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Chemical genetic screen in fission yeast reveals roles for vacuolar acidification, mitochondrial fission, and cellular GMP levels in lifespan extension

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Summary

The discovery that genetic mutations in several cellular pathways can increase lifespan has lent support to the notion that pharmacological inhibition of aging pathways can be used to extend lifespan and to slow the onset of age-related diseases. However, so far, only few compounds with such activities have been described. Here, we have conducted a chemical genetic screen for compounds that cause the extension of chronological lifespan of *Schizosaccharomyces pombe*. We have characterized eight natural products with such activities, which has allowed us to uncover so far unknown anti-aging pathways in *S. pombe*. The ionophores monensin and nigericin extended lifespan by affecting vacuolar acidification, and this effect depended on the presence of the vacuolar ATPase (V-ATPase) subunits Vma1 and Vma3. Furthermore, prostaglandin J_2 displayed anti-aging properties due to the inhibition of mitochondrial fission, and its effect on longevity required the mitochondrial fission protein Dnm1 as well as the G-protein-coupled glucose receptor Git3. Also, two compounds that inhibit guanosine monophosphate (GMP) synthesis, mycophenolic acid (MPA) and acivicin, caused lifespan extension, indicating that an imbalance in guanine nucleotide levels impinges upon longevity. We furthermore have identified diindolylmethane (DIM), tschimganine, and the compound mixture mangosteen as inhibiting aging. Taken together, these results reveal unanticipated anti-aging activities for several phytochemicals and open up opportunities for the development of novel anti-aging therapies.

Key words: autophagy; chronological lifespan; mitochondria; *S. pombe*; vacuole.

Introduction

A central goal in aging research is to determine the molecular mechanisms that contribute to aging. Age-related processes are controlled by genetic as well as environmental factors. One prominent way of extending lifespan in a wide range of model organisms is through dietary restriction (DR), and accordingly, mutations in nutrient sensing pathways extend lifespan from yeast to primates (Fontana *et al.*, 2010). In worms, flies, and mice, mutations that decrease the activity of the insulin/IGF-1 pathway cause longevity (Kenyon, 2010). Furthermore, target of rapamycin (TOR) kinases are major sensors of amino acids and nutrients, and inhibition of TOR signaling increases lifespan in many species (Fontana *et al.*, 2010). DR further leads to the activation of AMP kinase, which subsequently extends lifespan.

These nutrient sensing kinases influence aging through cellular transcriptional programs as well as through mitochondrial respiration. In the course of ATP production, the mitochondria produce reactive oxygen species (ROS) that can damage cellular components (Kenyon, 2010). Intriguingly, however, depending on the circumstances, both inhibition and enhancement of respiration cause longevity (Lee *et al.*, 2010), suggesting a 'mitohormetic' model for aging (Pan, 2011). A further enzyme class involved in aging is the sirtuin family of NAD^+-dependent histone deacetylases. Increased activity of selected sirtuins in *S. cerevisiae* (Sir2) (Sinclair & Guarente, 1997) and male mice (SirT6) promotes longevity (Kanfi *et al.*, 2012), but the precise mechanism is unclear, and there has been conflicting evidence for the effect of other sirtuins in some model organisms (Burnett *et al.*, 2011).

The identification of genetic anti-aging factors has provided opportunities for developing pharmacological interventions that delay aging and alleviate age-related diseases. Inhibition of nutrient signaling pathways, foremost TOR kinases, increases lifespan in a variety of organisms. Best known among these is the TOR inhibitor rapamycin, but other direct (e.g., wortmannin, caffeine) or indirect (methionine sulfoximine) TOR inhibitors have also been reported to counteract aging (Powers *et al.*, 2006; Fontana *et al.*, 2010). Furthermore, resveratrol is being widely discussed as an anti-aging drug based on the initial observation that it is an activator of the sirtuins, that it extends lifespan in yeast (Howitz *et al.*, 2003), nematodes, and flies (Wood *et al.*, 2004), and that it has beneficial health effects in mice (Baur *et al.*, 2006). Whether these biological effects result from the direct activation of sirtuins, or rather via an indirect mechanism of inhibiting cAMP-degrading phosphodiesterases (Park *et al.*, 2012), remains to be determined. Also, questions have arisen regarding the effect of resveratrol on aging in yeast (Kaeberlein *et al.*, 2005) as well as in some other model organisms (Bass *et al.*, 2007).

In addition to resveratrol and rapamycin, a limited number of compounds have been reported to cause longevity in some model systems, including spermidine through inhibition of histone acetyltransferases and lithocholic acid by suppression of lipid-induced necrosis in yeast (Goldberg *et al.*, 2010).

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In general, two mechanistically distinct types of lifespan exist: replicative lifespan (RLS) and chronological lifespan (CLS). In this study, we sought to identify compounds that extend CLS in the fission yeast *Schizosaccharomyces pombe*. *S. pombe* is particularly well suited for the study of CLS, because aging in fission yeast is not caused by acetic acid as a by-product of fermentative metabolism (Zuin *et al.*, 2010), and *S. pombe* does not exhibit age-dependent adaptive regrowth in aging cultures (Chen & Runge, 2009). Anti-aging pathways in *S. pombe* have been studied in less detail than in *Saccharomyces cerevisiae*, but include DR, the inhibition of the signaling kinase Sck2 (a homolog of *S. cerevisiae* Sch9), and the inhibition of glucose-mediated nutrient signaling via the Git3/PKA pathway (Roux *et al.*, 2010).

Here, we screened a library of natural compounds for substances that extended CLS in *S. pombe*. With this, we pursued two goals: 1) to identify previously unrecognized anti-aging activities of compounds with known cellular mechanisms and thus to uncover novel anti-aging pathways and 2) to identify lifespan-extending drugs in *S. pombe* that may serve as lead compounds for the treatment of age-related conditions in metazoans. Significantly, we report seven compounds and one compound mixture to exert lifespan-extending activity, which reveal roles for vacuolar acidification, mitochondrial fission, and homeostasis of intracellular guanosine monophosphate (GMP) levels in determining longevity in fission yeast.

Results

Identification of compounds that delay aging in *S. pombe*

We sought to identify compounds that increase CLS in *S. pombe* by performing a chemical genetic screen. In order to facilitate high-throughput screening, we adopted the method of lifespan determination by counting the number of colony forming units (CFUs) within an aging yeast culture (Roux *et al.*, 2010) to the format of a 96-well microtiter plate (Fig. 1A). Using this assay, we were able to recapitulate the well-documented effect of DR on lifespan extension in *S. pombe* (Chen & Runge, 2009). When cells were grown in the standard glucose concentration of 3%, cell survival decreased by day 7, and hardly any cells survived beyond day 10 (Fig. 1A). Conversely, when grown under conditions of DR (0.5% glucose), cells readily survived up to day 10 and beyond. Furthermore, an increased glucose concentration (5%) shortened their survival (Fig. 1A).

