tanshinone-induced longevity, we tested the CLS extension capacity of cryptotanshinone at 78 nM in different media (Fig. 2). This concentration was chosen because it does not inhibit cell growth or reduces biomass production and can extend CLS significantly. To our surprise, the data showed that cryptotanshinone extended CLS in several media (Fig. 2), especially in the low-glucose (0.5 %, CR condition, Fig. 2b), high-glucose (8 %, Fig. 2d), and buffered media (pH 6.0) (Fig. 2e, f). However, it could not extend lifespan in a medium in which the total amino acid amount was reduced (Fig. 2h). Taken together, these results indicate that cryptotanshinone-induced longevity greatly depends on media compositions. Among them, amino acids are important factors in influencing the lifespan extension capacity of cryptotanshinone. It is already established that CLS is severely compromised if cells are grown on synthetic-defined (SD) medium that can cause starvation due to deficiency of essential amino acids (Gomes et al. 2007; Boer et al. 2008). In the measurements presented here, this effect appears to be overriding CLS-extending effect of cryptotanshinone.

Essential amino acid sufficiency is required for cryptotanshinone-induced longevity

In order to examine how the amino acid compositions affect lifespan-extending activity of cryptotanshinone, seven media with different proportions of essential amino acids (EAAs) and nonessential amino acids (NEAAs) were designed and tested (Fig. 3, Supplementary Fig. S3a, S3b). A standard SD medium normally contains 14 amino acids and two bases, adenine and uracil (Sherman 1991; Murakami et al. 2008). Among these compounds, only histidine, leucine, lysine, and uracil are essential as auxotrophy-complementing amino acid for the wild-type (WT) yeast strain *S. cerevisiae* BY4742 (*MATα his3*Δ1 *leu2*Δ0 *lys2*Δ0 *ura3*Δ0) (Table 1). The medium with onefold EAA and fivefold NEAA (1E5N) produced longer CLS than the other media in the WT strain and 5E5N has the shortest CLS (Fig. 3a, Supplementary Fig. S3a, S3b). However, when cryptotanshinone was added to these media, the CLS changed greatly. Our results showed that EAA sufficiency was required for cryptotanshinone-induced CLS extension in the WT yeast based on the following observations: (1) cryptotanshinone could not extend CLS in low EAA media (0.2E0.2 N or 0.2E1N); (2) increasing NEAA did not improve the efficacy of cryptotanshinone when the EAA was maintained at the same level (e.g., 1E0.2 N, 1E1N, 1E5N); (3)

