Autophagy compensates impaired energy metabolism in CLPXP-deficient Podospora anserina strains and extends healthspan

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Summary
The degradation of nonfunctional mitochondrial proteins is of fundamental relevance for maintenance of cellular homeostasis. The heteromeric CLPXP protein complex in the mitochondrial matrix is part of this process. In the fungal aging model Podospora anserina, ablation of CLPXP leads to an increase in healthy lifespan. Here, we report that this counterintuitive increase depends on a functional autophagy machinery. In PaClpXP mutants, autophagy is involved in energy conservation and the compensation of impairments in respiration. Strikingly, despite the impact on mitochondrial function, it is not mitophagy but general autophagy that is constitutively induced and required for longevity. In contrast, in another long-lived mutant ablated for the mitochondrial PaIAP protease, autophagy is neither induced nor required for lifespan extension. Our data provide novel mechanistic insights into the capacity of different forms of autophagy to compensate impairments of specific components of the complex mitochondrial quality control network and about the biological role of mitochondrial CLPXP in the control of cellular energy metabolism.

Key words: aging; autophagy; CLPXP protease; energy metabolism; mitochondria; Podospora anserina.

Introduction
Mitochondria are eukaryotic organelles involved in various essential functions including iron/sulfur cluster synthesis, lipid and amino acid metabolism, copper homeostasis, and energy transduction. Maintenance of mitochondrial homeostasis crucially depends on the control of protein quality by repair enzymes, chaperones, proteases, and autophagy. One mitochondrial matrix protease is ‘caseinolytic protease’ (CLP). This protein is active in prokaryotes as well as in mitochondria and plastids of most eukaryotes. All characterized CLP proteases have the same basic structure and function. They consist of two heptameric rings with individual units of the CLP serine protease and form a proteolytic core cylinder, which is able to degrade small peptides and some unfolded proteins (Gottesman et al., 1997). Larger proteins which are degraded by CLPP are recognized, unfolded, and delivered to the proteolytic chamber of CLPP by CLPX, a hexameric AAA+ chaperone that together with two CLPP rings constitutes the CLPXP multiprotein complex. Until today, the precise biological role of this complex is only initially elucidated.

In the nematode Caenorhabditis elegans, CLPP was demonstrated to signal mitochondrial perturbation to the nucleus (Haynes et al., 2007) and induce the so-called mitochondrial unfolded protein response (UPRmt). In mammals, the role of CLPP in the control of UPRmt is less clear and controversially discussed. For instance, it was found that ClpX is selectively upregulated during myogenesis in mice, demonstrating the initiation of a CLPXP-dependent UPRmt-like response (Al-Furoukh et al., 2015). In contrast, in a mouse model lacking a mitochondrial tRNA synthetase, it was shown that the UPRmt is independent of CLPP (Seierling et al., 2016).

In humans, a change in CLPXP abundance is associated with the development of different diseases. Perrault syndrome is one of these diseases, which is characterized by ovarian failure and sensorineural deafness (Jenkinson et al., 2013). ClpP null mice are a good model for this disease. They display prominent phenotypes including complete infertility and auditory deficits, growth retardation, induction of inflammatory factors, and resistance to ulcerative dermatitis (Gispert et al., 2013). Another example is Friedreich ataxia (FRDA), a neurodegenerative disease caused by failure to assemble Fe-S clusters due to defects in the mitochondrial iron chaperone frataxin (Puccio & Koenig, 2002). In a cardiac conditional FRDA mouse model with a tissue-targeted frataxin deficiency in striated muscles, the proteolytic component CLPP is upregulated at mid-stage of the disease (Guillon et al., 2009). Finally, a role of CLPP in a number of cancers is suggested by an altered abundance of ClpP transcripts in different human cancer tissues, which may result from altered metabolic states in cancer cells (Goard & Schimmer, 2014; Cole et al., 2015; Seo et al., 2016).

In a previous study with the fungal aging model Podospora anserina, deletion of PaClpP, a gene encoding the proteolytic subunit of CLPXP, led to a counterintuitive pronounced increase in the healthy lifespan (‘healthspan’). In this mutant, lifespan increase is not linked to impairments in viability parameters (e.g., reduced growth rate and fertility) as it is typical for many long-lived P. anserina mutants in which the composition of the mitochondrial respiratory chain is affected (Schechhuber & Osiewacz, 2008). The phenotype of the PaClpP deletion mutant can be reverted by the human CLPP homolog (Fischer et al., 2013). A recent ‘CLPP substrate trapping assay’ resulted in the identification of 19 potential PaCLPP substrates and 47 interaction partners (Fischer et al., 2015). Most of them are proteins involved in the control of energy metabolism including components associated with components of metabolic pathways in mitochondria (e.g., pyruvate dehydrogenase complex, tricarboxylic acid cycle, subunits of electron transport chain complex I). These data suggest a function of mitochondrial CLPXP in the maintenance of energy metabolism. The same general role was concluded for mammalian CLPP (Cole et al., 2015; Seo et al., 2016; Szczepanowska et al., 2016). In addition, a moderate respiratory deficiency in CLPP knockout mice linked to ineffective mitochondrial protein synthesis caused by decreased

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amounts of fully assembled mitochondrial ribosomes (S5S, mitoribo-
somes) was reported (Szczepanowska et al., 2016). Overall, the data
support a basic functional conservation of CLPXP from fungi to
mammals.