We subsequently used this assay to screen a small, commercially available library of 522 natural products (Table S1) for substances that increased the CLS of *S. pombe*. Compounds were added at day 0 (initial inoculation of the microculture) to the growth medium (3% glucose) at a concentration of 4 $\mu g\ mL^{-1}$ or 20 $\mu g\ mL^{-1}$. Because the compounds in the library have different molecular weights, these concentrations corresponded to an average of 10 and 50 μM, respectively, which are concentrations frequently chosen for substance screens (Bedalov *et al.*, 2001). Thus, the cells were exposed to the substance during the whole course of the aging experiment. Cells were transferred back to agar plates after days 3, 5, 7, 10, and 12, etc., and the formation of cell spots after incubation of the plates was documented. In an initial screen, candidate compounds were identified that prolonged cell viability in aging cultures as measured by this assay. Their effect on lifespan extension was subsequently validated using a range of compound concentrations (see below), which led us to identify 19 compounds that increased CLS in *S. pombe* (Table 1).

Among these compounds was wortmannin, a known inhibitor of phosphoinositide 3-kinases and TOR kinases (Fig. 1B). As the inhibition of TOR kinases in *S. cerevisiae* and other organisms extends lifespan (Fontana *et al.*, 2010), we surmise that wortmannin causes lifespan extension in *S. pombe* due to its inhibitory effect on these kinases, and we therefore did not pursue it further.

Here, we concentrated on the further analysis of eight life-extending compounds that showed strong (++) or very strong (+++) CLS extension in the microtiter dish assay (Table 1): acivicin, 3,3′-diindolylmethane (DIM), mangosteen (a compound mixture, here referred to as a compound for simplicity), monensin, mycophenolic acid (MPA), nigericin, prostaglandin J_2 (PGJ_2), and tschimganine (Fig. S1). We have not pursued the eleven other lifespan-extending compounds (Table 1), because their effect on aging was mild. Nicotine only increased lifespan at a relatively high dose (1 mg mL^{-1}) and for this reason was not further investigated.

We tested several concentrations of the eight compounds for CLS extension and used the lowest possible concentration that did not cause a growth defect in subsequent experiments (Fig. S2). For instance, tschimganine showed the most significant lifespan extension at a concentration of 4 $\mu g\ mL^{-1}$, but caused a growth delay at 20 $\mu g\ mL^{-1}$ (Fig. S2). Thus, all eight compounds caused a marked increase in longevity in the microtiter aging assay (Fig. 1C). To our knowledge, these eight compounds have no apparent structural similarity to known life-extending molecules (Fig. S1), thus suggesting that they may modulate longevity by so far unknown mechanisms.

Because the compounds cause an effect on aging, we surmise that they are able to cross the cell wall and plasma membrane and affect an intracellular target. Furthermore, none of the compounds affected aging by changing the pH of the growth medium (data not shown). Of note, previous studies have shown that aging in *S. pombe*, unlike in *S. cerevisiae* (Longo *et al.*, 2012), is insensitive to acetic acid in the medium (Zuin *et al.*, 2010).

We further sought to validate the effect of seven of these compounds on lifespan extension by determining their effect on the viability of *S. pombe* cell cultures as determined by measuring CFUs (the effect of PGJ_2 was measured using a modified assay because of limited compound availability). In this assay, all seven compounds caused an increased longevity of the *S. pombe* strain that was comparable to that of the absence of Sck2 (Fig. 1D), thus confirming their effect on lifespan extension in an independent assay. A modified version of this assay was employed for PGJ_2 (Fig. 1E, see Supplementary Information for experimental procedure), which confirmed its effect on longevity.

Because DR induces longevity in many organisms, it was possible that the lifespan-extending compounds effectively were mimetics of DR conditions. All eight compounds were able to increase the lifespan of cells under DR (1% glucose) (Fig. 2A), indicating that