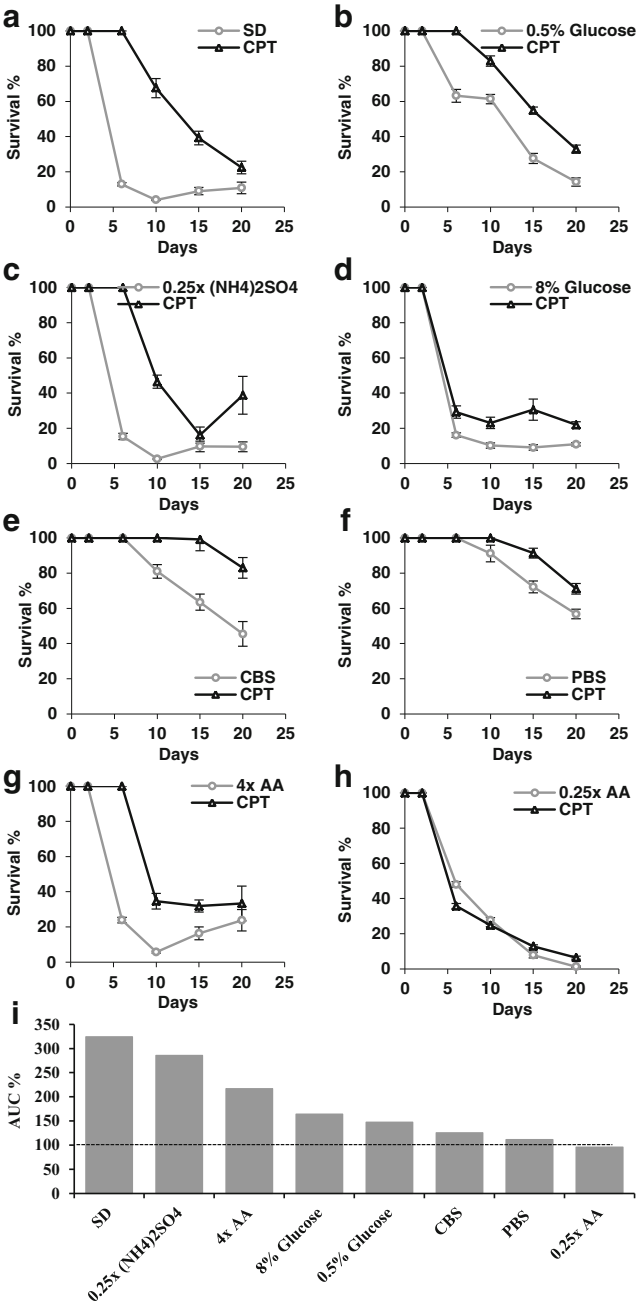

Fig. 2 Cryptotanshinone (CPT)-induced CLS extension is prevented by amino acids restriction. **a**–**h** Survival curve (mean+SEM, $n=8$) of wild-type strain BY4742 cultured in eight SD based media with or without cryptotanshinone (78 nM, 0.2 % methanol). They are SD medium with 2 % glucose (**a**), 0.5 % glucose as CR condition (**b**), 0.25-fold ammonium sulfate (**c**), 8 % glucose (**d**), SD medium prepared with citrate phosphate buffer solution (CBS, Na_2HPO_4, and citric acid, pH 6.0; **e**) or phosphate buffer solution (PBS, Na_2HPO_4 and NaH_2PO_4, pH 6.0; **f**), fourfold total amino acids (**g**), and 0.25-fold total amino acids (**h**). **i** Relative AUC comparison (AUC of compound/AUC of untreated×100 %) in the eight media. Cryptotanshinone dissolved in methanol, 2 μL of 39 μM compounds was added in 1 mL growth medium at the time of cell inoculation. The complete compositions of standard SD medium are listed in Table 1

although CLS changed slightly when EAA increased, the efficacy of cryptotanshinone improved significantly (e.g.,

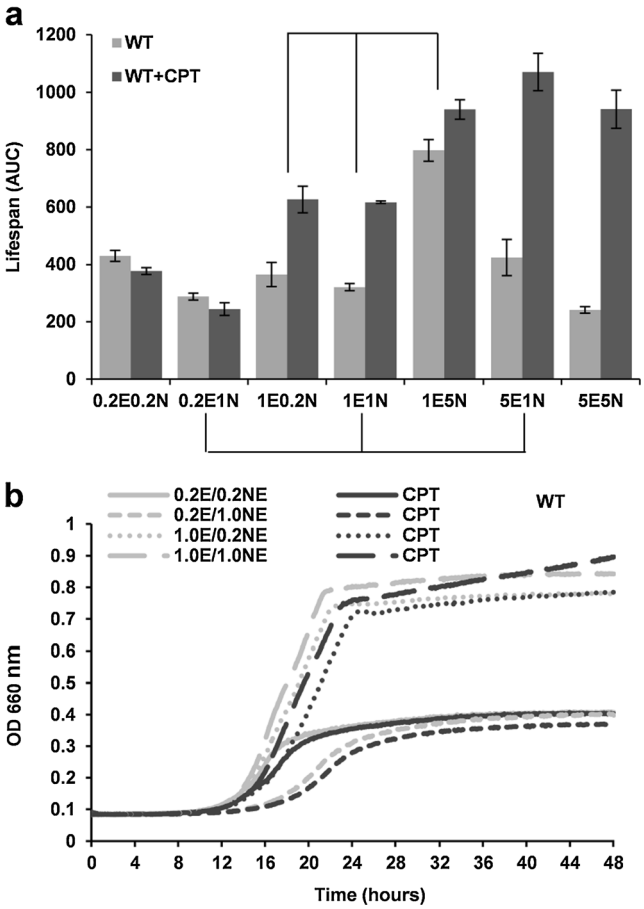

Fig. 3 **a** EAA sufficiency was required for cryptotanshinone-induced CLS extension. Yeast was grown in the seven media with or without cryptotanshinone (78 nM) and *error bars* represent SEM within six replicates; for survival curves, see Fig. S3. **b** Cryptotanshinone did not inhibit the yeast cell growth. The growth curve of yeast cultured in four SD based media with different amounts of EAA and NEAA. The growth curves showed that yeast cells could proliferate well with cryptotanshinone (CPT, 78 nM) in different media since the lag time (≈12 h) of each curve had no significant changes. *0.2E0.2 N* represents SD medium containing 0.2-fold EAA and 0.2-fold NEAA. The EAA and NEAA compositions are listed in Table 1

Table 1 Composition of synthetic-defined (SD) medium used for yeast chronological lifespan analysis

Component	Concentration
Glucose	20 g/L
Yeast nitrogen base (−AA/−AS)	1.7 g/L
Ammonium sulfate	5 g/L
Amino acids (×1)	
Essential	
Uracil	100 mg/L
L-Histidine	100 mg/L
L-Leucine	300 mg/L
L-Lysine-HC1	150 mg/L
Non-essential	
Adenine	80 mg/L
L-Arginine	40 mg/L
L-Aspartic acid	100 mg/L
L-Glutamic acid	100 mg/L
L-Methionine	80 mg/L
L-Phenylalanine	50 mg/L
L-Serine	400 mg/L
L-Threonine	200 mg/L
L-Tryptophan	200 mg/L
L-Tyrosine	40 mg/L
L-Valine	150 mg/L
L-Isoleucine	60 mg/L

0.2E1N, 1E1N, 5E1N); and (4) the ratio of EAA and NEAA concentrations could alter CLS, but the addition of cryptotanshinone changed this consequence and led to longer CLS in higher EAA media (e.g., 0.2E, 1E, 5E) (Fig. 3a). Moreover, cryptotanshinone did not suppress cell growth and biomass production in different media (Fig. 3b).

Next, we examined the effect of individual amino acids on the efficacy of cryptotanshinone (Fig. 4). Consistent with the analysis above that restriction of EAA prevents cryptotanshinone induced longevity, the addition of cryptotanshinone in the media with 0.1-fold histidine (Fig. 4e) and uracil (Fig. 4m) showed less CLS extension than in the NEAA (Fig. 4o). It should be highlighted that leucine and lysine were not shown here as the low concentration of these two amino acids led to low biomass production and an accelerated loss of viability.

