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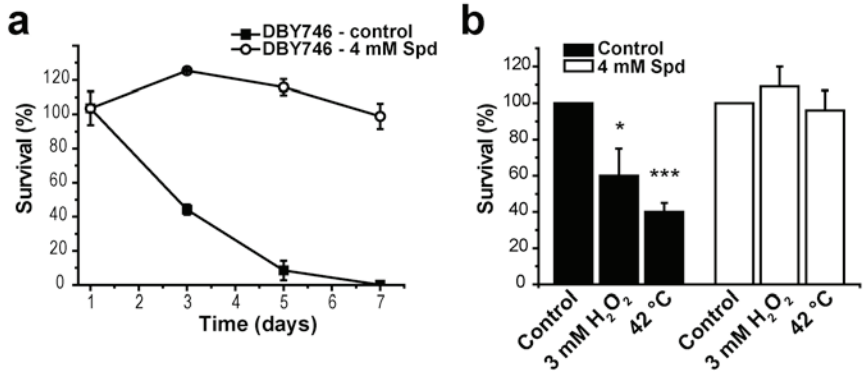

Figure S1 Spermidine treatment of yeast results in strong resistance against heat shock and hydrogen peroxide treatment. **(a)** Survival determined by clonogenicity during chronological ageing of wild type yeast (DBY746) with (o) and without (▪) addition of 4 mM spermidine at day 1. Data represent means ± SEM (n = 4). **(b)** Survival of pre-aged wild type cells stressed for 4 h with hydrogen peroxide (3 mM H_2O_2) or heat shock (42 °C) compared to unstressed cells. Cells were chronologically aged until day 24 with or without addition of 4 mM spermidine. Data represent means ± SEM (n = 4). *$p < 0.05$ and ***$p < 0.001$

