Bulk autophagy induction and life extension is achieved when iron is the only limited nutrient in *Saccharomyces cerevisiae*

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Abstract
We have investigated the effects that iron limitation provokes in *Saccharomyces cerevisiae* exponential cultures. We have demonstrated that one primary response is the induction of bulk autophagy mediated by TORC1. Coherently, Atg13 became dephosphorylated whereas Atg1 appeared phosphorylated. The signal of iron deprivation requires Tor2/Ypk1 activity and the inactivation of Tor1 leading to Atg13 dephosphorylation, thus triggering the autophagy process. Iron replenishment in its turn, reduces autophagy flux through the AMPK Snf1 and the subsequent activity of the iron responsive transcription factor, Aft1. This signalling converges in Atg13 phosphorylation mediated by Tor1. Iron limitation promotes accumulation of trehalose and the increase in stress resistance leading to a quiescent state in cells. All these effects contribute to the extension of the chronological life, in a manner totally dependent on autophagy activation.

Introduction
Iron is an essential metal for the majority of cellular types. It is required for a number of metabolic processes such as respiration, proteins, lipids or ribosome metabolism, DNA biosynthesis and repair and others [1, 2]. Since iron can be potentially toxic for cells, mechanisms tightly controlling its homeostasis are required. In budding yeast, Yap5, Aft1 and Aft2 are the classical iron regulators. Yap5 is a transcriptional factor responding to high levels of cytosolic iron [3] whereas Aft1 and its paralogue Aft2 mediate the responses to iron starvation [4, 5, 6]. Aft1 localization is determinant for its transcriptional function being localized in the nucleus when iron is scarce and translocate to the cytoplasm when iron is replenished [7]. Iron depletion causes health problems in humans such as cardiovascular diseases or anaemia. Some researchers have described the activation of autophagy in response to iron deprivation as a positive mechanism to recirculate iron [8, 9]. Sensing and transducing the signal of the iron status to different cellular targets is essential to achieve a correct cellular homeostasis, hence, Hog1, Snf1, Ras2/PKA and Pkc1 signalling pathways have been directly or indirectly related to iron homeostasis (reviewed in [10]. The stress-activated protein kinase (SAPK) Hog1 (p38 in humans) phosphorylates and regulate Aft1 activity [10]. The kinase Snf1 belongs to the AMPK family, it has been demonstrated its participation
in the regulation of the iron regulon during diauxia through Aft1 [11] and also a role for Snf1 in iron resistance has been reported [12]. Our group has demonstrated that Slt2 the MAP kinase of the PKC1-MAPK pathway, also regulates Aft1 under iron deprivation conditions (submitted). Mitochondria and vacuole are both organelles in which iron is accumulated; this localisation probably contributes to the cellular sensing of iron. TOR is a known regulator of cellular metabolism and proliferation in all the known eukaryotic models.

Nutrient starvation provokes changes in the metabolism and cell cycle and under certain circumstances causes a G1 arrest and entrance in a long-lived quiescent state [13, 14]. When cells are deprived of several biomolecules autophagy activation contributes to recycle nutrients (reviewed in: [15, 16]). Many autophagy mutants exhibit a reduction in their chronological life span [17]. These findings have led to several studies demonstrating that autophagy can play a positive role in longevity (for reviews: [18, 19, 20]).

TORC2 exerts a control in mitochondrial respiratory function, affecting calcineurin [21]. During amino acid starvation, TORC2 positively regulates autophagy by inhibiting the activity of the calcium-regulated phosphatase calcineurin and activating the general amino acid control (GAAC) [22]. In addition, TORC2, controls ATG8 expression upon repression of the heterodimer Zinc-finger transcription factors Msn2 and Msn4 [23] (Vlahakis et al., 2017b).