Cryptotanshinone requires Tor1 and Sch9 for CLS extension

To elucidate the genetic mechanism of cryptotanshinone-induced CLS extension, we focused on those evolutionarily conserved and nutrient-sensing longevity pathways from yeast to humans. In yeast, the Tor/Sch9 pathway was thought previously to be a highly conserved nutrient-sensing pathway that regulates longevity among different species (Fontana et al. 2010). Sch9 is proposed as a major nutrient-sensing factor to regulate cell growth, cell size, and stress resistance through controlling protein synthesis. Absence of Sch9 activity causes a small-sized phenotype and distinct growth defect, while increasing the lifespan (Fabrizio et al. 2001; Kaeberlein et al. 2005; Urban et al. 2007; Huber et al. 2009). We previously demonstrated that *sch9*Δ is more responsive to nutrients than WT, *tor1*Δ, and *sir2*Δ (Wu et al. 2013). Thus, we first examined the effect of cryptotanshinone on *sch9*Δ lifespan in SD media with different ratios of EAA and NEAA. Similar to the observation in WT, the amino acid composition changed CLS of *sch9*Δ, but cryptotanshinone did not increase the lifespan significantly even in the media containing a high concentration of EAA (Fig. 5a, Supplementary Fig. S3c,

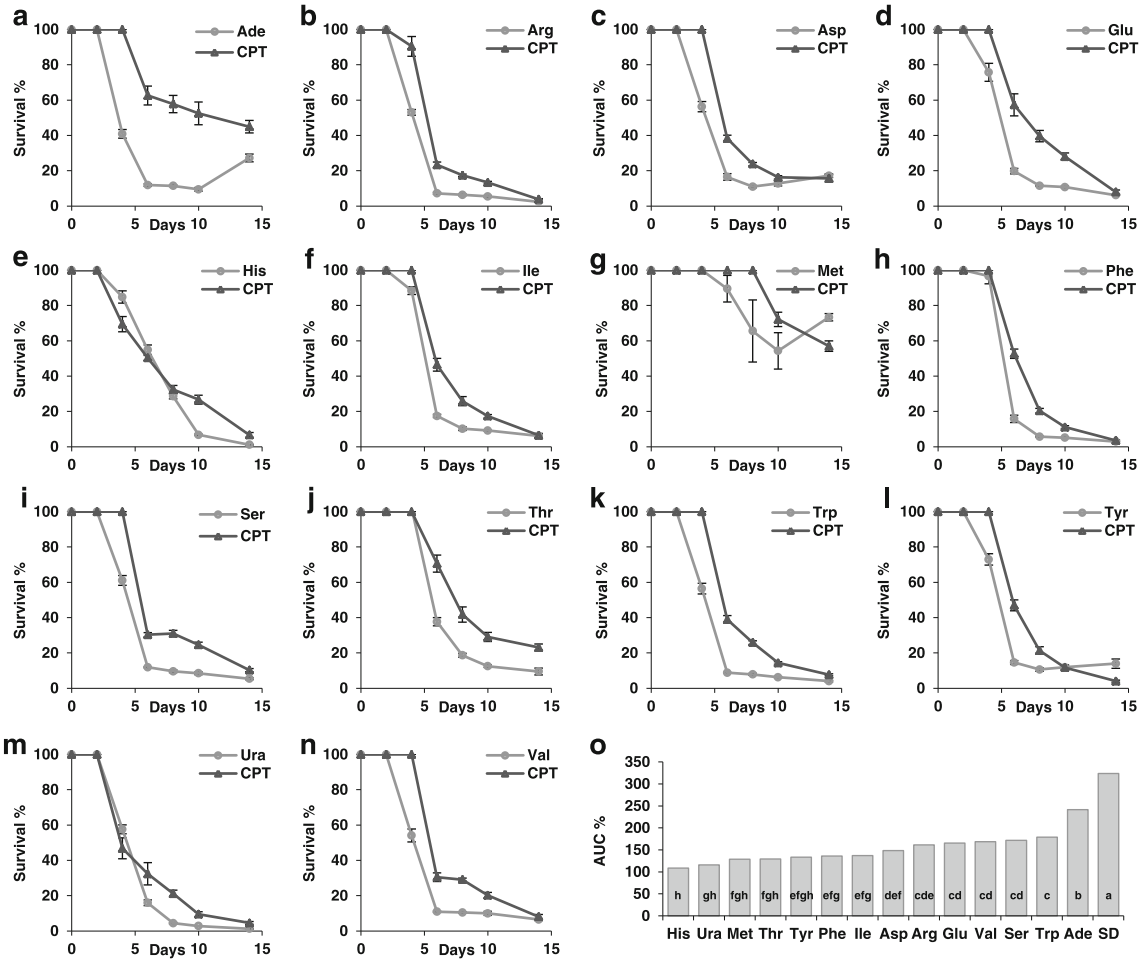

Fig. 4 Effect of individual amino acid restriction on cryptotanshinone-induced CLS extension. **a**–**n** Survival curve (mean, n=6) of wild-type strains cultured in SD based media containing 0.1-fold individual amino acid with/without cryptotanshinone. **o** Relative AUC comparison (AUC of compound/AUC of untreated×100 %) in different media. Data were expressed as mean (n=6) and compared using Duncan's multiple range test at $P<0.05$. *Different lowercase letters* in columns indicate significant difference

S3d). It implicates that cryptotanshinone-induced longevity requires Sch9 activity.