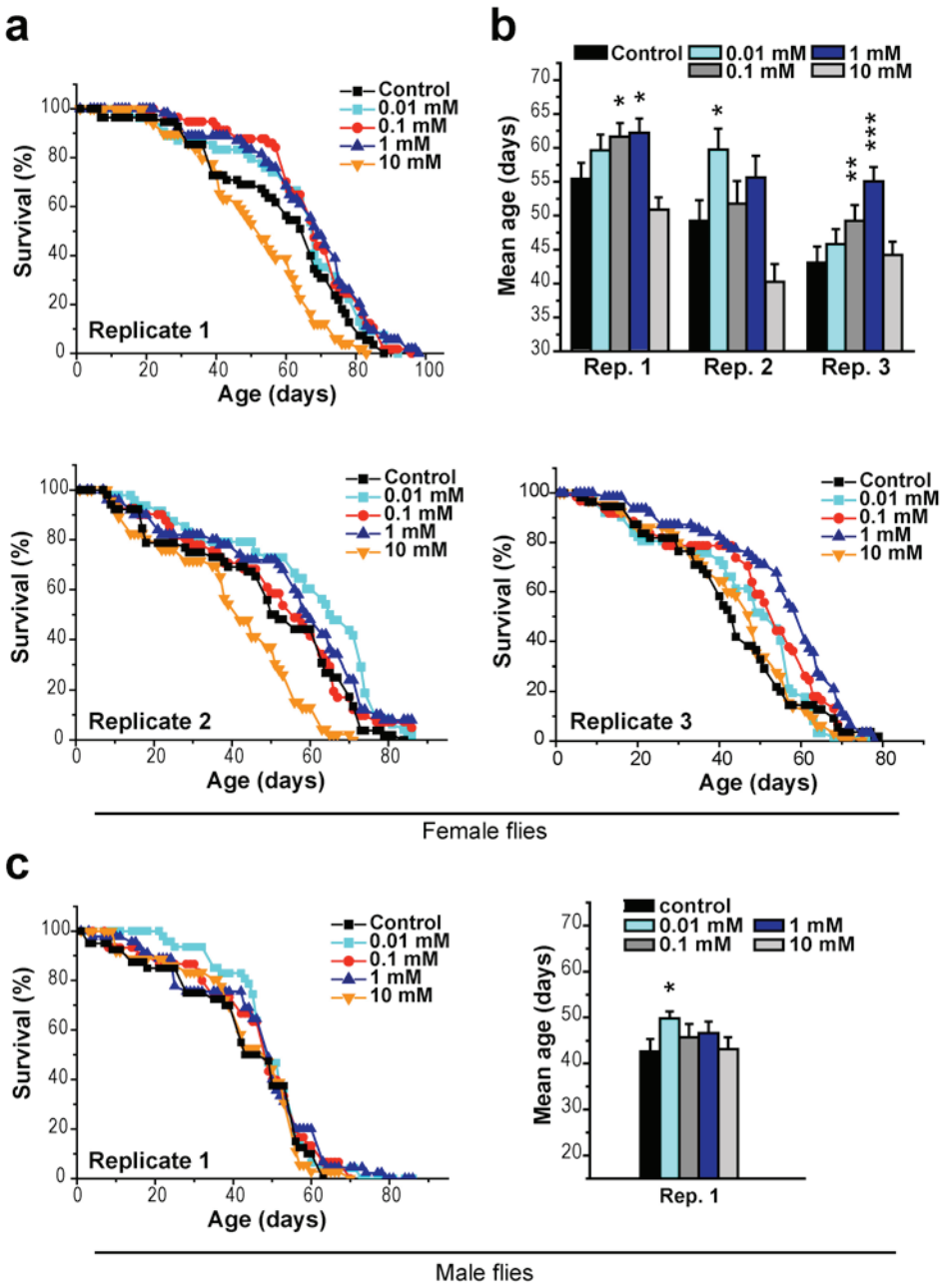

Figure S2 Administration of spermidine extends the life span of *Drosophila melanogaster*. **(a)** Replicates of female *Drosophila melanogaster* ageing experiments with and without (▪) supplementation of normal food with various concentrations of spermidine. **(b)** Mean life spans calculated from the experiments shown in (a). Data represent means ± SEM of 40-60 flies. *p < 0.05, **p < 0.01 and ***p < 0.001. **(c)** *Drosophila melanogaster* ageing experiment of males with and without (▪) supplementation of normal food with various concentrations of spermidine and respective mean life spans. Data represent means ± SEM of 40-60 flies. *p < 0.05.

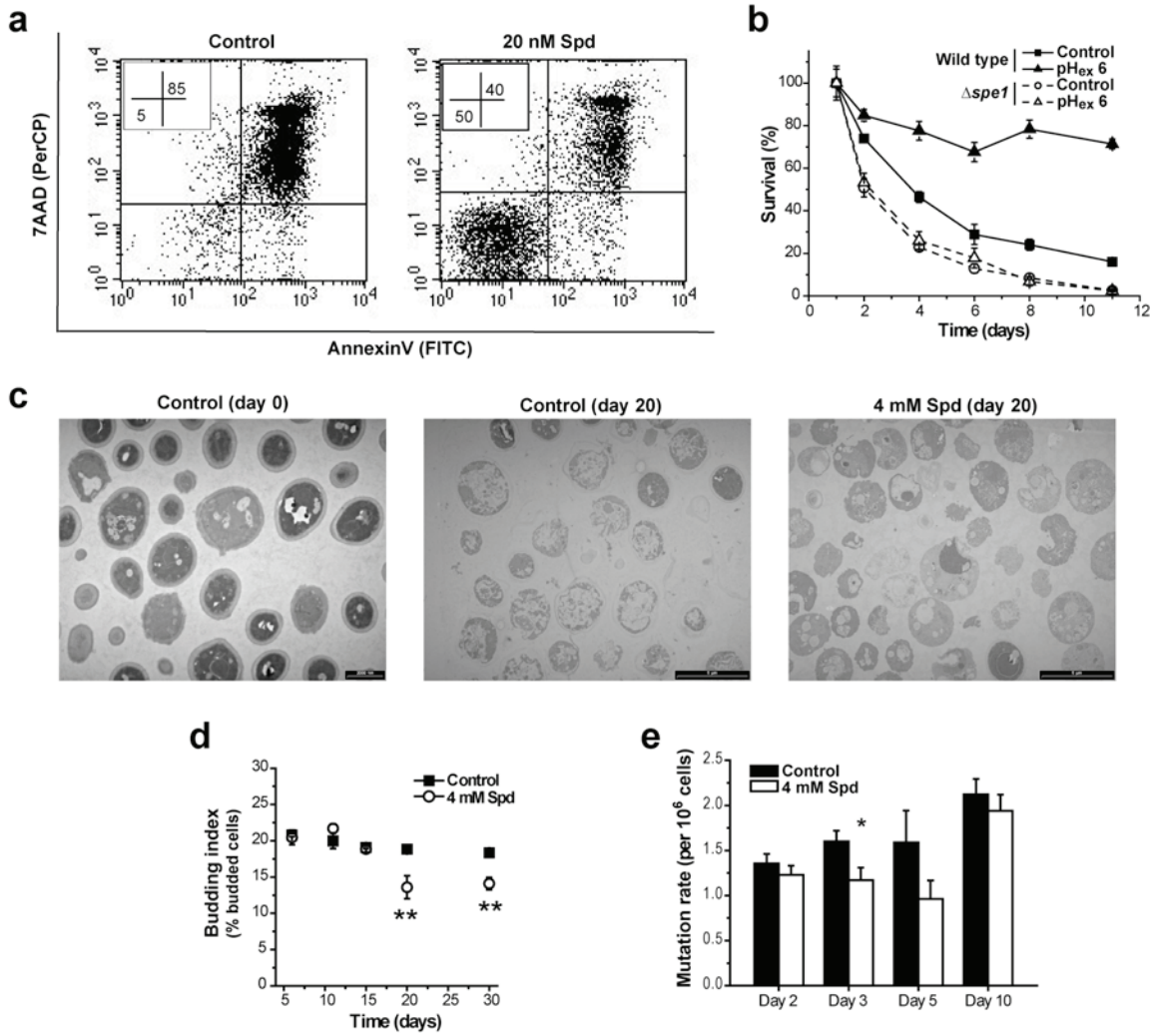

Figure S3 Spermidine inhibits necrotic cell death of aeging yeast and human PBMC. **(a)** Quantification (FACS analysis) of phosphatidylserine externalisation (FITC channel) and loss of membrane integrity indicative of necrosis (PerCP channel) using AnnexinV/7-ADD costaining of 12 days old human PBMC. Unstained cells were considered as viable. Dot plots with 30,000 cells evaluated of a representative experiment are shown. Numbers indicate the percentage of cells located in their respective gate. **(b)** Chronological ageing of wild type (closed symbols) and *Δspe1* (open symbols) with (▲,Δ) and without (▪,o) adjustment of extracellular pH to 6 (± 0.5). Data represent means ± SEM (n = 3). **(c)** Overview pictures of electron microscopy of 20 days old wild type yeast cells aged with or without (control) treatment of 4 mM spermidine and of healthy young cells. Higher resolution images of representative cells are shown in Figure 3E. **(d)** Budding index of wild type cells at indicated time points during chronological ageing with (o) or without (▪) application of 4 mM spermidine. Data represent means ± SEM (n = 3) with at least 500-1000 cells evaluated for each replicate. **p < 0.01. **(e)** Mutation rate per 10^6 living cells determined by canavanine resistance of wild type cells at indicated time points during chronological ageing with (open bars) or without (closed bars) application of 4 mM spermidine. Data represent means ± SEM (n = 5). *p < 0.05