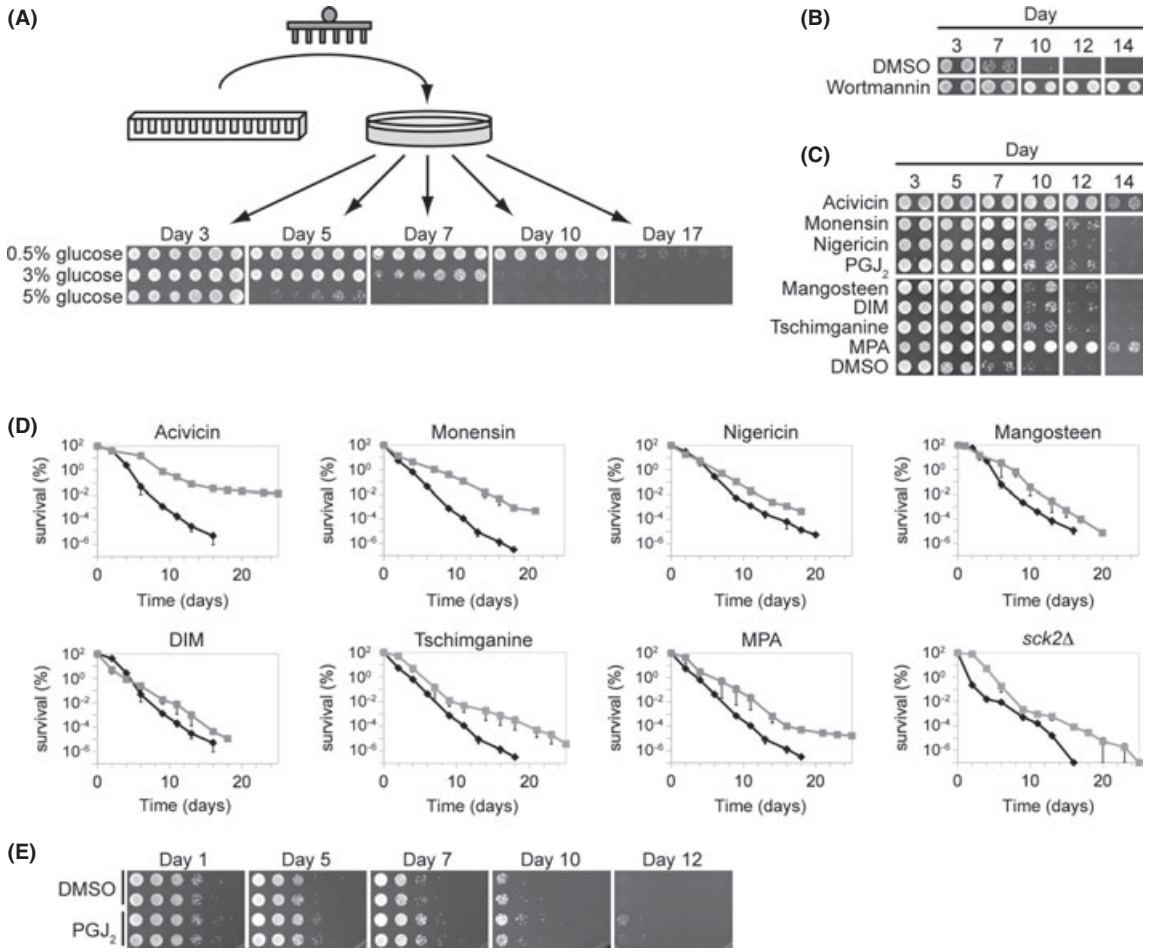

Fig. 1 Chemical genetic screen for lifespan-extending compounds in *Schizosaccharomyces pombe*. (A) Establishment of a microtiter assay for lifespan measurement that recapitulates the effect of dietary restriction on aging in fission yeast. *S. pombe* wild-type (wt, AEP57) cells were cultured in wells of a microtiter plate in supplemented SD medium containing 0.5, 3, or 5% glucose (one row of wells contains the same glucose concentration), and aliquots were transferred to agar plates using a replica tool at days 3, 5, 7, etc., of cultivation. Plates were incubated for 2 days at 30°C and documented. (B) Wortmannin, an inhibitor of phosphoinositide 3-kinases and TOR kinases, caused lifespan extension (2 μg mL^{-1}) as measured with the microtiter assay presented in (A). Dimethyl sulfoxide (DMSO) was used as a solvent control. (C) Lifespan extension was induced by 20 μg mL^{-1} acivicin, 4 μg mL^{-1} monensin, 2 μg mL^{-1} nigericin, 20 μg mL^{-1} prostaglandin J_2 (PGJ_2), 50 μg mL^{-1} mangosteen, 20 μg mL^{-1} diindolylmethane (DIM), 4 μg mL^{-1} tschimganine, and 20 μg mL^{-1} mycophenolic acid (MPA). DMSO served as a solvent control. (D) Lifespan-extending effect of the indicated compounds in the standard CLS assay. Viability at day 0 (=reaching stationary phase, approximately 30 h after inoculation) was set to 100%, and subsequent survival in the aging cultures was normalized to day 0. Lifespan extension of an *sck2*Δ strain (AEP62) is shown for comparison. Compound concentrations are as in C. Gray curves represent compound-treated/*sck2*Δ cells, while the black curves display the controls (DMSO/wt). The black bars represent the standard deviation. (E) Lifespan extension by PGJ_2. Cells were cultured in liquid medium and exposed to 20 μg mL^{-1} PGJ_2 or the solvent control (DMSO). Aliquots were sampled at the indicated times, serially diluted and spotted on complete medium. Plates were grown for 2 days at 30°C.

they functioned in pathways that were parallel to or partially overlapping with those of DR. All compounds also increased lifespan of cells with overnutrition (5% glucose, Fig. 2B), showing that they were able to counteract the negative effects of a high-caloric diet in yeast.

Aging in *S. pombe* is delayed in the absence of Sck2 and Pka1 (Roux *et al.*, 2006). Notably, while *sck2*Δ caused an extended lifespan as compared to wild-type (wt) cells, administration of the compounds even further increased the lifespan of the *sck2*Δ strain (Fig. 2C), which suggested that none of the compounds acted in aging by inhibiting Sck2. The effect in the *pka1*Δ strain background was less homogeneous. Tschimganine and nigericin caused lifespan extension of *pka1*Δ cells (Fig. 2D), indicating that their effect on aging was independent of Pka1. Furthermore, *pka1*Δ itself causes a slight growth defect as compared to wt strains, and four substances, DIM, mangosteen, acivicin, and MPA, exacerbated this growth defect, indicating that they acted in pathways parallel to Pka1. Furthermore, monensin and PGJ_2 were unable to further increase longevity of the *pka1*Δ strain, suggesting that their effect in aging was epistatic to the Git3/PKA nutrient signaling pathway.

Many long-lived mutants in *S. cerevisiae* cause increased resistance to heat shock and oxidative stress (Fabrizio *et al.*, 2010), and suppression of DNA damage may cause longevity. We therefore tested whether the anti-aging compounds affected the response of cells to these treatments as well as to DNA damage by methylmethane sulfonate (MMS). Notably, with the exception of monensin and tschimganine, all compounds caused increased resistance to oxidative stress (Fig. 3). In contrast, only DIM, mangosteen, and

Table 1 Compounds extending chronological lifespan in *Schizosaccharomyces pombe*[†]

CAS No.	Common name	Concentration (μg mL^{-1}) [‡]	Effect[§]
42228-92-2	Acivicin*	20	+++
495-02-3	Auraptene	4	(+)
1968-05-4	3,3′-Diindolylmethane*	20	++
518-17-2	Evodiamine	2	(+)
548-83-4	Galangine	4	(+)
83807-40-3	Geranylgeranoic Acid	4	(+)
77029-83-5	Hypocrellin A	2	(+)
	Mangosteen*	50	++
22373-78-0	Monensin Sodium*	4	++
24280-93-1	Mycophenolic Acid*	20	+++
54-11-5	(-)- Nicotine	1000	++
28380-24-7	Nigericin Sodium*	2	++
481-42-5	Plumbagin	4	+
60203-57-8	Prostaglandin J_2*	20	++
83-79-4	Rotenone	4	+
515-03-7	Sclareol	4	(+)
38970-49-9	Tschimganine*	4	++
2001-95-8	Valinomycin	2	(+)
19545-26-7	Wortmannin	2	+++

[†]Compounds are presented in alphabetical order. Compounds marked with an asterisk (*) were investigated in more detail in this study.
[‡]Concentration at which maximal CLS extension without an effect on cell growth was observed (Fig. S2).
[§](+) slight increase, + moderate increase, ++ strong increase, +++ very strong increase of CLS as assayed in the microtiter dish assay. Slight increase: CLS extension by 1–2 days; moderate increase: extension by 2–3 days; strong increase: extension by 4–5 days; very strong increase: CLS extension by more than 5 days as compared to DMSO-treated control.