Next, we tested the effect of cryptotanshinone on *tor1*Δ strain cultured in SD medium containing 0.1, 0.5, 2, and 5 % glucose. The data showed that cryptotanshinone could prolong lifespan in media with different levels of glucose (Fig. 5c, Supplementary Fig. S4a). This further indicates that cryptotanshinone-induced lifespan extension is not dependent on glucose concentration. Although this result suggests that the action of cryptotanshinone is independent of CR (0.5 % glucose), it is possible that cryptotanshinone could enhance the resistance of yeast against the toxic metabolites (e.g., ROS and organic acids) at high glucose levels. In contrast, cryptotanshinone could not prolong the lifespan of *tor1*Δ strain (Fig. 5d, Supplementary Fig. S4b). Overall, our results suggest that the CLS-extending effect of cryptotanshinone is overridden by the loss of either Sch9 or Tor1.

Tanshinone I, Tanshinone IIA, and cryptotanthinone extend yeast lifespan via similar mechanisms

To determine whether the tanshinones induce longevity via other mechanisms, tanshinone IIA (1.25 μM), cryptotanshinone (78 nM), and a mixture containing tanshinone I (1.7 μM), tanshinone IIA (0.42 μM), and cryptotanshinone (26 nM) were chosen, and their effects on CLS of wild-type BY4742, *sch9*Δ, and *sir2*Δ cultured in the standard SD medium were evaluated (Fig. 6). Tanshinone IIA (1.25 μM) and cryptotanshinone (78 nM) extended lifespan by equal amounts (Fig. 6a), but the mixture did not show a longer lifespan compared with that of individual compounds, which means that tanshinones have no additive or synergistic effect on yeast longevity. In addition, the three selected compounds could not extend CLS in *sch9*Δ, while they prolonged the *sir2*Δ lifespan significantly (Fig. 6b, c). Altogether, these