TORC1 is down regulated in conditions of iron limitation [24]. The inactivation of the complex retards aging in Saccharomyces cerevisiae [25]; interestingly this mechanism has been demonstrated to be evolutionary conserved in many eukaryotic models and human cells (for a review [26]). TOR plays a crucial role in regulating autophagy in all eukaryotic models [27]. TORC1 is a negative regulator of autophagy [28]. Mild inactivation of TORC1 stimulates macroautophagy and extends life span [17]. TORC1 activity is registered through its multiple downstream effectors [29]. Sfp1, one of the downstream effectors of TORC1, is a transcriptional factor that regulates both RP and RiBi genes [30, 31]. When TORC1 is active, Sfp1 has a nuclear localisation whereas in conditions of TORC1 inactivation, Sfp1 is translocated to the cytoplasm. Non starved cells sequester Msn2/Msn4 in the cytoplasm upon their hyperphosphorylation mediated by TORC1 and cAMP-PKA. Nutritional starvation causes inactivation of both pathways leading to Msn2/Msn4 dephosphorylation and nuclear translocation, leading to the induction of the expression of a wide number of genes by the heterodimer transcription....
factor [32, 33]. Rtg1 is a basic helix–loop–helix/Zip transcription factor that along with Rtg3 and Rtg2 integrates the pathway termed retrograde regulation [34, 35]. Rtg1 is localized to the cytoplasm when cells grow in the presence of all the required nutrients in logarithmic phase. Several stimuli such as TORC1 inactivation, nutrient deprivation or mitochondrial dysfunction provoke Rtg1 translocation to the nucleus (reviewed in [36]). When active, TORC1 hyperphosphorylates Atg13, thus inhibiting autophagy [28]. TORC1 inactivation leads to Atg13 dephosphorylation that triggers Atg1 kinase activity then leading to the formation of the complex Atg13/Atg1/Atg17/Atg29/Atg31 activating the autophagy process [37, 38].

In this study, we demonstrate that iron deprivation activates macroautophagy in growth conditions in which iron is the only limiting nutrient. Tor2-Ypk1 is required to detect and transmit the signal of iron deprivation allowing Tor1 to dephosphorylate Atg13 and induce the autophagy mechanism. Snf1 and Aft1 participate in detecting the signal of iron repletion leading to Tor1 induction and the consequent repression of autophagy. Iron limitation causes an early entrance in quiescence and extension of chronological life, conditioned to the simultaneous activation of the autophagy machinery. The results presented here suggest a coordinate linkage between nutritional signalling pathways, iron homeostasis and autophagy that favours life extension.

**Materials and methods**

**Yeast strains and plasmids**

*Saccharomyces cerevisiae* strains are listed in Table 1. All the strains named GSL are derivatives of the CML128 background. New null mutants described in this study were obtained by a one-step disruption method that uses the NatMx4 or KanMx4 cassettes [51]. Strains GSL199, 226, 297, 313, 325, 352, 374 and 382 were constructed upon integration of plasmid pGFP-Atg8 (original name: pHab142), previously digested with Stu1, in the *URA2* locus. Strains, GSL395, 398, 399 and 401 were constructed upon integration of plasmid pAtg1-HA previously digested with BstEII. The plasmid pAtg1-HA was obtained upon Atg1 cloning into the Pme1 and PstI sites of the integrative vector pMM351 [48].

Plasmid descriptions are listed in Table 2. Each particular ORF was amplified by PCR from genomic DNA to be directionally cloned in the specific plasmid.
**Media, growth conditions and reagents**

Yeasts were grown at 30ºC in SD medium (2% glucose, 0.67% yeast nitrogen base that lacked the corresponding amino acids for plasmid maintenance) plus amino acids [52]. Iron depletion conditions (-Fe) consisted on SD medium whose nitrogen base component was free of iron plus the addition of 80 µM of 4,7-diphenyl-1,10-phenanthrolinedisulfonic acid (BPS) (Sigma,146617) [42]. Iron was added as ammonium iron (III) sulphate hexacahydrate [NH₄Fe(SO₄)₂•6H₂O] (+Fe; F1543; Sigma) at a final concentration of 10mM.

We present a list of reagents detailing final concentrations in culture media and from which company they were purchased: Cycloheximide 150mg/ml (SIGMA, C4859); Rapid alkaline phosphatase (Roche, 105677520001). N-acetylcisteine: NAC 5 mM (Sigma, A9165); FM464 30 µg/µL (Invitrogen, T-3166); Rapamycin 200 ng/ml (Sigma, R0395); CaCl₂·2H₂O 100 mM (SERVA 15587); H₂O₂ 0.5 mM (Sigma, H1009); Sorbitol 0.8 M (Sigma, S6021); Dihydroethidium DHE 50 µM (Sigma, D7008); DAPI 2 mg/mL (Sigma, D9541); Erythromycin 0.5 mM (Sigma, E6376); Glycerol 3% (Fisher scientific, 800689). Cell cultures were exponentially grown at 600 nm [O.D₆₀₀] of 0.6.

**Calcineurin activity**

To determine calcineurin activity we used a calcineurin-dependent response element (CDRE) lacZ reporter described in [53] and β-galactosidase assay was determined according to the protocol previously publish by our group in [42].