The observed healthspan extension in the PaClpP deletion strain raises
the question about the molecular basis of this unexpected phenotype.
Here, we demonstrate that the loss of functional PaCLPXP causes
imperfections in mitochondrial respiratory chain and induces compens-
satory responses. Moreover, we describe that lifespan extension in the
ΔPaClpX and ΔPaClpP single mutants, and the ΔPaClpX/ΔPaClpP double
mutant (hereafter termed ΔPaClpXP) depends on a functional autophagy
machinery.

Results

Deletion of PaClpXP alters the composition of mitochondrial
electron transport chain complexes and affects mitochondrial
oxygen consumption

The recent identification of PaCLPP substrates and interaction partners
suggested a role of PaCLPXP in the control of mitochondrial metabolic
pathways (Fischer et al., 2015). To verify this function experimentally, we
analyzed mitochondrial respiration in the wild type and the ΔPaClpXP
double mutant. In a first set of experiments, we determined the relative
mitochondrial oxygen consumption rate (OCR) in isolated mitochondria.
In this study, state 4 OCR of the wild type was set to 100%. Interestingly,
when comparing both strains using pyruvate/malate as substrates of
complex I (C I), mitochondrial oxygen consumption was reduced in the
mutant in both state 4 and state 3 (Fig. 1a). Such a decrease in state 3
was also observed when succinate was additionally added as a substrate
for complex II (C II; CI and CII respiration) and when complex I was
inhibited by rotenone (C I respiration) (Fig. 1a). These results indicate a
general and not complex-specific decline of mitochondrial respiration in
the ΔPaClpXP strain.

Next, we analyzed mitochondrial respiration at the organismic level
using mycelia of the wild type and ΔPaClpXP. Normally, wild-type strains
of P. anserina respire via a standard cytochrome-c oxidase (COX, complex IV)-dependent respiratory chain. However, when this pathway
is impaired for any reason, a compensating mechanism is induced
leading to the expression of a gene coding for an alternative oxidase
(AOX; Borghouts et al., 2001; Scheckhuber et al., 2011). To elucidate
the contribution of these two terminal oxidases in the ΔPaClpXP mutant,
we measured the ratio between complex IV (C IV)- and AOX-dependent
OCR in mycelia of both strains. The basal OCR per mg dry weight of the
wild type was set to 100%, and the relative inhibition of COX by cyanide
or AOX by salicylhydroxyacidic acid (SHAM) was calculated (Fig. 1b).
Interestingly, compared to the wild type, COX-dependent respiration is
reduced and respiration via AOX is increased by approximately 15% in
ΔPaClpXP (Fig. 1b). Basal OCR was not significantly changed compared
to wild type (Fig. 1c). We verified these conclusions by western blot
analysis which demonstrated that not only enzymatic activity but also the
abundance of the AOX is increased in ΔPaClpXP in comparison with
wild type (Fig. S2).

To test whether the observed change in OCR results from the
decreased abundance of individual respiratory complexes, blue native
polyacrylamide gels (BN-PAGE) with crude mitochondrial extracts of
ΔPaClpXP and the wild type (Fig. 1d) were analyzed. Concordantly,
there is a decrease in the amount of C I and C V (Fig. 1d,e), as well as a
slight mobility shift of C IV visualized by Coomassie staining. In addition,
we analyzed the ‘in-gel’ activity of C V. In ΔPaClpXP, C V activity appears
to be reduced (Fig. 1d,e). Unexpectedly, but in concordance with the
healthy phenotype of the mutant, ATP content was unaltered in the
ΔPaClpXP strain (Fig. 1f), indicating that the observed changes in the
mitochondrial respiratory chain are compensated. This conclusion is
supported by the demonstration that neither superoxide levels (NBT
staining), a ROS generated at the mitochondrial respiratory chain, nor
hydrogen peroxide levels (DAB staining) generated by superoxide
dismutation differ in ΔPaClpXP compared to wild type (Fig. 1g).