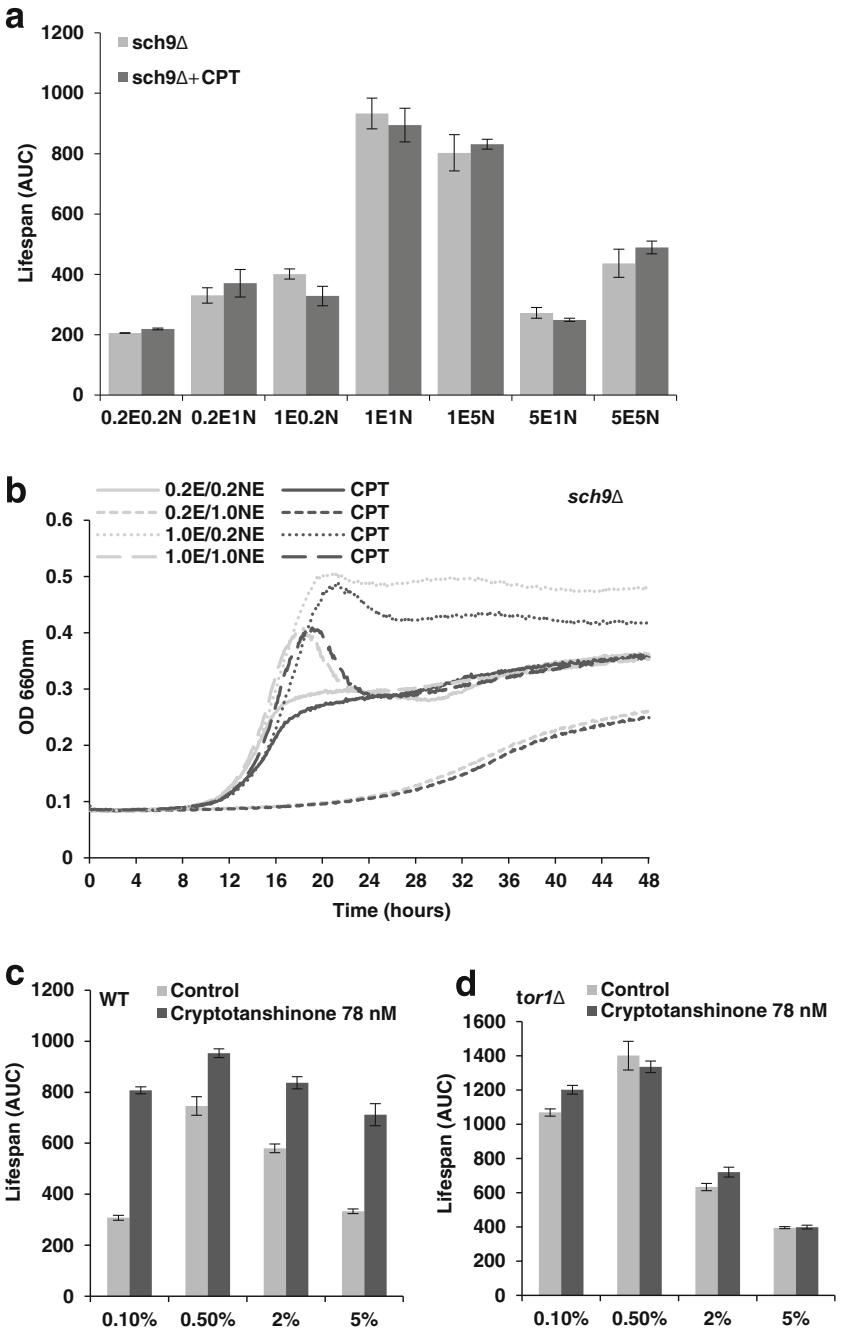

Fig. 5 Cryptotanshinone induced lifespan extension depends on Sch9 and Tor1 activity. **a** Deletion of *SCH9* prevented cryptotanshinone-induced CLS extension in different media. The yeast was grown in the seven media with or without cryptotanshinone (78 nM; mean± SEM, $n=6$); for survival curves, see Fig. S3. **b** Cryptotanshinone did not inhibit cell growth in *sch9*Δ strain. **c**, **d** Cryptotanshinone induced longevity was independent of glucose levels and was prevented by deletion of *TOR1*. Wild-type and *tor1*Δ cultured in SD medium containing 0.1, 0.5, 2, and 5 % glucose with or without cryptotanshinone (78 nM; mean± SEM, $n=6$); for survival curves, see Fig. S4. *0.2E0.2 N* represents SD medium containing 0.2-fold EAA and 0.2-fold NEAA. The EAA and NEAA compositions are listed in Table 1

observations indicate that tanshinones require Sch9 for CLS extension and they may act on similar mechanisms. The structural similarity of these compounds may indicate that they act on a similar mechanisms and prevent potential synergistic effects.

Gcn2 regulates essential amino acids and cryptotanshinone-induced CLS extension

In addition to the Tor-Sch9 nutrient-sensing pathway, general amino acid control (GAAC) is an important nutrient-sensing pathway in the regulation of yeast growth and metabolism. It is also noteworthy that GAAC could be a major factor of the Tor/Sch9 pathway, and Gcn2 may play a central role in the integration of GAAC and Tor/Sch9 pathways (Staschke et al. 2010). Gcn2 is one of the major evolutionarily conserved protein kinases in response to nutritional cues, especially amino acid starvation (Wilson and Roach 2002). The starvation causes activation of Gcn2, which subsequently phosphorylates eukaryotic initiation factor-2 (eIF2). As a result, initiation of general protein synthesis is repressed. This change enables cells to conserve resources and have time to

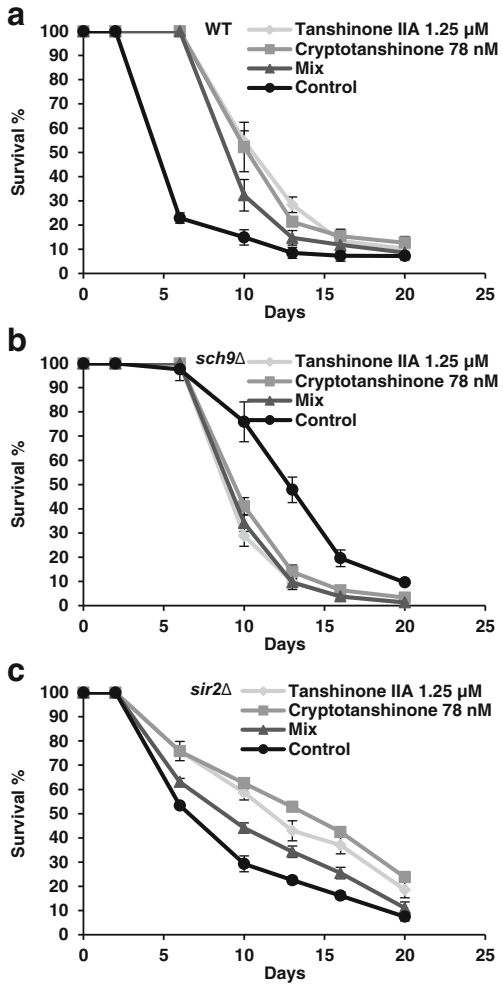

Fig. 6 Tanshinones have no synergetic effect on yeast longevity via similar mechanisms. **a** Tanshinones had no synergetic effect on CLS extension. Tanshinone IIA (1.25 μM), cryptotanshinone (78 nM), and a mixture containing tanshinone I (1.7 μM), tanshinone IIA (0.42 μM), and cryptotanshinone (26 nM) were tested in wild-type BY4742 grown in standard SD medium. **b** Deletion of *SCH9* prevented CLS extension by the Danshen compounds. **c** Tanshinones extended CLS in dependence of the deletion of *SIR2*. **d** Comparison of CLS of wild type, *sch9*Δ, and *sir2*Δ treated with tanshinones. AUC represents the survival integral for lifespan comparison (mean±SEM, $n=6$)

reconfigure the transcriptome to alleviate nutrient stress (Staschke et al. 2010). Concomitantly, Gcn2 phosphorylation also elevates Gcn4 activity, a transcription activator of a large number of genes subject to the GAAC, many of which are involved in amino acid biosynthesis (Hinnebusch 2005).

Based on the above reasoning, it is possible that Gcn2 could be involved in the regulation of cryptotanshinone-induced CLS extension. We measured CLS of *gcn2*Δ in the seven media and found that a higher EAA concentration had longer CLS and NEAA had less contribution to CLS extension than EAA (Fig. 7a, Supplementary Fig. S3e, S3f), which means Gcn2 mainly regulated EAA and may partly prevent CLS extension in WT and *sch9*Δ strains grown in the media with a high level of EAA (Figs. 3a and 5a). We also found that cryptotanshinone are more effective to extend lifespan of WT in 5E1N than that in 1E5N (Fig. 3a), while cryptotanshinone cannot extend lifespan of *gcn2*Δ in 1E5N and slight increase in 5E1N. Obviously, 1E5N and 5E1N are greatly different in amino acid composition. Cryptotanshinone extends CLS in WT and the efficacy is mainly dependent on EAA concentration (Fig. 3a). Thus, this observation is consistent with the conclusion that EAA is a key determinant for cryptotanshinone activity. Furthermore, our data showed that the deletion of *GCN2* could impair the efficacy of cryptotanshinone relative to WT in normal and high EAA media (Figs. 3a and 7a). Thus, we may conclude that the CLS-extending effect of cryptotanshinone is partly overridden by loss of Gcn2 in EAA sufficient media.