**Vacuole and dihydroethidium staining**

For vacuole visualization, cells were stained with FM4-64 (N-(3-triethylammoniumpropyl)-4-(p-diethylaminophenylhexatrienyl) pyridinium dibromide, and to determine cellular oxidation we used dihydroethidium. Both protocols were previously described by our group in [41].

**Glucose and trehalose determinations**

We followed the directions detailed in [54].

**The index of respiratory competence (IRC) and mitochondria mutation frequency assay**
The IRC value was calculated as the ratio between the number of colony-forming units (CFUs) observed on plates containing a non-fermentable medium YPEG (glycerol) vs a fermentable medium YPD (glucose). Mitochondria mutation frequency assay was determined as the ratio between the CFUs counted on YPEG plates plus erythromycin vs the number of CFUs counted in YPD plates. Both protocols were carried out according to [55].

**Cell survival and chronological life span**

To assay cell viability cells were grown to mid log phase O.D\textsubscript{600}: 0.6 in SD medium supplemented with the required amino acids. Viability was registered through serial dilutions and plated by triplicate onto YPD plates.

We measured the chronological life span (CLS) in the different strains based on the survival of populations of non-dividing yeast cells according to [42]. The viability was scored by counting the number of cells able to form colonies, CFU (colony-forming units). Cultures were started at an O.D\textsubscript{600}: 0.6. The same number of cells collected from each culture were plated in triplicated into YPD plates and allowed to grow at 30ºC for 3-4 days. CLS curves were plotted with the corresponding averages and standard deviations from three independent experiments.

**Protein extraction and immunoblot analyses**

Total yeast protein extracts were prepared as previously described in [42]. The antibodies for western blotting were as follows: anti-HA 3F10 (no. 12158167001; Roche Applied Science), was used at a dilution of 1:2,000 in 0.25% non-fat milk and the corresponding secondary was goat anti-rat IgG horseradish peroxidase conjugate (no. AP136P, Millipore). Anti-GFP (no. 632381; Living Colours) was used at a dilution of 1:2,000 and anti-Phospho-glycerate kinase (459250, Invitrogen) used at a dilution 1:1,200, both with the secondary antibody anti-Mouse (LNA931v/AG, GE Healthcare). Anti-Phospho-eIF2α (Ser51) (3597S, Cell Signalling) at a dilution 1:1,000 and anti-Phospho-AMPKα (Thr172) (167253S, Cell Signalling) at a dilution of 1:1,000 both with the secondary antibody anti-Rabbit (LNA934v/AG, GE Healthcare). They were used as indicated by the manufacturers.

The protein-antibody complexes were visualized by enhanced chemiluminescence, using the Supersignal substrate (Pierce) in a Chemidoc (Roche Applied Science).
Autophagy activity
For monitoring autophagy, we used the protocol described by [56] and modified by [43].

Results
Iron deprivation activates autophagy flux in conditions in which the only limiting nutrient is iron.

Iron is an essential element for eukaryotic cells. In a previous study we have demonstrated that iron deprivation provoked a descent in TORC1 activity [24], therefore we wondered whether iron availability also determined the onset of autophagy, in conditions in which iron is the only nutritional restriction imposed during exponential phase.

We decided to analyse autophagy progression through the immunological detection of free GFP from GFP-Atg8 genomic fusion [57]. GFP accumulation was only clearly detected in wt cells depleted for iron in exponential conditions; however, the free GFP band was undetectable in atg7 and atg1 mutants (Fig 1A). As expected, GFP moiety was undetectable in cells growing exponentially in control SD medium not depleted for iron in all the strains tested (Fig 1A). The increase in autophagy flux [58] detected in iron depletion conditions (Fig 1B) was indicative of induction of bulk autophagy. Moreover, total Atg8 also experienced an increase in wt cells growing in iron depletion conditions (Fig 1C). These results were confirmed upon observation in the fluorescence microscope of GFP accumulation in vacuoles (the dye FM4-64 accumulates in the vacuolar membranes) in wt cells growing exponentially in iron starved medium (Fig 1D), as opposed to atg7 and atg1 mutants in which GFP-Atg8 showed a disperse localisation through the cytoplasm and occasionally a punctated localisation to the PAS (Fig 1D and E). In order to discard that this would be a specific effect of the background we used different backgrounds and observed that in all of them iron deprivation induced autophagy flux in exponentially growing cells (not shown). We also observed the appearance of PAS (preautophagosome sites) detected upon GFPAtg8 (Fig 1D white arrows and E) and Atg13GFP (Fig 1F) microscopic observation, indicative of activation of autophagy flux. We ruled out the contribution of specialized autophagy in the vacuolar accumulation of GFP derived from GFP-Atg8 by analysing atg11 and atg32 mutants (Supplementary Material)
We used the enzymatic pho8Δ60 assay [59, 56] to quantify autophagy and confirmed that iron deprivation provoked an increment of autophagy in cultures not limited for nutrients other than iron, as compared to SD control cultures (Fig 1G).