In ΔPaClpXP, mitophagy is not induced as an adaptive
response to impairments in protein quality control

In a parallel study, we demonstrated that mitophagy is upregulated
during aging in a PaSod3 deletion mutant of P. anserina and compen-
sates the loss of this mitochondrial quality control component (Knup-
ertz et al., 2017). We therefore tested whether induction of mitophagy
plays also a role in the different PaClpXP deletion mutants. As the
microscopy analysis of autophagosomes does only provide evidence
for the initiation of autophagy but can neither discriminate between
nonselective and selective autophagy, we performed a biochemical
analysis (Melling-Wesse et al., 2002; Kanki et al., 2009). In
this approach, the fate of a reporter protein fused to GFP is followed. Upon
degradation of a reporter protein via autophagy, the GFP part of the
fusion protein, which remains stable in the vacuole, can be detected as
‘free GFP’ by western blot analysis. We used strains expressing a
PaSod3::Gfp fusion gene encoding a mitochondrial protein as a marker
for mitophagy (Fig. S1A). Although mitochondrial function is compro-
mised in the analyzed PaClpXP mutants, and in marked contrast to what
we found in a PaSod3 deletion mutant (Knuppertz et al., 2017), there is
no difference in mitophagy levels in wild type and PaClpXP deletion
mutants, neither at young nor at advanced age (Fig. 2a,b). We verified
this result using a previously generated PaSOD33126L::Gfp mitochondrial
reporter strain (Fig. S1B; Knuppertz et al., 2017), in which PaSOD3 is
enzymatically inactive (Figs 2a,b and S3). Interestingly, in total protein
extracts, the PaSOD33126L::Gfp full-length protein is underrepresented
and only detectable when large amounts of protein are used in western
blot analysis. However, as demonstrated in previous studies, the full-
length fusion protein is clearly visible in mitochondrial extracts (Knup-
ertz et al., 2017) and the ‘free GFP’ observed in the different strains
results from the degradation of the fusion protein via autophagy/1
mitophagy (Knuppertz et al., 2014, 2017).

To exclude the possibility that mitophagy is defective in the different
PaClpXP deletion strains, we attempted to induce mitophagy in the
different PaClpXP deletion mutants. After testing a number of different
components and conditions (i.e., FCCP, nitrogen starvation, rotenone),
we identified the alkylating agent and carcinogen methyl methanesul-
fonate (MMS) as an autophagy- and mitophagy-inducing compound in
P. anserina wild type (Fig. S4A–E). MMS treatment was found to
increase ‘free GFP’ in the different ΔPaClpXP mutants which express
the mitophagy marker gene PaSod33126L::Gfp (Fig. 3C). These data
clearly demonstrate that the general capacity to induce mitophagy is not
affected in strains ablated for the different PaCLPXP components.
Interestingly, relative growth rate of ΔPaClpXP under MMS treatment
is significantly reduced when compared to MMS-treated wild type,
suggesting that the induction of mitophagy negatively affects vegetative
growth of the ΔPaClpXP strain (Fig. 2e).

Next, we tested the impact of lowering mitophagy beyond the
basic levels found in the ΔPaClpXP mutant. Therefore, we combined
the PaClpXP deletion with the deletion of PaCypD, a gene coding for
the mitochondrial peptideyl prolyl-cis, trans-isomerase cyclophilin D

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(CYPD) known to be involved in the regulation of mitochondrial permeability transition (mPT) regulation, programmed cell death (Brust et al., 2010), and mitophagy (Carreira et al., 2010; Kramer et al., 2016). In the latter mutant, mitophagy levels are lower than the basal levels normally observed in \textit{P. anserina} strains (Kramer et al., 2016). Compared to the \textit{PaClpXP} deletion strain, the \textit{\Delta PaClpXP}/\textit{\Delta PaCypD} mutant (Fig. S1C) is characterized by an increased lifespan (Fig. 2d), demonstrating that lifespan extension in \textit{\Delta PaClpXP} is thus not dependent on the induction of mitophagy. Moreover, as indicated by the increased lifespan, in this mutant, it appears to be