Cryptotanshinone extend lifespan in sod2Δ

Reactive oxygen species (ROS)-initiated irreversible cellular damage is the cornerstone of free radical theories of aging, and enzymatic antioxidants, particularly superoxide dismutase (SOD), are critical in protection of ROS damage to cells (Harman 1956; Finkel and Holbrook 2000). Yeast has two SOD genes, cytoplasmic copper-zinc superoxide dismutase (SOD1) and mitochondrial manganese superoxide dismutase (SOD2). The lack of either of the two SODs resulted in decreased lifespan enormously, and the deletion of *SOD2* has a shorter lifespan than the deletion of *SOD1* (Unlu and Koc 2007). *SOD2* was proposed as a downstream target of Tor/Sch9 nutrient signaling pathway for longevity extension by decreasing in part ROS levels in yeast (Fabrizio et al. 2003; Fontana et al. 2010; Pan et al. 2011). Thus, it is interesting to ascertain whether cryptotanshinone reduces ROS stress to extend CLS. Surprisingly, although cryptotanshinone could not reduce the intracellular ROS level in *sod2*Δ mutant at early stationary phase day 2, it extended CLS in the *sod2*Δ strain that has shortened lifespan significantly in the standard SD medium due to impaired superoxide detoxification in the cell (Fig. 8a, b) (Pan et al. 2011).

Discussion

In this study, we used a high-throughput assay in yeast chronological aging model to screen anti-aging compounds from natural products, and discovered that cryptotanshinone from *S. miltiorrhiza* Bunge has strong lifespan extending activity in relatively low concentrations (e.g., 78 nM). We found that cryptotanshinone has a stronger efficacy than tanshinone IIA and tanshinone I. Compared to other reported anti-aging compounds, such as resveratrol, rapamycin, lithocholic acid, and spermidine (Howitz et al. 2003; Powers et al. 2006; Eisenberg et al. 2009; Pan et al. 2011; Burstein et al. 2012),

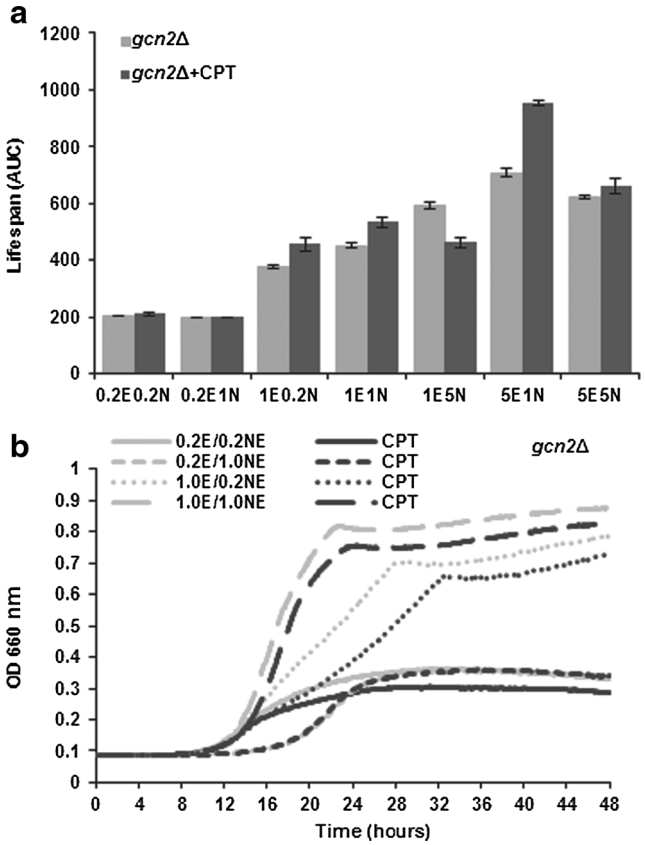

Fig. 7 Gcn2 regulated amino acids homeostasis to extend lifespan and impaired cryptotanshinone-induced longevity in different media. **a** The yeast were grown in the seven media with or without cryptotanshinone (78 nM; mean±SEM, $n=6$); for survival curves, see Fig. S3. **b** Cryptotanshinone did not inhibit cell growth in *gcn2*Δ strain. *0.2E0.2 N* represents SD medium containing 0.2-fold EAA and 0.2-fold NEAA. The EAA and NEAA compositions are listed in Table 1

cryptotanshinone is promising as anti-aging medicine based on four reasons:

1. Ideal pharmaceutical properties. Cryptotanshinone meets Lipinski's rule of five (Lipinski 2004) of drug evaluation, which states that, in order for molecules to penetrate cell membranes and be absorbed into the human body, a drug molecule should have no more than one violation of the following: (a) no more than five hydrogen bond donors, (b) no more than ten hydrogen bond acceptors, (c) a molecular mass less than 500 Dalton, and (d) an octanol-water partition coefficient (log(P)) not greater than five. Cryptotanshinone has no hydrogen bond donors, two hydrogen bonding acceptors due to two C=O groups, a molecular weight smaller than 500 (296 g/mol), and an octanol-water partition coefficient of three.
2. High activity. Cryptotanshinone can extend yeast CLS more than two times in a standard SD medium at low concentration (78 nM) that does not inhibit cell growth or supress biomass production (Figs. 3b, 5b, and 7b). In contrast, resveratrol extends yeast replicative lifespan at much higher concentration (10 μM) and does not extend CLS even at 100 μM (Howitz et al. 2003); rapamycin prolongs yeast CLS at 200 nM but it greatly inhibits cell growth (Pan et al. 2011).
3. Multifunctionality. Cryptotanshinone not only exerts longevity extension in diverse media and strains as shown in this study, but also processes numerous other bioactivities assessed in different models including human cells (Don et al. 2007; Chen et al. 2010, 2012; Mei et al. 2010; Park et al. 2012a). Aging is a very complex and dynamic process involving multiple factors and their interactions; thus, a compound that is multi-targeting of these factors or pathways might be desired as an anti-aging candidate. Resveratrol seems to be a good example but it has low efficacy (Park et al. 2012b).
4. Action on evolutionarily conserved genetic pathway. Our data documented that EAA, rather than other nutrients, is the key nutrient factor that is required for cryptotanshinone to induce lifespan extension. Since almost all living cells require EAA for protein synthesis and survival, we hypothesize that cryptotanshinone could be applied in diverse cell lines for lifespan extension. Moreover, cryptotanshinone was reported to inhibit the mammalian target of rapamycin complex 1 (mTORC1)-mediated phosphorylation of ribosomal p70 S6 kinase 1 (S6K1 having similar functionality to Sch9) and eukaryotic initiation factor 4E binding protein 1 (4EBP1) in a concentration- and time-dependent manner (Chen et al. 2010). Numerous investigations have shown that these proteins as the key mediators are involved in regulation of aging and aging-related diseases in mammal. Thus, cryptotanshinone could have the potential to increase lifespan via reducing the highly conserved nutrient-sensing mTOR pathway activity, which is a control point of lifespan of in higher organisms (Fontana et al. 2010; Johnson et al. 2013).

It is well known that *tor1*Δ and *sch9*Δ are responsive in amino acid or glucose sensing (Fontana et al. 2010; Longo et al. 2012). We cannot rule out the possibility that *tor1*Δ and *sch9*Δ have similar behavior in response to different conditions. For example, *sch9*Δ shows longer lifespan, smaller cell size, slower growth rate, and stronger stress resistance than *tor1*Δ even in the same medium (Wei et al. 2008, 2009). This may be due to the fact that Tor2 can function, in redundancy to Tor1, in the TORC1 complex. In the present study, we tested *sch9*Δ in different amino acid concentrations and *tor1*Δ in different glucose concentrations to further confirm the fact that cryptotanshinone-induced longevity requires Tor1 and Sch9 in multiple media. We do not intend to elucidate the genetic interactions between *SCH9* and *TOR1*, as it has been well

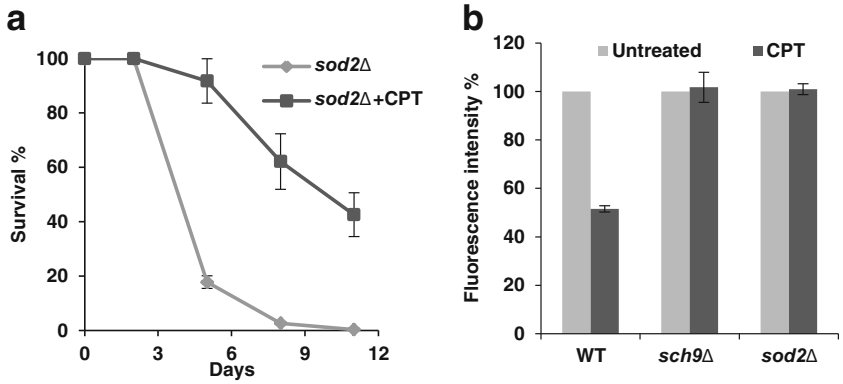

Fig. 8 Cryptotanshinone mediates reactive oxygen species (ROS) production. **a** Cryptotanshinone extends CLS of *sod2*Δ strain (mean±SEM, $n=6$). **b** Intracellular ROS levels of wild-type, *sch9*Δ, and *sod2*Δ strains grown in standard SD medium with/without cryptotanshinone were quantified by a Bio-Tek plate reader. The ROS probe H_2DCFDA was used. DCF fluorescence was measured at 520 nm with excitation at 480 nm. Relative fluorescence intensity (fluorescence intensity of compound/fluorescence intensity of untreated×100 %) at day 2 was presented. *Error bars* are mean±SD, $n=4$

studied (Wei et al. 2008, 2009). Furthermore, our previous data shows that *sch9*Δ are more responsive in amino acid, glucose, and other nutrient (e.g., YNB) sensing (Wu et al. 2013).