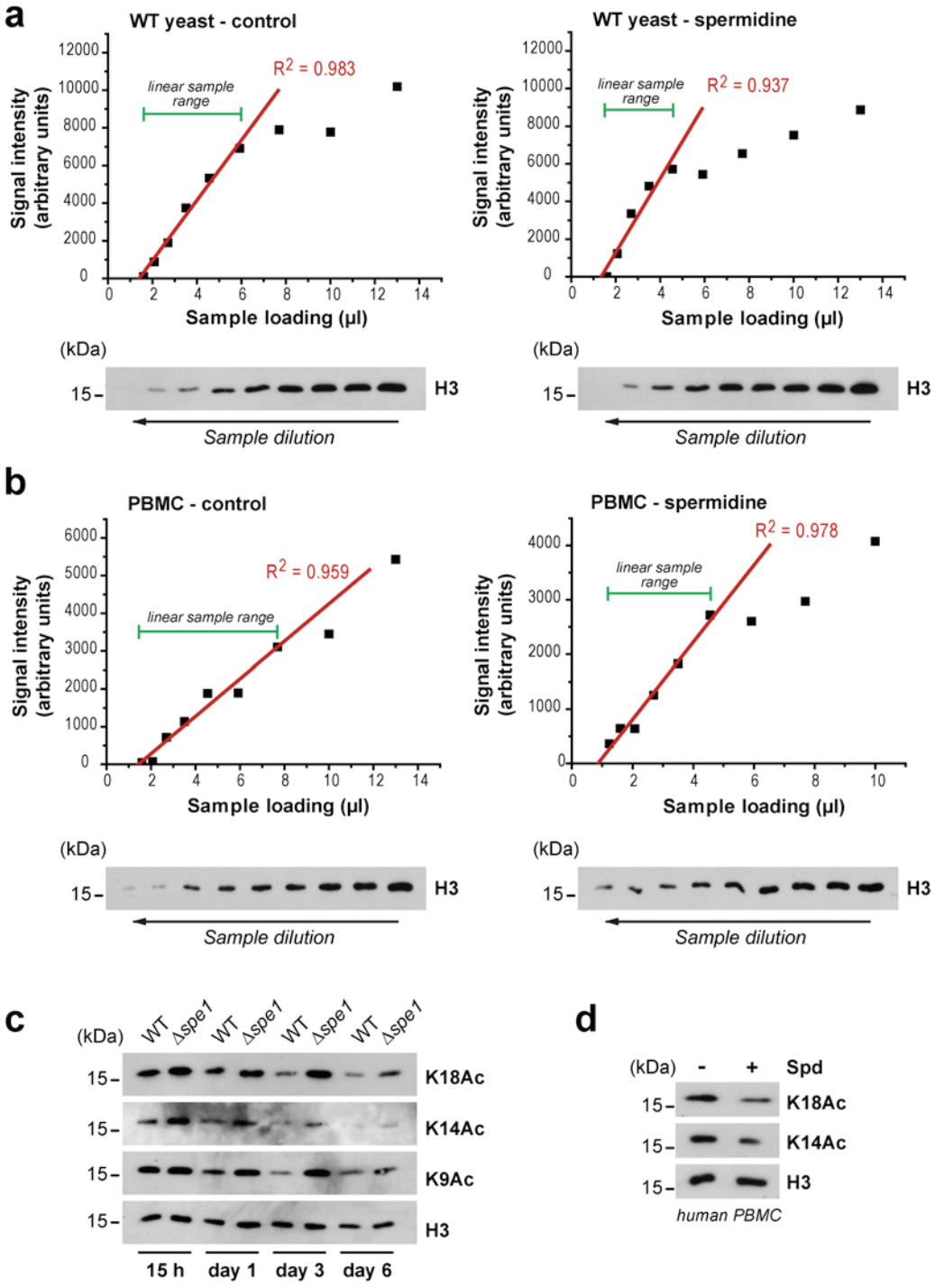

Figure S4 Serial dilutions were initially applied to immunoblot analysis in order to determine the linear range of sample loading and quantification by densitometry. **(a, b)** Representative examples of the identification of the linear range for quantitative immunoblot analyses are presented. Linear regression was performed after densitometry of shown immunoblot analyses from yeast (**a**) and PBMC (**b**) protein extracts aged with or without (control) application of spermidine for 16 days (**a**) or 6 days (**b**) in order to determine the linear range of sample loading and quantification. Blots were probed with an antibody specific for total histone H3. Subsequently, samples were applied to immunoblot analysis exclusively within this linear range for the quantification of spermidine effects on histone acetylation using acetylated lysine specific antibodies (see Methods for details). **(c)** Representative immunoblot analysis of wild type and *Δspe1* yeast cell extracts 15 hours past inoculation and after 1, 3 and 6 days of chronological ageing. Blots were probed with antibodies against total histone H3 or H3 acetylation sites at the indicated lysine residues. **(d)** Immunoblot analysis of human PBMC extracts after 6 days of ageing in the presence (+) or absence (-) of 20 nM spermidine. A representative blot is shown.