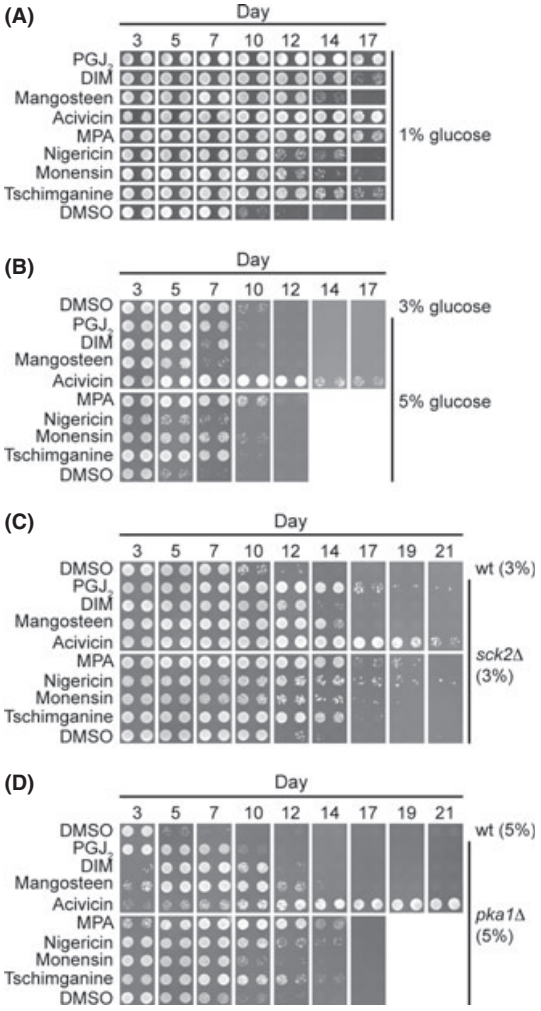

Fig. 2 The lifespan-extending compounds acted independently of dietary restriction and Sck2 and partially depended on Pka1. (A) Effect of the indicated compounds on aging under dietary restriction (1% glucose) in a wt strain (AEP57). Concentrations of substances are as in Fig. 1C. (B) Effect of the compounds in overnutrition conditions (5% glucose). Compound concentrations as in Fig. 1C. (C) Lifespan-extending effect of the indicated compounds (concentrations as in Fig. 1C) in an *sck2*Δ strain (AEP62). Aging of a wt strain (AEP60) is shown for comparison. (D) Effect of lifespan-extending compounds in a *pka1*Δ strain (AEP117) under overnutrition conditions (5% glucose).

tschimganine caused heat-shock resistance, whereas monensin, nigericin, MPA, and acivicin had the contrary effect and resulted in increased sensitivity to heat shock and additionally led to increased sensitivity to MMS (Fig. 3). Therefore, as has been reported in earlier studies for genetic mutations (Fabrizio *et al.*, 2010), there was little concordance between the effect of the compounds on lifespan extension and on stress response or DNA damage response.

In summary, the above results showed that two compounds, monensin and PGJ_2, may influence aging by inhibiting the PKA signaling pathway, whereas the other compounds acted in pathways parallel to the kinases Pka1 and Sck2 as well as to DR, suggesting that they functioned by novel anti-aging mechanisms. We next sought to gain more mechanistic insight into the aging pathways affected by these compounds.

Anti-aging effects of monensin and nigericin reveal a role for vacuolar acidification in aging

Comparison of the chemical structures of the lifespan-extending compounds showed that two of them, monensin and nigericin, have similar structures (Fig. S1). Both compounds are ionophores that mediate Na^+/H^+ and K^+/H^+ exchange. As a consequence, they are able to disturb the membrane potential and thus the function of several intracellular compartments, including Golgi and the vacuole (Dinter & Berger, 1998). Notably, treatment with the Golgi-disturbing agent brefeldin A had no effect on lifespan (data not shown), indicating that endoplasmic reticulum (ER) stress alone was not sufficient to extend lifespan. Importantly, in *S. cerevisiae*, monensin treatment is epistatic to the function of the vacuolar H^+ ATPase (V-ATPase) (Gustavsson *et al.*, 2008), indicating that its most prominent effect in yeast is its interference with the maintenance of an acidic pH in the vacuole.

In *S. pombe*, the genes *vma1*$^+$ and *vma3*$^+$ code for subunits A and C of the V-ATPase (Iwaki *et al.*, 2004), which is required for vacuolar acidification, vacuole morphology, and endocytosis. If monensin and nigericin influence lifespan by disturbing the pH of the vacuole, then one might expect that disturbance of vacuolar acidification may similarly affect lifespan and that this effect should be epistatic to the ionophores. Interestingly, we observed that

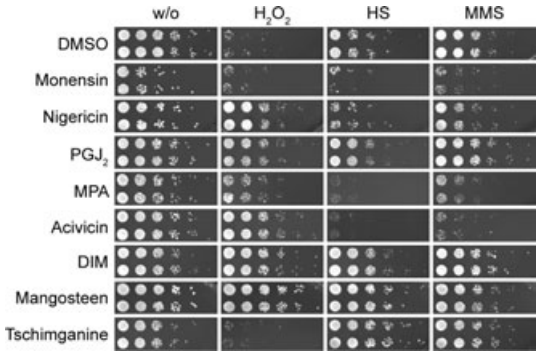

Fig. 3 Effect of anti-aging compounds on sensitivity to oxidative stress, heat shock, and DNA damage. A wt strain (AEP57) was treated with H_2O_2 (150 mM) for 1 h, heat shocked (HS) at 48°C for 1 h or treated with methyl-methane sulfonate (MMS), 0.2%] for 45 min. Serial dilutions were spotted onto full medium plates and grown for 2 days at 30°C.

*vma1*Δ and *vma3*Δ both caused a shortening of the *S. pombe* lifespan (Fig. 4A), indicating that the absence of an acidic pH in the vacuole or changes in vacuolar morphology enhanced aging. This was in agreement with observations in *S. cerevisiae* that the deletion of genes implicated in protein targeting to the vacuole (*VPS* genes) caused a shortening of lifespan (Fabrizio *et al.*, 2010). Significantly, in these vacuolar mutants, both monensin and nigericin were no longer able to cause a prolonged lifespan (Fig. 4A). This showed that the effect of these compounds on aging required the activity of the V-ATPase.