We have shown that deleting Tor/Sch9 signaling pathway in yeast eliminates CLS extension by cryptotanshinone. Sod2 was proposed as a downstream of Sch9; double deletion of *SCH9* and *SOD2* abolished CLS extension in *sch9*Δ (Fabrizio et al. 2003; Fontana et al. 2010). Our results demonstrate that removing mitochondrial Sod2 cannot prevent cryptotanshinone-induced lifespan extension and cryptotanshinone does not diminish the intracellular ROS level in *sod2*Δ mutants based on the fluorescence intensity of the redox sensitive probe (Fig. 8), but significantly reduce the oxidative stress status in the WT strain. For the WT strain, cryptotanshinone may inhibit the activity of Tor/Sch9 signaling, and subsequently reduce total ROS production (Supplementary Fig. S5) (Fontana et al. 2010). Consistently, there is no change in the ROS level in *sch9*Δ strain with/without cryptotanshinone treatment (Fig. 8b). On the other hand, it is possible that H_2DCFDA detects general ROS stress (including superoxide, hydrogen peroxide, and hydroxyl radicals) but not specifically superoxide radical, which is a major signaling ROS mediated by SOD2 in mitochondria. The change of superoxide stress level in *sod2*Δ is thus not possible to be distinguished by fluorescence intensity for the general ROS status of the cells. The alternative possibility is that cryptotanshinone may extend CLS of *sod2*Δ via other mechanism. Previous studies have revealed that cryptotanshinone mediates the AMPK pathway, PI3K pathway and endoplasmic reticulum (ER) stress (Kim et al. 2007; Mei et al. 2010; Park et al. 2012a), which might contribute to lifespan extension. It would be worthwhile to probe whether mitohormesis is a possible mechanism underlying cryptotanshinone extension of CLS (Ristow and Zarse 2010; Pan 2011).

The EAA concentration in a medium is a key nutrient parameter in affecting lifespan-extending capacity of cryptotanshinone in WT yeast and in determining CLS of *gcn2*Δ. We have demonstrated that EAA and NEAA composition changes the lifespan of WT and *sch9*Δ significantly (Figs. 3 and 5) and EAA is a determining factor for biomass production in different strains (Figs. 3b, 5b, and 7b). On the other hand, EAA restriction (0.2-fold EAA of normal) greatly decreases biomass and restriction of both EAA and NEAA (0.2EAA/0.2NEAA) does not inhibit cell growth in comparison to that under normal conditions, while EAA and NEAA imbalance (0.2EAA/1NEAA) delays cell growth in WT, *sch9*Δ, and *gcn2*Δ trains (Figs. 3b, 5b, and 7b). To our knowledge, only few previous studies reported that a higher EAA concentration causes a longer CLS and higher biomass production in yeast chronological aging model (Gomes et al. 2007; Boer et al. 2008; Alvers et al. 2009). Although the individual EAA concentration differs from ours, the results are in agreement with our observation.

Gcn2 regulates amino acid homeostasis and protein synthesis by modulating amino acid biosynthesis in response to different amino acids deprivation in yeast (Hinnebusch and Natarajan 2002). So far, there are two known ways to regulate Gcn2 activity. Firstly, amino acid starvation causes accumulation of uncharged tRNAs that bind to Gcn2 protein kinase and subsequently activates Gcn2. Secondly, rapamycin activates Gcn2 by inhibiting TORC1 even in amino acid-depleted cells (Hinnebusch 2005). In this study, we document for the first time that Gcn2 regulates EAA and NEAA homeostasis to alter lifespan and the deletion of *GCN2* causes yeast cells to become amino acid insensitive, which leads to longer lifespan and prevents cryptotanshinone induced lifespan extension in EAA sufficient media (Fig. 7).

Although it is not clear whether cryptotanshinone affects Gcn2 directly, our data suggest that cryptotanshinone appears to reduce Tor/Sch9 pathway activity and, in turn, elevate Gcn2 activity (Supplementary Fig. S5). It is consistent with previous studies shown that Gcn2 is at downstream of TOR and

upstream of Gcn4 linking TOR pathway and GAAC pathway (Steffen et al. 2008; Staschke et al. 2010). Growth in media with amino acid imbalance can elicit the pathway. Activation of GAAC decreases CLS while suppression of GAAC prolongs CLS in minimal medium (Alvers et al. 2009). Testing of the CLS of *gcn4*Δ strain cultured in the different media we used with or without cryptotanshinone would be informative to find out if Gcn4 is indeed a potential longevity factor for chronological and replicative lifespans and a master regulator of gene expression for GAAC in yeast (Steffen et al. 2008; Alvers et al. 2009). Gcn2 was examined herein because our aim is to delineate genes that are conserved aging factors. The domain structure of Gcn2 is highly conserved and it functions as both general and gene-specific translational control in fungi, insects, and mammals (Hinnebusch 2005). Taken together, it is clear that Gcn2 has a longevity-regulating function.

In conclusion, our findings demonstrate that cryptotanshinone-induced lifespan extension is dependent on the nutrient compositions of media, especially EAA concentration. Restriction of total amino acids or EAA and deletion of *TOR1*, *SCH9*, or *GCN2* prevent longevity extension by cryptotanshinone. An increased lifespan of *sod2*Δ by cryptotanshinone and intracellular ROS level suggests that cryptotanshinone might mediate ROS stress resistance. Based on these results, we propose that cryptotanshinone targets partly Tor1, Sch9, Gcn2, and Sod2 (Supplementary Fig. S5) to prolong the lifespan of yeast at nanomolar concentrations. The highly conserved genes that cryptotanshinone is targeting merit future investigation of longevity activity of cryptotanshinone in higher organisms and its molecular mechanisms. The structure and activity dependency of the tanshinones is noteworthy and warrants more investigation in order to establish a correlation and discovery of even more potent anti-aging compounds.

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