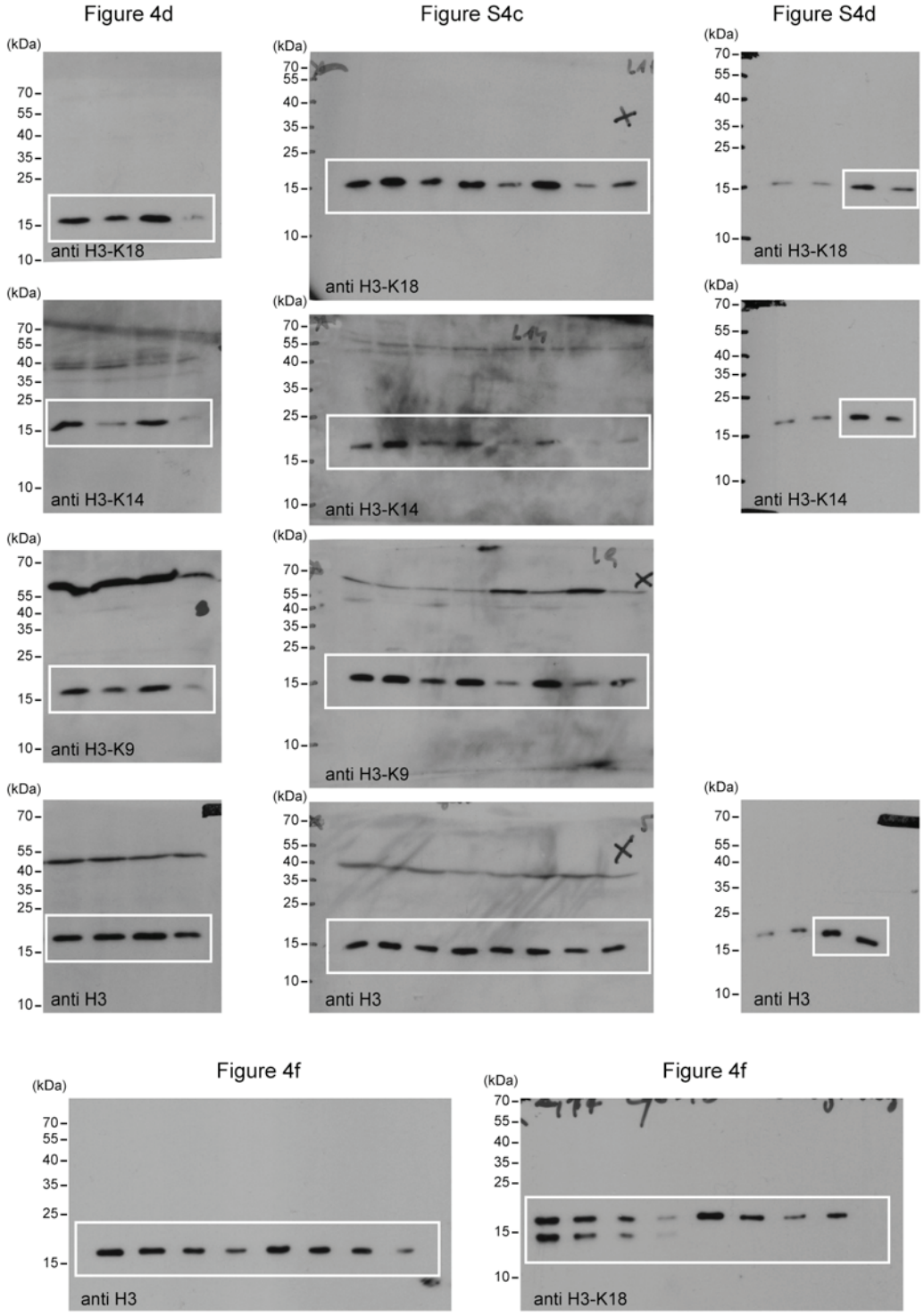

Figure S5 Full scans of key western blots presented in this study. Rectangles delimit cropped areas used in the indicated figures.