As the absence of V-ATPase accelerated *S. pombe* aging, this suggested that the opposite, namely increased V-ATPase function, may have the reverse effect and cause lifespan extension. Indeed, overexpression of *vma1*$^+$ from the heterologous *nmt1*$^+$ promoter attenuated aging (Fig. 4B), and as above, the presence of monensin was unable to further increase lifespan in this context.

We further sought to verify the effect of monensin and nigericin on vacuolar acidification by measuring the accumulation of the fluorescent dye quinacrine in acidic compartments. Untreated young wt cells showed maximal vacuolar fluorescence, and no further increase was observable in young cells treated with the compounds (Fig. 4C). In contrast, as expected, *vma3*Δ cells lacking the V-ATPase showed no quinacrine staining. Interestingly, aged untreated cells showed a weaker quinacrine staining that was more dispersed and punctate, suggesting that the vacuoles became fragmented and that vacuolar acidity decreased with increasing age in *S. pombe* cells. Strikingly, aged cells treated with monensin or nigericin showed a distinct quinacrine staining in that the cells were strongly and uniformly stained and lacked the fragmentation of acidic compartments observed in untreated cells. Furthermore, the overexpression of *vma1*$^+$ showed the same staining pattern as monensin or nigericin treatment in aged cells (Fig. 4C). This indicated that the two compounds or *vma1*$^+$ overexpression counteracted the age-associated fragmentation of acidic compartments and the loss of acidity during aging.

In summary, our results showed that maintenance of the acidic pH of the vacuole and suppression of vacuolar fragmentation, either

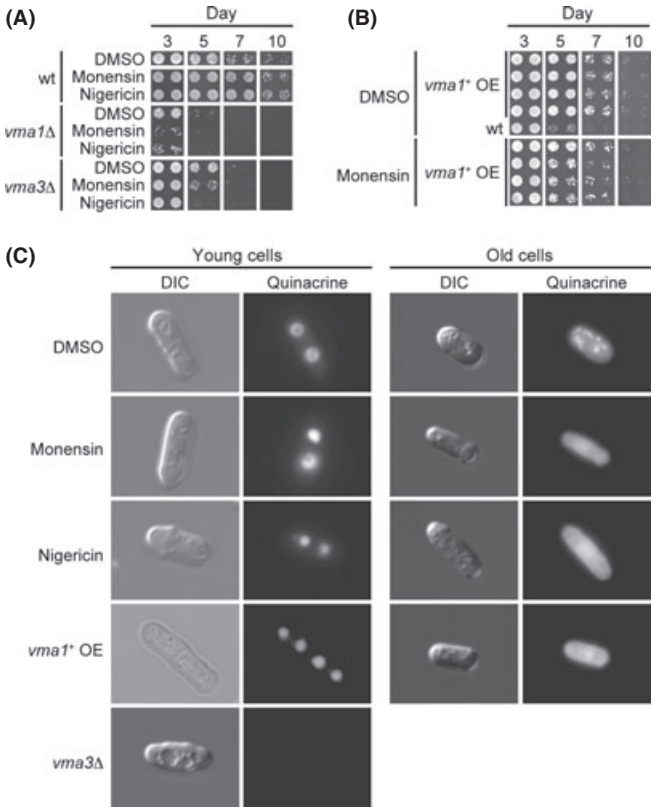

Fig. 4 Pharmacological or genetic disturbance of vacuolar acidification caused lifespan extension. (A) Lifespan extension by monensin and nigericin required a functional V-ATPase. Aging assays with wt (AEP67), *vma1*Δ (AEP68), and *vma3*Δ (AEP69) strains were performed in the presence of 4 μg mL^{-1} monensin or 2 μg mL^{-1} nigericin or with the solvent control (DMSO). *vma1*Δ caused lifespan shortening. (B) Overexpression of *vma1*$^+$ prolonged lifespan and was not further extended by treatment with monensin. A wt strain (AEP57) carrying an empty vector (pAE1429) or a plasmid with *vma1*$^+$ under control of the *nmt1*$^+$ promoter (pAE1958) was used in an aging assay with 4 μg mL^{-1} monensin or DMSO as a control. (C) Vacuolar fragmentation in aged cells was counteracted by treatment with monensin or nigericin, or by overexpression of *vma1*$^+$. Acidic compartments of young cells (exponential growth phase) or aged cells (day 3) were stained with quinacrine to visualize vacuoles. Monensin (4 μg mL^{-1}) or nigericin-treated cells (2 μg mL^{-1}) were compared with control cells treated with DMSO.

using the V-ATPase or by pharmacological intervention using monensin and nigericin, were able to extend lifespan in *S. pombe* cells, thus showing an unanticipated role for vacuolar pH homeostasis in aging.

Prostaglandin J_2 uncovers a role for mitochondrial fission in lifespan extension

In our screen, we identified the class 2 prostaglandin PGJ_2 to promote longevity in *S. pombe*. Of note, yeast cells do not produce prostaglandins. We also tested other prostaglandins (PGA_1, PGB_1, PGE_1, PGE_2, and $PGF_{2\alpha}$) for an effect on aging, but none of them caused lifespan extension (data not shown). Importantly, 15d-PGJ_2 has previously been shown to induce mitochondrial elongation in mammalian cells (Mishra *et al.*, 2010), and we therefore asked whether the lifespan-extending effect in yeast was related to this function.

Mitochondria are dynamic organelles that continuously fuse and divide. In mammals, the key molecules for mitochondrial fission are dynamin-related protein 1 (Drp1) and Fis1 (Scorrano, 2007). The reciprocal process, mitochondrial fusion, is controlled by mitofusin (Mfn) 1/2 and OPA1 (Scorrano, 2007). Accordingly, the induction of mitochondrial elongation by 15d-PGJ_2 in mammalian cells results from the inhibition of mitochondrial fission by covalent modification and inactivation of the fission protein Drp1 (Mishra *et al.*, 2010). In fission yeast, homologs of Drp1 and OPA1 are called Dnm1 and Msp1.

In order to test whether there is a relationship between the potential inactivation of the mitochondrial fission protein Dnm1 by PGJ_2 and lifespan extension, the CLS of *dnm1*Δ and *msp1*Δ cells was determined. Notably, the CLS of *dnm1*Δ cells was comparable to that of wt cells, while *msp1*Δ cells displayed a slightly reduced lifespan (Fig. 5A), indicating that impaired mitochondrial fusion accelerated aging. This was in agreement with the observation in *S. cerevisiae* that the absence of the OPA1/Msp1 homolog Mgm1 reduced lifespan (Scheckhuber *et al.*, 2011). Importantly, treatment with PGJ_2 resulted in increased lifespan of wt and *msp1*Δ cells, whereas the CLS of *dnm1*Δ cells was unchanged by PGJ_2 (Fig. 5B). These results indicated that lifespan extension by PGJ_2 required Dnm1 and that it thus acted in the Dnm1-dependent mitochondrial division pathway. However, *dnm1*Δ cells, which have a defect in mitochondrial fission, did not display an increased CLS compared to wt cells, suggesting that PGJ_2 affected other anti-aging pathways apart from mitochondrial fission.