**Tor1 and Tor2 regulate autophagy in response to iron availability during exponential phase.**

To elucidate whether the signal of iron deprivation flows from TORC1 inhibition to the different read-outs and to the machinery of autophagy we have analysed several TORC1 substrates in wt and atg7 mutant deficient in autophagy (since we observed the same results in atg7 and in atg1 mutants we do not show the results corresponding to the later): Msn2/4 [47, 60, 32, 33], Rtg1 [34, 35] Sfp1 [30, 31] GCN2/eIF2α [61, 62]. Under optimal growth conditions Rtg1 and Msn2/Msn4 are localized to the cytoplasm, whereas Sfp1 is located in the nucleus. Iron starvation provoked Rtg1 translocation to the nucleus and Sfp1 localisation to the cytoplasm (Fig 2A), however Msn2 was not affected by the scarcity of this metal in neither wt nor atg7 strains and remained localized in the cytoplasm (Fig 2A). Rtg1 localisation upon iron deprivation was not a consequence of mitochondrial dysfunction leading to oxidative stress since treatment with the antioxidant N-Acetyl cysteine was unable to induce the translocation of the transcription factor from the cytoplasm to the nucleus (Fig 2B). TORC1 and a variety of stresses cause an increased abundance of uncharged tRNAs that activate the kinase Gcn2, which in turns phosphorylates eIF2α to globally attenuate the protein synthesis. Our data indicate that TORC1 inactivation caused by iron depletion did not provoke eIF2α phosphorylation when no other nutrient was limiting (Fig 2C).

Atg13 phosphorylation by TORC1 inhibits autophagy [63]. TORC1 inactivation leads to Atg13 dephosphorylation which provokes detachment and activation of Atg1 by phosphorylation with the subsequent activation of autophagy. We observed Atg13 dephosphorylated whereas Atg1 became phosphorylated upon iron depletion in wt cells and also in the autophagy mutants atg1 and atg7 (Fig 2D), suggesting that in conditions of iron depletion with no other nutrient limitation, TORC1 is correctly inactivated and this inactivation is an event previous to the transmission of the signal to Atg13 and Atg1 proteins to activate the autophagy machinery. Consequently, in the absence of either Atg1 or Atg7, even when TORC1 is inactivated and Atg13 dephosphorylated the autophagy does not take place.
With the aim of completely inactivate TORC1 complex we treated control cultures growing exponentially with rapamycin and provoked both TORC1 inactivation and bulk autophagy and autophagy flux induction. However, in iron depleted cultures rapamycin did not cause any significant additional change taking into consideration that the starvation for this nutrient had previously caused the inactivation of TORC1 (Fig 2E). The autophagy analysis of *pho8Δ60* delivery to the vacuole confirmed our conclusions since rapamycin provoked an expected induction of autophagy in control conditions, but was unable to alter the already induced autophagy in iron starved conditions (Fig 2F). Our results support the hypothesis that iron deprivation leads to TORC1 inactivation during logarithmic phase, however this signal flows only to specific read-outs.