\textbf{Fig. 1} Mitochondrial function is partially impaired in \textit{\Delta PaClpXP} compared to wild type. (a) Relative oxygen consumption rate (OCR) of 7-day-old \textit{\Delta PaClpXP} and wild type mitochondria (for each strain, three mitochondrial preparations with three to six technical replicates were analyzed). State 4 OCR of the wild type was set to 100%. (b) The relative oxygen consumption of mycelia from 7-day-old wild type and \textit{\Delta PaClpXP} strains (three biological and four technical replicates) was analyzed after KCN (inhibition of C IV) or SHAM (inhibition of alternative oxidase (AOX)) addition. The basal oxygen consumption of the wild type was set to 100%, and the inhibition of the oxidases was calculated relatively. (c) Basal oxygen consumption rate (OCR) per mg dry weight from mycelia of 7-day-old wild type and \textit{\Delta PaClpXP} strains (three biological and four technical replicates). (d) BN-PAGE analysis of 100 \micro g mitochondrial protein extracts from 7-day-old wild type and \textit{\Delta PaClpXP} (three mitochondrial preparations). SC, supercomplexes; V2, complex V dimer; I1, complex I monomer; V1, complex V monomer; III2, complex III dimer; IV1, complex IV monomer. Changes in the composition are marked with arrows. Coomassie-stained gel (left) and ‘in-gel’ staining of C V (right) are demonstrated. (e) Quantification of relative complex I (CI, Coomassie-stained gel) and complex V (CV, CV-stained gel) protein levels of wild type and \textit{\Delta PaClpXP} mitochondrial extracts (\(n = 3\)) that were separated during a BN-PAGE analysis shown in (d). Protein abundance in the wild type was set to 1. Error bars correspond to the standard deviation. \(P\)-values were determined by Student’s \(t\)-test (\(P < 0.05\)). (f) ATP levels of 7-day-old wild type vs. \textit{\Delta PaClpXP} (three biological replicates with three technical replicates) were measured by a luminescence-based assay. Error bars correspond to the standard deviation, and \(P\)-values were determined with paired Student’s \(t\)-test (\(P > 0.05\)). (g) Determination of superoxide and hydrogen peroxide in 7-day-old wild type and \textit{\Delta PaClpXP} strains by NBT and DAB staining. (a–c) Error bars correspond to the standard deviation, and \(P\)-values were determined by two-tailed Mann–Whitney–Wilcoxon \(U\)-test. (a, b, e) *\(P < 0.05\), **\(P < 0.01\), ***\(P < 0.001\).
advantageous to protect mitochondria from degradation via mitophagy. However, this protection goes along with a slight reduction in growth rate (Fig. 2e). Supporting this hypothesis, a comparative western blot analysis of total protein extracts demonstrates that mitophagy is reduced in a \( \Delta PaClpXP/\Delta PaCypD/\Delta PaSod3::Gfp \) mutant compared to \( PaSod3::Gfp \) and \( \Delta PaClpXP/\Delta PaSod3::Gfp \) (Fig. 2f). This reduction is similar to that in the previously reported \( \Delta PaCypD/\Delta PaSod3::Gfp \) strain (Kramer et al., 2016). Interestingly, we found no obvious differences in the amount of superoxide but a slight decrease in hydrogen peroxide levels in a \( \Delta PaClpXP/\Delta PaCypD \) mutant compared to the wild type and the single deletion mutants that probably could be co-responsible for the healthier phenotype of this mutant (Fig. 2g).
Fig. 3  Autophagy is induced in a starvation-like response of *PaClpXP* mutant strains. LSFM of hyphae from 4 (a)- and 20 (b)-day-old wild type and Δ*PaClpX*, Δ*PaClpP*, and Δ*PaClpXP* strains expressing *Gfp::PaAtg8*. (c) Quantification of autophagosomes of 4- and 20-day-old wild type and Δ*PaClpX*, Δ*PaClpP*, and Δ*PaClpXP* strains expressing *Gfp::PaAtg8* (*n* = 10). *P*-values were determined between 4- and 20-day-old strains and between wild type and mutant of the same age. Error bars correspond to the standard error. *P*-values were determined by two-tailed Mann–Whitney–Wilcoxon U-test. (d) Monitoring autophagy by western blot analysis of 7- and 20-day-old *PaSod1::Gfp* compared to Δ*PaClpXP*/*PaSod1::Gfp*, Δ*PaClpP*/*PaSod1::Gfp*, and Δ*PaClpXP*/*PaSod1::Gfp* cultivated on CM medium. (e) Monitoring autophagy by western blot analysis of 7-day-old *PaSod1::Gfp* compared to Δ*PaClpX*/*PaSod1::Gfp*, Δ*PaClpP*/*PaSod1::Gfp*, and Δ*PaClpXP*/*PaSod1::Gfp* cultivated on CM or M2 medium normalized to the level of *PaSOD1*. Protein abundance in 7-day-old *PaSod1::Gfp* was set to 1. Error bars correspond to the standard deviation. *P*-values were determined by Student’s *t*-test. (g) Survival curves of the wild type (*n* = 25) and Δ*PaClpXP* (*n* = 25) cultivated on M2 vs. M2-. *P*-values were determined between wild type and Δ*PaClpXP* on M2 (P < 0.001), wild type and Δ*PaClpXP* on M2- (P < 0.001), wild type on M2 and M2- (P < 0.001), and Δ*PaClpXP* on M2 and M2- (P < 0.001) by two-tailed Mann–Whitney–Wilcoxon U-test. (h) ATP levels of 5-day-old wild type, Δ*PaClpXP*, and Δ*PaClpXP*Δ*PaAtg1* (three biological replicates with three technical replicates) were measured by a luminescence-based assay. Error bars correspond to the standard deviation, and *P*-values were determined with paired Student’s *t*-test. (c, f, h) * = *P* < 0.05, ** = *P* < 0.01, *** *P* < 0.001.
Nonselective general autophagy is increased and compensates for the defects in the energy metabolism of \textit{PaClpXP} deletion mutants