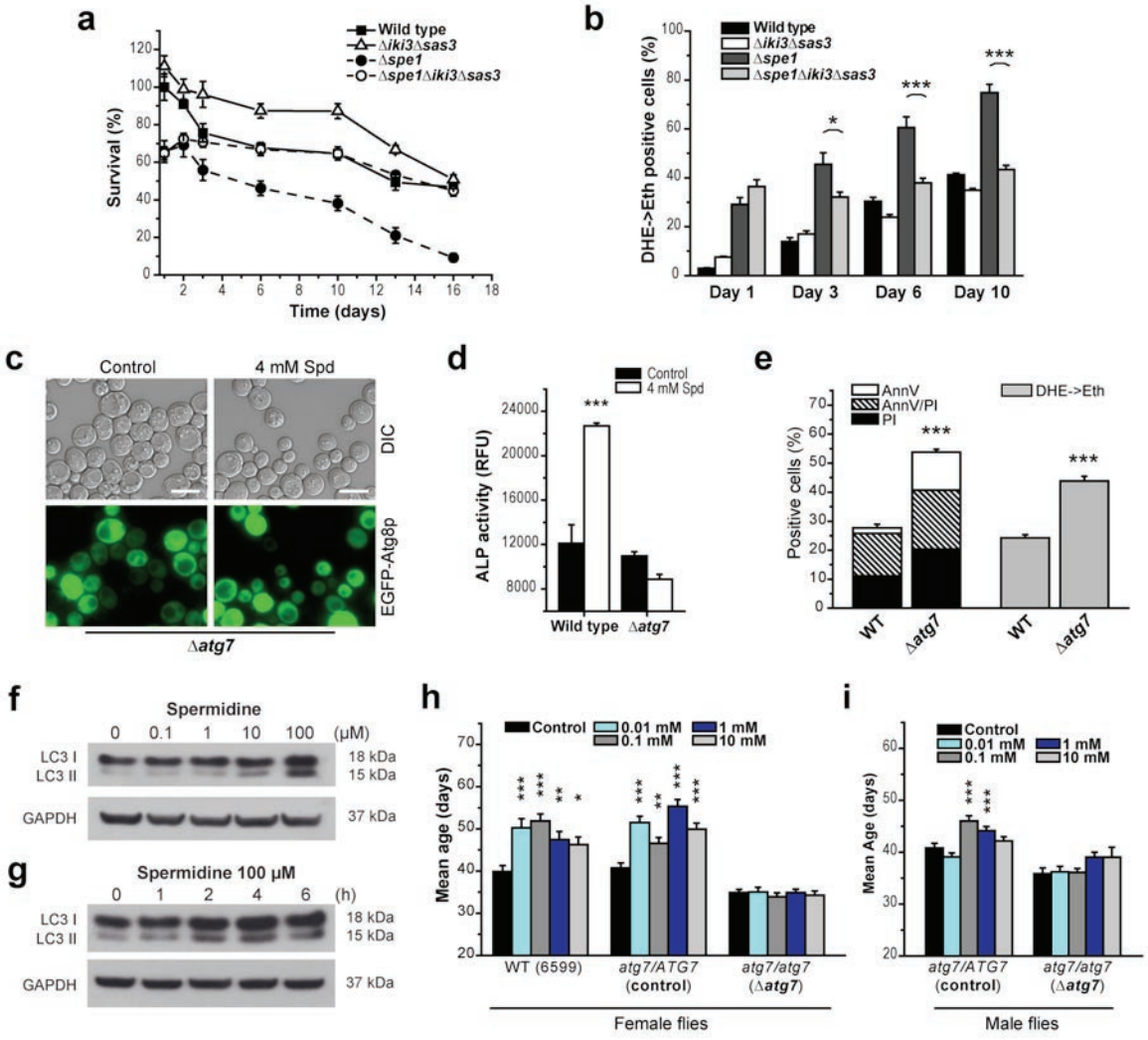

Figure S6 *ATG7*-dependent autophagy protects against age-induced programmed death and is critical for spermidine-mediated life span extension. **(a)** Chronological ageing of wild type (▪), *Δiki3Δsas3* (Δ), *Δspe1* (•) and *Δspe1Δiki3Δsas3* (o) cells. Data represent means ± SEM (n = 4). **(b)** Quantification (FACS analysis) of ROS accumulation using DHE staining of cells obtained from the chronological ageing experiment shown in (a). Data represent means ± SEM (n = 4). *p < 0.05, ***p < 0.00

Supplementary Table S1

Modulation of yeast chronological ageing upon single disruption of genes involved in histone acetylation and deacetylation.

The effects on chronological ageing observed in 28 single deletion strains of genes involved in histone acetylation (***bold italic*** characters) or deacetylation (*italic* characters) are presented. All strains were aged with and without application of 4 mM spermidine and survival was determined by clonogenicity. Deletion strains were assigned to one of six categories, depending on the effects on survival during ageing and the ability of spermidine to improve this survival.

Survival during chronological ageing (compared to WT)	**Pro-survival effect of spermidine application (compared to WT)**	**Single deletion of**
increased during early ageing (day 5 to 15)	reduced	***SAS3, IKI3, ELP3***[1]
strongly reduced	increased during early ageing (due to fast death of control cultures), BUT diminished or absent at later time points (day 15 to 25)	***GCN5, SGF73****, HDA2*
not affected	slightly reduced during early ageing (day 5 to15)	***AHC1***
slightly increased	not affected	*HDA1*
slightly reduced	not affected	***SPT10,*** *RXT2, SDS3, SAP30*
not affected	not affected	***HAT1, HPA2, HPA3, YNG1,*** *HDA3, HOS1, HOS2, HOS3, HOS4, RPD3, PHO23, SIR2, HST1, HST2, SET3, SIF2*

[1]The pro-survival effect of spermidine in the ***ELP3*** deleted strain was only reduced until day 10 of ageing. ***Bold italic*** characters indicate genes involved in the process of histone acetylation, while *italic* characters indicate genes involved in deacetylation.

Supplementary Table S2

Spermidine treatment induces the expression of autophagy-related genes during yeast ageing.

A subset of 50 genes related to macroautophagy, microautophagy or autophagy in general (all genes listed in GO:0016236, GO:0016237 and GO:0006914 obtained from SGD on June, 1st 2008) have been analysed (based on Affymetrix array data) for differential expression during chronological ageing of yeast upon treatment with 4 mM spermidine.