The above results suggested that PGJ_2 acted in aging by inhibiting mitochondrial fission. To further test this, we monitored mitochondrial morphology in young and aged cells in the presence or absence of PGJ_2. In young cells, a punctate pattern of mitochondrial staining was observed, regardless of whether cells were treated or not (Fig. 5B). In aged untreated cells, mitochondrial staining was weak and showed an altered distribution. Importantly, aged cells that were treated with PGJ_2 contained elongated, tubular mitochondria that were not observed in untreated cells. This demonstrated that PGJ_2 caused mitochondrial elongation, most likely by inhibiting fission.

Notably, prostaglandins in human cells have been reported to inhibit G-protein-coupled receptors (GPCR) (Narumiya *et al.*, 1999), indicating that PGJ_2 may also inhibit such (a) receptor(s) in yeast. Therefore, we analyzed the CLS of strains carrying a deletion of genes encoding GPCRs or proteins involved in G-protein signaling (Xue *et al.*, 2008). One such protein is the glucose receptor Git3 whose absence has previously been shown to increase CLS (Roux *et al.*, 2010). In our experiment, *git3*Δ cells were the only cells whose lifespan was increased compared to that of wt (Fig. 5C). Furthermore, PGJ_2 treatment caused no further lifespan extension in *git3*Δ cells, while it increased CLS in all other GPCR deletion strains (Fig. 5C). This suggested that PGJ_2 extended the CLS of fission yeast

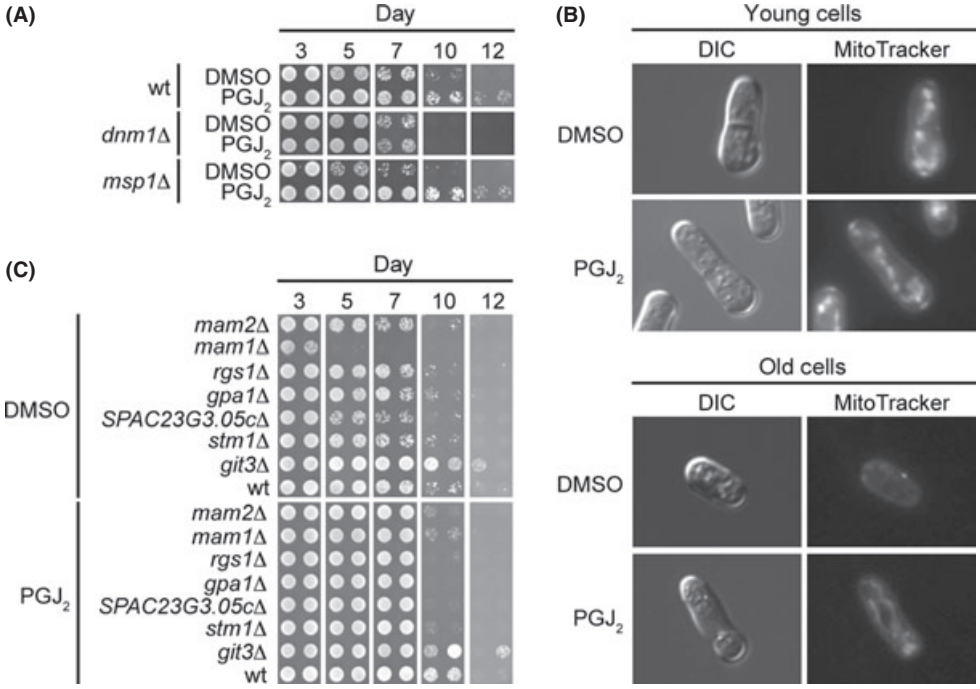

Fig. 5 Prostaglandin J_2 caused lifespan extension by inhibition of mitochondrial fission and the glucose receptor Git3. (A) The longevity-promoting effect of PGJ_2 required the mitochondrial fission protein Dnm1, but not the fusion protein Msp1. Aging assays were performed with wt (AEP57), *dnm1*Δ (AEP176), and *msp1*Δ (AEP177) in the presence of 20 μg mL^{-1} PGJ_2 or DMSO. (B) Treatment with PGJ_2 caused mitochondrial elongation. Young and aged wt cells (AEP57, at exponential growth phase or at day 1) treated with DMSO or 20 μg mL^{-1} PGJ_2 were stained with MitoTracker Orange to visualize mitochondria. (C) Induction of longevity by PGJ_2 required the G-protein-coupled glucose receptor Git3, but none of the other G-protein-coupled proteins in *Schizosaccharomyces pombe*. The indicated strains were used for aging assays in the presence or absence of PGJ_2 as indicated in (A).

by inhibiting Git3 in addition to its effect on mitochondrial fission. Because Git3 signals via Pka1 (Roux *et al.*, 2010), this was in agreement with our earlier observation of a Pka1 dependence of lifespan extension by PGJ_2 (Fig. 2D).

Disturbance of intracellular GMP levels by mycophenolic acid and acivicin counteracts aging

Interestingly, two compounds identified in the chemical genetic screen for anti-aging substances affected enzymes involved in the biosynthesis of GMP. Mycophenolic acid (MPA) is an inhibitor of inosine monophosphate dehydrogenase (IMPDH), the enzyme that controls the rate of GMP synthesis in the *de novo* pathway of purine synthesis (Fig. S4). Acivicin is a glutamine analog that selectively and irreversibly abolishes the glutaminase activity of GMP synthetase by a covalent modification (Nakamura *et al.*, 1995) (Fig. S4). Based on this, we hypothesized that these two compounds extended CLS by disturbing the cellular guanine nucleotide pool. This model suggests that interfering with the function of the respective GMP biosynthetic enzymes, IMPDH and GMP synthetase, should extend lifespan. However, deletion of the respective genes with this predicted function (*gua1*$^+$ and *gua2*$^+$) is lethal in *S. pombe* (Kim *et al.*, 2010), and no alleles are available, such that this hypothesis could not be tested.