Next, we set out to elucidate the mechanistic basis of the observed lifespan extension in the different \textit{PaClpXP} deletion mutants. From previous work, we know that autophagy is active as a longevity-assurance mechanism that is induced during aging (Knuppertz et al., 2014). Moreover, we found that mild stress can lead to a beneficial hormetic response leading to an increased lifespan (Knuppertz et al., 2017). To test such a role of autophagy in the \textit{PaClpXP} mutants, we quantified the abundance of autophagosomes microscopically in wild type, \textit{\textit{PaClpX}}, \textit{\textit{PaClpP}}, and \textit{\textit{PaClpXP}} strains which express the \textit{\textit{Gfp::PaAtg8}} fusion gene encoding the GFP-labeled autophagosomal marker \textit{PaAtg8} (Fig. S1D). Significantly, fluorescence microscopy revealed a high number of autophagosomes in all three \textit{PaClpXP} deletion mutants already at juvenile age when autophagosomes are almost absent from wild type cells (Fig. 3a,c). At advanced age, this number strongly increases in the wild type but only slightly in the \textit{PaClpXP} deletion mutants (Fig. 3b,c).

To analyze whether the identified autophagosomes deliver their cargo to vacuoles where it is subsequently degraded, we analyzed the fate of the cytoplasmic \textit{PaSOD1::GFP} fusion reporter protein biochemically in wild type and the different \textit{PaClpXP} deletion strains (Fig. S1E). While ‘free GFP’ was found to be strongly increased during aging of the wild type grown in complete (CM) liquid medium, autophagy in the \textit{PaClpXP} mutants was slightly increased in 7-day-old cultures compared to the wild type of the same age. However, there was no increase observed in 20-day-old strains (Fig. 3d,f), verifying that that 20-day-old strains of this long-lived mutant are not in the same biological late state of aging as the wild type.

Autophagy is known to be important for balancing energy sources at critical times in development and in response to nutrient availability. Concordantly, when we changed cultivation conditions and grew strains on M2 solid medium, a minimal medium that we used for the microscopic analysis and lifespan experiments, autophagy was strongly induced in the wild type. Moreover, autophagy in the different \textit{PaClpXP} deletion mutants was significantly higher on M2 compared to the corresponding wild type control (Fig. 3e,f). These results are in accordance with data from the microscopic analysis (Fig. 3a–c).

Subsequently, we investigated the impact of starvation on lifespan of the \textit{\textit{\Delta PaClpXP}} mutant in more detail. We determined the lifespan of both the wild type and the \textit{\textit{\Delta PaClpXP}} strain on standard M2 minimal medium and on M2 depleted for nitrogen and glucose (M2-) (Fig. 3g). Interestingly, the mutant strain lives much longer on M2- than on M2 medium, suggesting that the applied harsh nutritional starvation is beneficial for the mutant most likely via a stronger induction of autophagy. Notably, lifespan of the wild type on M2- medium is increased, similar to the lifespan of \textit{\textit{\Delta PaClpXP}} on M2 medium (Fig. 3g).

After the demonstration of the induction of general autophagy in the \textit{PaClpXP} deletion strains and the effects of nutritional starvation on lifespan, we next investigated whether autophagy contributes to energy conservation and ATP content in the \textit{PaClpXP} double mutant. We measured ATP content of the \textit{\textit{\Delta PaClpXP/PaAtg1}} strain in which autophagy is blocked and compared it to the \textit{PaClpXP} deletion strain and the wild type. Strikingly, we found that the wild type-like ATP content of the \textit{\textit{\Delta PaClpXP}} strain depends on a functional autophagy machinery (Fig. 3h).

To test whether the \textit{\textit{\Delta PaClpXP/PaAtg1}} mutant is characterized by a more profound alteration in the amount of mitochondrial respiratory chain complexes than the \textit{\textit{\Delta PaClpXP}} mutant, thus explaining the consequent changes in ATP levels upon block of autophagy, we performed an additional BN-PAGE experiment. We used mitochondrial extracts of 7-day-old wild type, \textit{\textit{\Delta PaClpXP/PaAtg1}} and \textit{\textit{\Delta PaAtg1}} strains. Interestingly, compared to wild type, we found no obvious differences in the amount of mitochondrial respiratory chain complexes in the autophagy-deficient \textit{PaAtg1} deletion mutant (Fig. S5). For the \textit{\textit{\Delta PaClpXP/\Delta PaAtg1}} mutant strain, we found the same characteristic differences in the protein amount of mitochondrial respiratory chain complexes as in the \textit{PaClpXP} deletion mutant (Fig. 1d and S5). These results indicate that the detected impairments in mitochondrial respiration are caused via the loss of the \textit{PaCLPXP} protease and not upon a modulated autophagy rate in this mutant. This lack of \textit{PaCLPXP} activity in \textit{P. anserina} is effectively compensated by autophagy, acting as a prosurvival pathway via the conservation of the cellular energy status.