Day 3	**Differential expression by spermidine**		**Day 10**			
Gene	Factor	P-value	Gene	Factor	P-value	
ATG10	1.39	0.012	*ATG10*	0.94	0.453	**macroautophagy**
ATG12	1.34	0.348	*ATG12*	1.23	0.134	
ATG13	1.33	0.044	*ATG13*	1.25	0.327	
ATG14	2.01	0.032	*ATG14*	1.42	0.048	
ATG16	2.03	0.194	*ATG16*	1.74	0.240	
ATG18	1.30	0.194	*ATG18*	1.76	0.030	
ATG20	1.22	0.684	*ATG20*	1.43	0.091	
ATG21	1.56	0.290	*ATG21*	1.74	0.057	
ATG29	1.86	0.316	*ATG29*	2.13	0.064	
ATG5	1.20	0.012	*ATG5*	1.43	0.018	
ATG7	1.64	0.144	*ATG7*	2.22	0.039	
PTC6	1.02	0.402	*PTC6*	1.02	0.925	
VPS15	1.48	0.241	*VPS15*	1.93	0.137	
VPS30	1.73	0.422	*VPS30*	1.73	0.015	
VPS34	1.03	0.869	*VPS34*	1.11	0.004	
CMD1	1.11	0.316	*CMD1*	1.00	0.723	**microautophagy**
GTR2	0.89	0.200	*GTR2*	0.48	0.192	
MEH1	1.08	0.541	*MEH1*	1.39	0.169	
NVJ1	1.41	0.388	*NVJ1*	1.69	0.159	
PEP4	0.84	0.394	*PEP4*	1.11	0.416	
SLM4	1.10	0.224	*SLM4*	2.08	0.064	
VAC8	1.03	0.852	*VAC8*	1.12	0.648	
VTC1	1.28	0.468	*VTC1*	1.33	0.125	
VTC2	0.97	0.768	*VTC2*	0.73	0.235	
VTC3	1.09	0.783	*VTC3*	0.90	0.656	
VTC4	1.45	0.440	*VTC4*	0.84	0.460	

Table continues on next page…

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Gene	Factor	P-value	Gene	Factor	P-value
ATG1	1.42	0.071	*ATG1*	1.12	0.089
ATG11	1.23	0.053	*ATG11*	1.67	0.163
ATG15	1.27	0.121	*ATG15*	1.20	0.035
ATG17	1.17	0.542	*ATG17*	1.32	0.267
ATG19	1.83	0.133	*ATG19*	3.12	0.128
ATG2	1.14	0.258	*ATG2*	1.48	0.141
ATG22	1.21	0.234	*ATG22*	1.10	0.622
ATG23	1.39	0.094	*ATG23*	1.09	0.223
ATG26	1.22	0.403	*ATG26*	1.26	0.040
ATG27	1.15	0.183	*ATG27*	1.13	0.257
ATG3	1.08	0.370	*ATG3*	1.55	0.097
ATG4	1.24	0.571	*ATG4*	1.07	0.365
ATG8	1.30	0.185	*ATG8*	0.97	0.436
ATG9	1.40	0.168	*ATG9*	1.87	0.177
CCZ1	1.54	0.150	*CCZ1*	1.06	0.495
CIS1	1.57	0.199	*CIS1*	1.50	0.275
GSG1	0.99	0.588	*GSG1*	0.87	0.527
SEC23	0.94	0.499	*SEC23*	1.25	0.289
IRS4	1.10	0.278	*IRS4*	0.76	0.085
MON1	1.49	0.516	*MON1*	1.09	0.417
SEC16	1.21	0.738	*SEC16*	0.86	0.155
SEC24	1.18	0.136	*SEC24*	0.95	0.359
TAX4	1.14	0.449	*TAX4*	1.09	0.401
VAM3	1.01	0.720	*VAM3*	0.94	0.383

microautophagy

Supplementary Table S3

Spermidine extends the life span of nematodes in a Beclin 1 dependent fashion.

C. elegans mean and maximum life spans are shown from N2 wild type and *bec-1* RNAi knockdown animals aged with or without supplementation of normal food with 0.2 mM spermidine (Spd).

Strain	**Mean life span ± SEM (days)**	**Max life span (days)**	**Deaths/total**	***P* value**
N2 (wild type)	22.1 ± 0.8	36	111/130	
N2 + 0.2 mM Spd	25.5 ± 1.3	41	110/130	< 0.0001 Compared to N2
bec-1(RNAi)	15.8 ± 1.5	26	110/130	
bec-1(RNAi) + 0.2 mM Spd	17.1 ± 1.6	29	109/130	< 0.0001 Compared to N2 + 0.2 mM Spd

Supplementary Table S4

Primers used for gene disruption and cloning.

Sequences of control primers kan-B, Leu-B (used for verification of *IKI3* and *SPE1* deletion) and Ura-C (used for verification of *SAS3* deletion) were according to Gueldener et al.(*2*).