Anti-aging properties of diindolylmethane, mangosteen, and tschimganine

We further considered the effects of the three remaining anti-aging compounds identified here, DIM, mangosteen, and tschimganine. DIM influences several different pathways that are linked to a delay of aging (Aggarwal & Ichikawa, 2005), and DIM is being used in clinical trials against multiple forms of cancer. Notably, it has been reported to increase the production of ROS, but also to protect against oxidative stress (Fan *et al.*, 2009). To test for an effect of DIM on ROS production in yeast cells, we stained DIM-treated wt cells with dihydrorhodamine 123 (DHR123), a cell-permeable fluorogenic probe that can be used to detect ROS (Roux *et al.*, 2006). Interestingly, DIM-treated cells in the exponential phase of growth showed increased ROS levels as compared to control cells, whereas ROS levels were decreased in stationary-phase cells (Fig. 6A), an effect that was not observed for any of the other lifespan-extending compounds reported here (data not shown).

Similar to DIM, mangosteen also has known anti-oxidative properties (Obolskiy *et al.*, 2009). It is a compound mixture extracted from the exocarp of the mangosteen fruit (*Garcinia mangostana*). Accordingly, like DIM, mangosteen caused a decrease in ROS levels in stationary-phase cells (Fig. 6A). However, in contrast to DIM, mangosteen did not lead to increased ROS levels in exponentially growing cells. We furthermore tested the individual effects of the four xanthones that are the main constituents of mangosteen, namely γ-mangostin, α-mangostin, gartanin, and 8-desoxygartanin (Obolskiy *et al.*, 2009), but none of them altered the CLS of *S. pombe* cells (data not shown), suggesting that another unknown constituent of mangosteen, or the interplay of single compounds, led to lifespan extension.

Tschimganine was first isolated from the roots of *Ferula tschimganica*. Except for an estrogenic activity (Ikeda *et al.*, 2002), little is known about this compound. Tschimgine and tschimganidine are two related compounds (Fig. 6B), and we therefore tested their effect on aging in *S. pombe*. Interestingly, tschimganidine caused lifespan extension, although to a lesser extent than tschimganine and at a higher compound concentration, whereas tschimgine showed no effect on lifespan (Fig. 6C). Because the only difference between tschimganine and tschimgine is the presence of a methoxy group in the former, this indicates that this chemical group contributes to the anti-aging effect of tschimganine. This group is contained in tschimganidine, but the bornyl substituent is replaced in tschimganidine by a more complex structure. One

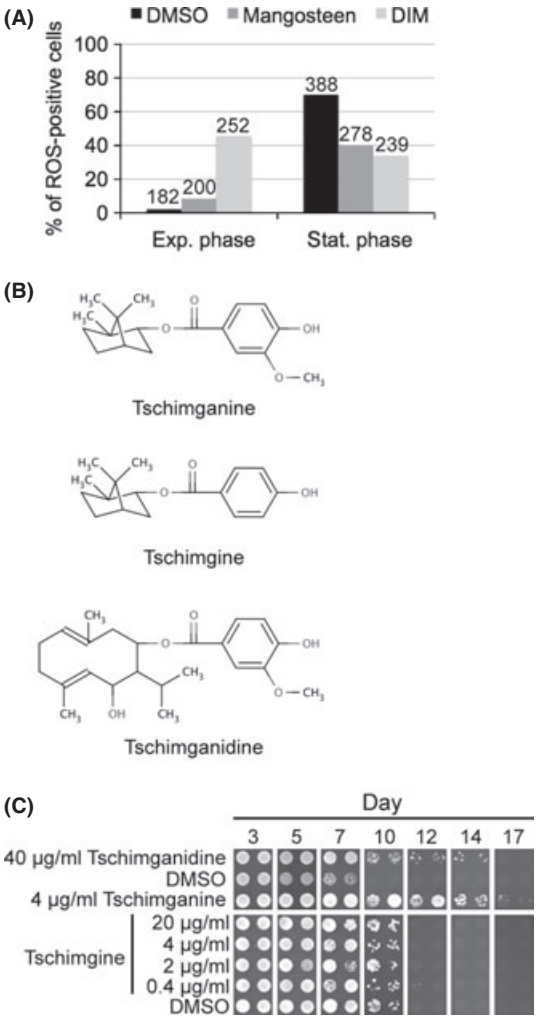

Fig. 6 Anti-aging properties of diindolylmethane (DIM), mangosteen, and tschimganine. (A) DIM induced the production of reactive oxygen species (ROS) in exponentially growing cells, and 20 µg mL^{-1} DIM and 50 µg mL^{-1} mangosteen caused a reduction in cells in stationary phase. wt cells (AEP57) in the respective growth phases were stained with DHR123. Numbers above the bars indicate the number of cells evaluated. (B) Chemical structure of tschimganine, tschimgine, and tschimganidine. (C) Tschimganidine, but not tschimgine, increased lifespan in fission yeast. The aging assay was performed as in Fig. 1C using the indicated compound concentrations.

possibility is that the bornyl moiety enhances membrane permeability, which may explain why tschimganine has more potent anti-aging properties than tschimganidine.

Discussion

In this study, we have conducted a chemical genetic screen for small molecules that increase CLS in *S. pombe* and have characterized the effect of seven compounds and one compound mixture in more detail. In doing so, we have identified lifespan-extending effects for compounds that affect vacuolar acidification, mitochondrial fission, Git3/PKA signaling as well as intracellular GMP levels, and thus have uncovered novel pharmacological ways of modulating cellular pathways that counteract aging.

Our screen yielded two related ionophores, monensin and nigericin, as anti-aging compounds. They acted by affecting the acidity of the vacuolar compartment and by counteracting vacuolar fragmentation in aging cells, thus reflecting a link between vacuolar biology and aging. In agreement with this, we found that overexpression of the *vma1*$^{+}$ gene, which encodes a subunit of the vacuolar ATPase, also extended lifespan and inhibited the loss/fragmentation of acidic compartments in aged cells. Vacuolar function is important to counteract aging, because it is necessary for the autophagic removal of damaged cellular components (Kenyon, 2010). Consistent with this, defects in autophagy shorten lifespan in yeast (Alvers *et al.*, 2009), and lifespan extension, for instance by reduced insulin/IGF signaling in worms, requires autophagy (Melendez *et al.*, 2003). Our results show that maintaining the acidic vacuolar pH delays senescence in *S. pombe*. Notably, these conclusions are in good agreement with a recent study showing that vacuolar acidity declines with replicative age in the yeast *S. cerevisiae*, and that overexpression of subunits of the V-ATPase (*VMA1* or *VPH2*) suppresses this decline and increases replicative lifespan (Hughes & Gottschling, 2012). One possibility is that autophagic processes in the cell thus are enhanced and that this then slows aging processes. However, whether monensin or nigericin can be used for lifespan extension in larger organisms or even in humans remains to be seen, because they are widely used as growth promoters in the poultry and cattle industry due to their antimicrobial effect against coccidiosis.