The healthy phenotype of \textit{PaClpXP} deletion strains depends on functional autophagy

Next, we investigated whether the healthy phenotype of the \textit{PaClpXP} deletion mutant depends on functional autophagy and therefore generated \textit{\textit{\Delta PaClpX/PaAtg1}}, \textit{\textit{\Delta PaClpX/PaAtg1}}, and \textit{\textit{\Delta PaClpXP/PaAtg1}} mutants, in which autophagy is completely blocked (Fig. S1F). Strikingly, in the double mutants, the longevity phenotype of \textit{\textit{\Delta PaClpX}}, \textit{\textit{\Delta PaClpP}}, and \textit{\textit{\Delta PaClpXP}} is reverted to wild type level (Fig. 4a,b,d,e,g,h). In addition, the growth rate is reduced in all mutants lacking ATG1 compared to the single deletion strains and the wild type (Fig. 4c,f,i). As lifespan and growth rate are decreased by the loss of autophagy, these data demonstrate that the longevity phenotype of the mutants, in which components of the \textit{PaCLPXP} complex are ablated, depends on a functional autophagy apparatus and the induction of autophagy.

Loss of PaIAP causes a lifespan prolonging phenotype that is unaffected by autophagy

After the demonstration of the induction of autophagy in mutants affected in one component of the mitochondrial proteolysis system, we next asked the question of whether or not such a response is general or whether it is specifically depending on the kind of perturbation. As an example of another mitochondrial protease, we chose \textit{PaIAP}, an i-AAA protease located in the inner mitochondrial membrane. Like in the different \textit{PaClpXP} deletion strains, lifespan of the \textit{PaIap} deletion strain is strongly increased (Weil et al., 2011). To test whether autophagy is linked to this phenotype, we measured autophagosome abundance in a \textit{PaIap/Gfp::PaAtg8} strain (Fig. S1D). Strikingly, compared to wild type, we did not observe significant differences in the amount of autophagosomes either in the young or in the advanced age (Fig. 5a, b). In addition, as demonstrated by the unaffected lifespan of a \textit{\textit{\Delta PaIap/\Delta PaAtg1}} double mutant (Fig. S1F), the increase in lifespan of \textit{\textit{\Delta PaIap}} is not dependent on a functional autophagy machinery (Fig. 5c,d). Relative growth rate is slightly reduced in the double mutant, identifying that the loss of \textit{PaAtg1} affects vital functions in \textit{\textit{\Delta PaIap}} (Fig. 5e). MMMS treatment of \textit{PaSod1::Gfp} compared to \textit{\textit{\Delta PaIap/PaSod1::Gfp}} (Fig. S1E) revealed that autophagy is inducible and not defective in \textit{\textit{\Delta PaIap}} (Fig. 5f). In addition, autophagy is unaltered during aging of the \textit{\textit{\Delta PaIap/PaSod1::Gfp}} strain compared to the wild type control (Fig. 5g, h). Overall, these initial cellular characteristics of \textit{\textit{\Delta PaIap}} compared to those of \textit{\textit{\Delta PaClpXP}} suggest that mitochondrial proteases have specific functions beside their role in degradation of damaged proteins.
Compromising their function does not induce one general but different, specific responses.

**Discussion**

Maintenance of cellular homeostasis is a key to keep biological systems functional and healthy. Impairments lead to aging and the development of disorders and severe diseases. A number of different surveillance pathways are active to balance cellular homeostasis over the lifetime of biological systems. These pathways may interact with each other allowing the compensation of impairments in one pathway or after overwhelming its capacity. The details about the underlying cross talks and their mechanistic basis are currently only initially elucidated.

Here, we reported novel data identifying autophagy, the vacuolar degradation of cytoplasmic components, as a mechanism involved in the compensation of impairments in mitochondrial protein quality control in a mutant of the fungal aging model *P. anserina* in which PaCLPP has been ablated and the lifespan is unexpectedly increased.

CLPP is the proteolytic component of a mitochondrial matrix protein complex consisting of CLPP and a chaperone, CLPX, responsible for delivering substrates to the peptidase for degradation. While the complex, or derivatives of it, is found in prokaryotes and mitochondria and plastids of most eukaryotes, it is remarkable that CLPP is missing in yeast. Certainly because of this lack, the biological role of CLPPX is only initially understood.