Primer for…	Sequence
loxP-*Ura3*-loxP cassette (*SAS3* deletion using pUG72)	5'-TTCCTTCTTCATTAATTAGTCTCCGTATAATTTGCAGATACAGCTGAAGCTTCGTACGC-3' 5'-ACATGTATATGCTTATATCCAATATATACCCATCGCCGCGCATAGGCCACTAGTGGATCTG-3'
loxP-*Leu2*-loxP cassette (*SPE1* deletion using pUG73)	5'-GTTCTACAACTTTTTCATAGTAATCAAAACCTTTGAATTTCAAACTTACTCAGCTGAAGCTTCGTACGC-3' 5'-CACCCCCTCCGTCTCTCTTGCGAAAGTCGTGGTTAAATATATCCTGCATAGGCCACTAGTGGATC-3'
Deletion control primers (forward)	5'-AGGCCAATTGAACAAGAAAT-3' (*SAS3*) 5'-GTACTAGTAGAGTTCAAGACA-3' (*IKI3*) 5'-AATTTTAATCTGCGCCGTGC-3' (*SPE1*)
Deletion control primers (reverse)	5'-GGATGTATGGGCTAAATG-3' (kan-B) 5'-TTGGCTAATCATGACCCC-3' (Ura-C) 5'-AGTTATCCTTGGATTTGG-3' (Leu-B)
pUG35-Ura/*NHP6A*	5'-ATCTGAATTCATGGTCACCCCAAGAGAAC-3' 5'-ATCTGAATTCAGCCAAAGTGGCGTTATATAAC-3'
pUG36-Ura/*ATG8*	5'-ATCTGAATTCATGAAGTCTACATTTAAGTCTGAATATCC-3' 5'-ATCTATCGATCTACCTGCCAAATGTATTTTCTCC -3'

Supplementary Table S5

Primers used for quantitative real time PCR (qPCR) and quantitative reverse transcription real time PCR (q-RT PCR).

Primer specific for…	Sequence
Gene (qRT-PCR)	
ATG7 (forward)	5'-TTGCAATACGATGTTCCTGACTTGA-3'
ATG7 (reverse)	5'-TGCTAGCTTACCTTGCACATTCCTT-3'
ATG11 (forward)	5'-CACTGCACCTACCCAGCAAGAA-3'
ATG11 (reverse)	5'-AGCAGCTGATCGGGAGGAATCT-3'
ATG15 (forward)	5'-AGGAAGAACACGCCATGTGGATA-3'
ATG15 (reverse)	5'-CAAAATCTTCCGGCTCTGTTTCA-3'
Promotor (qPCR)	
pATG7 (forward)	5'-CATGAGATTCCTTTGGACACCCTTT-3'
pATG7 (reverse)	5'-TGCATAACTTAAGACCCTTTCTGACGA-3'
pNMD3 (forward)	5'-TTGAAGCTCATCGCATTGGAAAAG-3'
pNMD3 (reverse)	5'-TGAATTCCATCCTTTTGTCAAATTCC-3'
pDBP8 (forward)	5'-TTTTGCCATAGAAGCCGTGAGAAG-3'
pDBP8 (reverse)	5'-GATGCTACGGAGATCTAAAGCGTC-3'
pMTG2 (forward)	5'-TTCACTTCCCCGAAATAGTACTGAAGGA-3'
pMTG2 (reverse)	5'-GCCGTAATTCTCTTTTGAAAACGCTAGA-3'
pCDC23 (forward)	5'-GCGTACATAAAAAGCACTTCGGGTA-3'
pCDC23 (reverse)	5'-TGTCGTCATTCATGGTTCTAAATGC-3'
pPRP8 (forward)	5'-ATTTAAAGTGACCATGGCAGAAGGA-3'
pPRP8 (reverse)	5'-GGCGGTAGTCCACTCATCTTTCTTT-3'
pSOL3 (forward)	5'-AACGCCGAATTTACAACTCGAAACT-3'
pSOL3 (reverse)	5'-CCATTGTCGGGGATAAAAAGGTAAG-3'
pTDA11 (forward)	5'-AGTGTAAGTATCACAAATCAAGCAAAACAA-3'
pTDA11 (reverse)	5'-ACCCTTCATCACCTTTGGAATCAC-3'

Supplementary Information

Supplementary Results and Discussion

Yeast Doubling Times and Depletion of Polyamines.

Polyamines are required for normal growth of yeast cells[1]. Therefore, *spe1* mutant cells were grown to stationary phase in a way that they retained sufficient polyamine concentrations when growing, but displayed maximal diminution of intracellular polyamines upon entry into stationary phase, where chronological ageing begins. As a result, Δ*spe1* cells arrested growth at 30-40% lower cell density compared to wild type cells. This was achieved by growing Δ*spe1* cells (that were kept on YPD plates before) for approximately 13-14 generations (including overnight culture) in normal SCD.

Decreased doubling times of Δ*spe1* cells (80.9 ± 0.3 min) compared to wild type (91.7 ± 0.2 min) (likely due to the requirement of polyamines during aerobic growth[1]) confirmed that the observed Δ*spe1* phenotypes were not due to an increased growth rate which could in principle cause accelerated ageing.

Cell death triggered by depletion of spermidine is necrotic, not apoptotic.

Depletion of polyamines (e.g. spermidine) by deletion of *SPE1*, coding for the enzyme that catalyses the rate limiting step of polyamine biosynthesis, precipitates premature ageing accompanied by markers of oxidative stress (see main text of the manuscript, Figure 2).