We furthermore report here that pharmacological inhibition of mitochondrial fission increased longevity in fission yeast. This observation is in agreement with work from *C. elegans* showing that inhibition of the mitochondrial fission factor DRP-1 exacerbates the lifespan-extending effect of reduced insulin/IGF growth signaling (Yang *et al.*, 2011). It furthermore mirrors work in *S. cerevisiae* and the filamentous ascomycete *Podospora anserina* showing that the absence of the Drp1 homolog Dnm1 increases lifespan (Scheckhuber *et al.*, 2007) and that attenuation of mitochondrial fission in *S. cerevisiae* by acetyl-L-carnitine counteracts aging (Palermo *et al.*, 2010). We here extend these findings by the discovery of a novel pharmacological means to extend lifespan by inhibiting mitochondrial fission. Interestingly, recent work indicates that there is cross-talk between vacuolar acidity and mitochondrial function and that mitochondrial fragmentation in aging cells can be counteracted by maintaining the acidic pH in the vacuole (Hughes & Gottschling, 2012). It is interesting to note that the chemical genetic screen reported here has revealed compounds acting on mitochondrial fission (PGJ_2) and vacuolar acidification (nigericin and monensin).

Our work furthermore has yielded two compounds that affect cellular GMP levels, MPA and acivicin, as counteracting aging in *S. pombe*. Both compounds inhibit enzymes involved in GMP biosynthesis, implicating that reducing intracellular GMP levels contributes to longevity. One possibility is that this causes an imbalance in the guanine nucleotide pool, which leads to impaired ribosome biogenesis and an altered translational program that affects aging. Alternatively, changes in the guanine nucleotide pool may be perceived by the cells as a signal of nutrient deprivation and thus may induce longevity pathways, much as is the case for DR.

Our screen has further highlighted three other compounds as displaying anti-aging activities, DIM, mangosteen, and tschimganine. While for the compounds described above we have been able to deduce a mechanism of action in longevity based on previous knowledge, this approach has not yielded specific insights for the remaining three compounds. Both DIM and mangosteen are described to have anti-oxidative properties, which we also observed. However, we also found DIM to increase ROS production in exponentially growing cells. One possibility is that DIM increases the cellular ROS level during exponential growth, which in turn activates the expression of stress response genes, thus resulting in increased oxidative stress resistance and a reduced cellular ROS level in stationary-phase cells. Interestingly, DIM is a major acid condensation product of indole-3-carbinol, which is found in *Brassica* vegetables. The proposed beneficial health effects of these vegetables may in part be attributed to DIM. DIM also has anti-proliferative effects on tumor cells and has been tested in clinical trials against certain cancer types, although so far with little effect (Castanon *et al.*, 2012). It will therefore be interesting to determine the molecular target of DIM that causes lifespan extension in yeast in order to see how this is relevant for its diverse effects in larger eukaryotes.

Similarly to DIM, mangosteen and other products of the mangosteen fruit (*Garcinia mangostana*) are sold as dietary supplements. Several parts of *G. mangostana* have been used for hundreds of years in traditional herbal medicine for a variety of medical conditions, based on their antibacterial, antifungal, and anti-inflammatory properties (Obolskiy *et al.*, 2009). Whether any of these are related to the anti-aging activity we describe here will be of future interest.

As for tschimganine and tschimganidine, very little is known about their biological activities. They can be found in Iranian propolis (bee glue), which is widely used in folk medicine; they have antibacterial activity and cytotoxic activity in several cancer cell lines (Sahranavard *et al.*, 2009), and tschimganidine is a phytoestrogen (Ikeda *et al.*, 2002). Thus, we here have identified an unanticipated activity for these substances that holds promise for their further development as drugs that delay age-related diseases. Identifying their molecular mechanism of lifespan extension will be paramount for their further medical use.

In summary, our approach of using a cellular readout for identifying biological anti-aging activity has proven successful in revealing unexpected effects for compounds with known cellular targets. We thus show that pharmacological modulation of vacuolar acidification, inhibition of mitochondrial fission, and perturbation of intracellular GMP levels slow down the aging process in *S. pombe* cells. We furthermore have identified three lifespan-extending compounds whose mechanism we have not been able to deduce. This circumstance reveals the flip side of the coin of discovering activities using a biological readout in that the compounds exert a clear biological effect, but the molecular mechanism of this effect, in the absence of any other information about the compound, cannot easily be determined. Nonetheless, these substances represent interesting new starting points for the development of interventions to slow down the aging process and the onset of age-related diseases.

Experimental procedures

Yeast strains, media, and measurement of chronological lifespan

Schizosaccharomyces pombe strains used in this study and growth conditions are described in Supporting Information. CLS was measured either according to Chen & Runge (2009) or using a high-throughput method developed in this study. Briefly, overnight cultures of yeast strains grown in supplemented SD medium (Supporting Information) were used to inoculate medium in a 96-well microplate. The compounds (final concentration as indicated in the text and figure legends) were added to the medium immediately before adding the yeast cells. For overnutrition or DR, 5% and 0.5% or 1% glucose were used, respectively. Aliquots of the cultures were taken at regular intervals, spotted onto *S. pombe* full medium (YES) plates and grown for 2 days at 30°C. The density of the grown cells per spot reflects the viability of the single aging cultures.

In vivo staining of ROS accumulation, acidic compartments, and mitochondria

Fluorescence staining of cells for ROS accumulation using dihydrorhodamine 123 (DHR123) was performed as described (Roux *et al.*, 2006). Stained cells were counted by using ImageJ 1.39. The staining of acidic compartments was performed using quinacrine dihydrochloride as described earlier (Liu *et al.*, 1997), except that quinacrine was dissolved in YES medium. Mitochondria were stained using 150 nM MitoTracker Orange. All fluorescence stainings were analyzed under a Zeiss Axio Imager.M1 fluorescence microscope using the AxioVision Software.

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Author contributions

JS performed the experiments. JF, JS, and AEM conceived and planned the experiments. JS and AEM wrote the manuscript.

Conflict of interest

The authors declare no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Fig. S1 Chemical structure of CLS-extending compounds.

Fig. S2 Determination of the optimal concentration for CLS extension of the indicated compounds in *S. pombe*

Fig. S3 Inhibitors of GMP biosynthesis cause lifespan extension.

Table S1 Compounds tested in this study for anti-aging effects (Biomol, Evonik)