Importantly, in our study, we found that nonselective autophagy is upregulated in the Δ*PaClpP* and the Δ*PaClpX* single mutants and the *PaClpXP* double deletion mutant. Moreover, we demonstrated that lifespan extension of this mutant depends on a functional autophagy machinery. Certainly, more data are required to unravel the details about which components of this machinery lead to healthspan extension. However, our current view of a protective role for autophagy in the *PaClpXP* deletion strain is in concordance with previous results that uncovered general autophagy as a longevity-assurance mechanism in *P. anserina* (Knuppertz et al., 2014). In wild type aging of this aging model, general autophagy becomes upregulated in the later phases of life when the mitochondrial energy metabolism is severely impaired due to the accumulation of mitochondrial dysfunction (Osiewacz & Kimpel, 1999; Borghouts et al., 2001). Significantly, the deletion of genes coding for the two components of the PaCLPPX led to the constitutive induction of general autophagy. The identification of CLPPX substrates and interaction partners in *P. anserina* and in mammalian cells suggests that this protease is specifically involved in the control of the mitochondrial energy metabolism (Cole et al., 2015; Fischer et al., 2015; Szczepanowska et al., 2016). It thus appears that both during wild type aging and as a result of PaCLPPX ablation, the resulting perturbation of the energy metabolism is the trigger for autophagy induction. In this process, sensing of the cellular nutrient status by adenosine monophosphate-activated protein kinase (AMPK) and subsequent signaling to conserve and generate ATP by initiating different responses such as increasing glucose uptake, glycolysis, fatty acid oxidation, halting protein synthesis, and the induction in alternative oxidation (Borghouts et al., 2001; Jones et al., 2005; Kelekar, 2005; Shaw, 2009) may also play a role in the *PaClpXP* deletion strains but remain to be experimentally addressed in future experiments.

Concordantly, our current study revealed that the ablation of components of PaCLPPX indeed affects mitochondrial respiration. We found changes in mitochondrial respiratory chain complex I, IV, and V composition and an overall decreased OCR, while ATP content is unaltered compared to the wild type. Interestingly, we demonstrated that ATP content is significantly decreased in Δ*PaClpXP*Δ*PaAtg1* when autophagy is defective (summarized in Fig. 6). These findings agree with those in yeast, where general autophagy is induced in strains in which mitochondrial function is affected by respiratory inhibitors (Defieux et al., 2013). Moreover, our results are in concordance with those of previous studies on mammalian cell culture showing that the loss of autophagy reduces ATP levels (Hubbard et al., 2010; Tang & Rando, 2014). Along these lines, we conclude that autophagy in *PaClpXP* deletion mutants is induced as the result of decreased mitochondrial ATP generation and that the bioenergetic demands of the mutants are balanced by increased autophagy leading to a hormetic response and an increased lifespan. Such a response was recently also observed in *P. anserina* wild type, in which mitophagy was induced via the application of mild oxidative stress (Knuppertz et al., 2017).

In striking contrast to what we found for the different *PaClpXP* mutants, the increase in lifespan of a *Palap* deletion mutant, in which another mitochondrial protease is ablated (Weil et al., 2011), is independent of autophagy. The underlying compensation mechanism in this mutant is currently unclear and remains to be unraveled. In yet another *P. anserina* mutant, we found that the induction of mitophagy efficiently compensates the ablation of the mitochondrial superoxide dismutase, a component of the ROS scavenging system (Knuppertz et al., 2017). In this mutant, superoxide was identified to trigger the induction of mitophagy.

Overall, our data show that impairments of components of the mitochondrial quality control network do not generally lead to the induction of general autophagy as a basic cellular compensation mechanism but to specific responses, which depend on the nature of the perturbation. The underlying specific signaling mechanisms are currently