Enhanced generation of oxygen radicals upon *SPE2* deletion has also been observed in growing cells[2]. Since oxidative stress can cause apoptosis in yeast[3, 4] we determined apoptotic markers of wild type and polyamine depleted Δ*spe1* cells. Surprisingly, the frequency of apoptotic events (that is cells that exhibit DNA-fragmentation detectable by TUNEL or phosphatidylserine externalisation detectable with Annexin V) was not affected by *SPE1* deletion (Fig. 2e). Instead, we observed an increase in necrotic, PI positive cells in Δ*spe1* cultures compared to wild type controls (Fig. 2e). Accordingly, deletion of apoptotic effector molecules (including the yeast caspase, Yca1p[5]; apoptosis-inducing factor, Aif1p[6]; endonuclease G, Nuc1p[7]; or the serine protease HtrA2/OMI, Nma111p[8]) in the background of Δ*spe1* did not prevent the ageing-associated death that was accelerated by polyamine depletion (data not shown). Again, the necrotic phenotype of Δ*spe1* cells was completely abolished by application of 0.1 mM spermidine (Fig. 2d, e). We therefore conclude that depletion of intracellular polyamines can precipitate premature chronological ageing via non-apoptotic, presumably necrotic death of yeast cells.

Spermidine prolongs life span in various ageing models in a pH-independent fashion

Administration of spermidine to chronologically ageing yeast or alkalinisation of the medium with NaOH both prolonged yeast life span and increased the extracellular and cytosolic pH (data not shown). However, the life span-extending effect of alkalinisation is strictly dependent on intracellular polyamines, as it was only observed in wild type, not in Δ*spe1* cultures (Fig. S3b). It is important to note that in all other models used (including yeast replicative life span), the effect of spermidine was pH independent. In

fact, the pH was either titrated to control levels upon addition of spermidine to yeast media or to the drinking water of mice or simply did not change the pH of mammalian cell culture media, worm or fly media (which were all sufficiently buffered).

Recently, it has been reported that one of the major causes of yeast chronological ageing is the excessive production of acetic acid[9]. As none of the other ageing models used in this study were associated with such an (extreme) extracellular acidification, it seems to be a unique circumstance for the yeast system. Nevertheless, the ageing mechanisms identified by this system are transferable to higher eukaryotes including mammals[10-12].

Spermidine application counteracts age-induced necrotic cell death.

Upon spermidine application, chronological ageing yeast exhibited a drastic reduction in markers of necrosis and oxidative stress as compared to untreated controls (Fig. 3a-c). In contrast, externalisation of phosphatidylserine, an early apoptotic marker (Annexin V^+ PI^- cells), remained largely unaltered. Instead, loss of membrane integrity due to primary necrosis (PI positivity) and late apoptosis resulting in secondary necrosis (Annexin V^+ PI^+) was reduced from 50% to less than 10% in spermidine-treated cultures as late as after 18 days of ageing (Fig. 3c). We conclude that death associated with chronological ageing of yeast is mainly mediated by spermidine-inhibitable necrosis-like cell death.

Inhibition of cell death rather than regrowth of death-resistant mutants mediate life span extension upon spermidine application.

To verify that the improved survival of spermidine-treated cultures is indeed due to cell death inhibition rather than regrowth of mutants[13], we determined the budding index and

mutation frequency during the course of ageing (Fig. S3d). While application of spermidine did not affect the percentage of budded cells until day 15, it actually reduced the budding index by ~30% at later points in the experiment (Fig. S3d). This has also recently been connected to chronological life span extension[14]. The mutation frequency, which was monitored by assessing the appearance of canavanine resistant mutants[13], was slightly reduced by spermidine (Fig. S3e), indicating that spermidine might actually increase genomic stability. Thus, these observations raise the possibility that epigenetic modifications rather than genetic changes are responsible for the positive effects of spermidine on longevity.

Inhibition of histone acetyltransferases is responsible for spermidine mediated longevity.

As the role of the Sir2p deacetylase is well established in replicative ageing[15, 16], we tested its potential involvement in polyamine-promoted longevity during chronological ageing. Deletion of *SIR2* did not abrogate the ability of spermidine to extend the chronological life span (data not shown; Table S1). Thus, the observed hypoacetylation during chronological life span extension is not due to the sole induction of Sir2p activity. Similarly, deletion of each of the other known yeast sirtuins (*HST1, HST2, HST3, HST4*) did not affect longevity upon spermidine application (Table S1). This result is compatible with previous findings suggesting that chronological life span extension is not mediated by Sir2p activity[12] nor by any of the other yeast sirtuins[17].

Theoretically, spermidine treatment could lead to hypoacetylation either via activation of histone deacetylases or via inhibition of histone acetyltransferases (HATs). We therefore

investigated whether the disruption of each of the 28 genes involved in histone (de)acetylation would affect chronological ageing in the presence or absence of spermidine. The anti-ageing (pro-survival) effect of spermidine was partially abrogated only upon deletion of genes involved in the process of histone acetylation, e.g. Δ*iki3* and Δ*sas3* (Table S1). These findings suggest that inhibition of histone acetyltransferases rather than activation of histone deacetylases is responsible for histone H3 hypoacetylation and life span extension upon spermidine application.

Autophagy is required for maximum longevity of chronologically ageing yeast.

Autophagy has been suggested to play an important role in various scenarios of longevity[18-20]. Accordingly, autophagy-deficient Δ*atg7* cells exhibited higher death rates than wild type cells (due to necrosis as well as apoptosis) during yeast chronological ageing (Fig. S6e). Deletion of *ATG7* also compromised the life span extending effects of spermidine application in yeast (Fig. 6d, e), although survival could be protracted by spermidine early during ageing (Fig. 6d). This indicates that autophagy is crucial for maintaining full viability during polyamine-dependent life span extension. Similar results were observed when autophagy was blocked by deletion of *ATG6* or *ATG8* instead of *ATG7* (data not shown). Since Δ*atg7* cells could still be rescued early during ageing, autophagy-independent "backup" mechanisms may also be elicited by spermidine.

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