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Fig. 5 The healthy phenotype of ΔPaIap is not autophagy dependent. (a) LSFM of hyphae from 4- and 20-day-old wild type and ΔPaIap strains expressing Gfp::PaAtg8. (b) Quantification of autophagosomes of 4- and 20-day-old wild type and ΔPaIap strains expressing Gfp::PaAtg8 (n = 10). P-values were determined between 4- and 20-day-old strains and between wild type and mutant of the same age. Error bars correspond to the standard error. (c) Survival curves of the wild type (n = 27), ΔPaAtg1 (n = 27; P < 0.001), ΔPaIap (n = 46), and ΔPaIap/ΔPaAtg1 (n = 38; P < 0.01). (d) Relative mean lifespan of ΔPaAtg1 (n = 27), ΔPaIap (n = 46), and ΔPaIap/ΔPaAtg1 (n = 38) resulting from the comparison of the mean lifespan of each strain with the mean lifespan of the wild type (n = 27, set to 100%). (e) Relative mean growth rates of ΔPaAtg1 (n = 27), ΔPaIap (n = 46), and ΔPaIap/ΔPaAtg1 (n = 38) derived from the comparison of the mean growth rate of each strain with the mean growth rate of the wild type (n = 27, set to 100%). (f) Monitoring mitophagy during MMS treatment (0.09% for the last 5 h of cultivation) by western blot analysis of 7-day-old PaSod1::Gfp compared to ΔPaIap/PaSod1::Gfp. (g) Monitoring autophagy by western blot analysis of 7-day, 10-day, 20-day, and 24-day-old PaSod1::Gfp compared to ΔPaIap/PaSod1::Gfp. (h) Quantification of ‘free GFP’ protein levels of 7-day (n = 4), 10-day (n = 4), 20-day (n = 4), and 24-day (n = 4)-old PaSod1::Gfp vs. ΔPaIap/PaSod1::Gfp normalized to the level of PaSOD1. Protein abundance in 7-day-old PaSod1::Gfp was set to 1. Error bars correspond to the standard deviation. P-values were determined by Student’s t-test. (b, d, e) P-values were determined by two-tailed Mann-Whitney-Wilcoxon U-test (* = P < 0.05, ** = P < 0.01, *** = P < 0.001).
only at the beginning to be understood. They certainly hold the key to understand the basis of the different responses. Apart from this basic relevance, our work provides novel data about the role of CLPXP, which emerges to be of important relevance for aging and specific adverse effects such as Perrault syndrome or different forms of human cancer (Fischer et al., 2013; Gispert et al., 2013; Jenkinson et al., 2013; Cole et al., 2015; Deepa et al., 2016; Seo et al., 2016). Studies using the P. anserina model system will continue to contribute to the elucidation of the cellular functions of this component of the mitochondrial quality control network and of its interactions with other cellular surveillance pathways.

**Experimental procedures**

An expanded section describing experimental procedures is available in Data S1.

**P. anserina strains**

Podospora anserina wild type strain ‘s’ (Rizet, 1953), the PaSod1::Gfp, PaSod3::Gfp (Zintel et al., 2010) and ΔPaClpX, ΔPaClpP (Fischer et al., 2013, 2015), ΔPalap (Weil et al., 2011) and ΔPaCypD (Brust et al., 2010) as well as the ΔPaAtg1, Gfp::PaAtg8 (Knuppertz et al., 2014), PaSod3H26L::Gfp, Gfp (Knuppertz et al., 2017) strains, and newly generated mutants were used. These are as follows: ΔPaClpP/ΔPaClpX (simplified as ΔPaClpXP), ΔPaClpXPΔPaAtg1, ΔPaClpXPΔPaAtg1, ΔPaClpXPΔPaAtg1, and ΔPaClpXPΔPaSod1::Gfp, ΔPaClpXPΔPaSod1::Gfp, ΔPaClpXP/ΔPaSod1::Gfp, ΔPaClpXP/ΔPaSod3::Gfp, ΔPaClpXP/ΔPaSod3H26L::Gfp, ΔPaAtg8, ΔPaClpP/ΔPaAtg8 and ΔPaClpXP/ΔPaAtg8, ΔPalap/ΔPaSod1::Gfp, ΔPalap/Gfp::PaAtg8, ΔPalapΔPaAtg1, and ΔPaClpXP/ΔPaCypD. Double mutants were obtained after crossing the single-mutant strains (e.g., ΔPaClpXP with ΔPaClpP) and selection of strains from the progeny containing both mutations (e.g., ΔPaClpXP).

**Statistical analysis**

For statistical analyses of lifespan, growth rate, oxygen consumption measurements, and autophagosome determination, two-tailed Mann–Whitney–Wilcoxon U-test was used. For the statistical analysis of protein amounts during western blot analyses and ATP measurements, we used paired Student’s t-test. The respective samples were compared with the appropriate wild type sample. For all analyses, the minimum level of statistical significance was set at $P < 0.05$ (not significant different means $P > 0.05$; significant different (*) means $P < 0.05$; highly significant different (**) means $P < 0.01$; very highly significant different (***) means $P < 0.001$).

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Conflict of interest
None declared.

Author contributions
HDO and LK designed this study and wrote the manuscript. LK performed the experiments. HDO supervised the study.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article.

**Fig. S1** Southern blot analyses of genomic DNA for verification of different *P. anserina* mutant strains.

**Fig. S2** Determination of the mitochondrial AOX protein amount in ΔPaClpXP and the wild type.

**Fig. S3** Western blot analysis using the *PaSod3H26L::Gfp* mitophagy reporter strain.

**Fig. S4** Methyl methanesulfonate (MMS) as a tool to study mitophagy induction in *P. anserina*.

**Fig. S5** BN-PAGE analysis of mitochondrial extracts from ΔPaClpXP/ΔPaAtg1 and ΔPaAtg1 compared to wild type.

**Data S1** Supporting experimental procedures.