We decided to test other possible signalling pathways related to nutritional responses that could be related to detection and transduction of the iron starvation signalling, these are, TOR2/Ypk1, Ras2, Gcn2 and Snf1. TOR2 signals to the mitochondrial function to positively regulate autophagy [50]. Ypk1 is the most relevant target of TORC2 for this mechanism, therefore we deleted *YPK1* as a surrogate for TORC2 disruption, as wisely indicated in [50]. We could observe that the absence of Ypk1 abrogated the autophagy flux induced by iron starvation in exponentially growing cells (Fig 3A and B). In addition, from the observation of TORC1 substrates upon iron deprivation, we concluded that in *ypk1* strain Tor1 activity was reduced only for some substrates, Rtg1 and Sfp1 (Fig 3C) but contrary to that observed in wild type cells, neither Atg1 became phosphorylated nor Atg13 became dephosphorylated (Fig 3D). We ruled out the possibility that iron deprivation would be provoking mitochondrial damage since in both a *rho0* strain and upon antymicine treatment (not shown) activation of the autophagy flux was clearly detected (Fig 3E and F). Consequently, we do not believe that the absence of autophagy activity detected in *ypk1* mutant upon iron deprivation is due to mitochondrial damage. Consequently, our results are consistent with the absence of autophagy flux observed in *ypk1* mutant (Fig 3A) and indicate that in the absence of TOR2/Ypk1 the iron starvation signal cannot be transmitted to Atg13 and Atg1 to activate the autophagy machinery through Tor1. We added rapamycin to both wt and *ypk1* cultures to fully inactivate TORC1 complex in both wt and *ypk1* strains. Upon TORC1 inactivation with rapamycin, we observed autophagy activation in *ypk1* strain, both in conditions of iron deprivation or not. In accordance with these observations Atg13 became dephosphorylated and Atg1 phosphorylated in all the samples treated with rapamycin (Fig 4A). These results suggest that in conditions of iron deprivation,
TORC1 is active for specific substrates in the absence of Ypk1, nevertheless, TORC1 is inhibible upon treatment with the macrolide rapamycin which functions independently of iron signalling. Concerning TORC1 read-outs, we observed that Rtg1 was localized in the cytoplasm and Sfp1 was localized to the nucleus in 100% of the cells of both strains, indicating that TORC1 complex was completely inactivated (Fig 4B). Taking altogether our results we can conclude that Ypk1 is required to transmit the iron starvation signalling to the autophagy machinery through Tor1.

Ypk1 function has been associated to Gcn2 activation with the subsequent induction of autophagy in conditions of amino acids starvation [50]. We could determine that autophagy regulation upon iron deprivation when amino acids are not limiting does not involve Gcn2 activity since no eIF2α phosphorylation was detected (Fig 4C). Ypk1 signalling regulates autophagy by repressing the activity of calcineurin and thus inducing Gcn2 activity [50]. Calcineurin activity was measured using a CDRE-driven lacZ reporter, as described previously [53, 50]. Calcineurin activity was not reduced in iron starvation conditions, moreover, it was even higher than the activity quantified in SD control conditions, although much lower than that determined in ypk1 mutant cells (Fig 4D). Moreover, gcn2 mutant did not present any defect in autophagy activation in iron limiting conditions (Fig 4E). Taking into consideration that Msn2 is not translocate to the nucleus upon iron starvation (Fig 2A), we discard the possibility that neither Msn2/Msn4 [23] nor calcineurin/Gcn2 could be the Tor2 targets that govern autophagy induction when iron is limited in the cultures. Our results suggest that Tor2 activity is required in order to transmit the signal to the autophagy machinery through Tor1 when iron is limiting, suggesting that Tor1 inactivation as a consequence of iron depletion does not activate bulk autophagy without Tor2 activity. The absence of Ras2 kinase also presented results related to GFP accumulation from GFPAtg8 and Atg13 and Atg1 phosphorylation similar to those observed in wt cells (Fig 4E). A certain level of autophagy was already detected in the mutant tor1 growing in control SD medium that was significantly increased when cultures were grown in iron starvation conditions (Fig 4E). In addition, the absence of Snf1 caused a significant increase in Atg8 expression upon iron deprivation (Fig 4E) suggesting that the AMPK is playing a negative role with respect to the synthesis of Atg8.

Aft1 is the transcriptional factor involved in regulating iron homeostasis [5]. We observed that aft1 deletion did not substantially differ from those results obtained with the wt strain with respect to the autophagy activation in response to iron depletion, since
autophagy flux, GFP accumulation in the vacuoles, Atg13 and Atg1 phosphorylation patterns were similar to that detected in wt cells upon iron starvation (Fig 4F). However, upon iron depletion the levels of total protein Atg8 suffered a significant increase in the absence of Aft1, suggesting that under this nutritional circumstances Aft1 negatively affects Atg8 synthesis (Fig 4F).

Taken altogether these results we conclude that when iron is the only scarce nutrient in the culture medium Tor2 activity is required in order to induce bulk autophagy through Tor1 inactivation.

**Snf1 through Aft1 are both involved in signal detection of iron availability to down regulate autophagy through Tor1**

Iron replenishment to exponential growing cultures previously grown without iron, caused a remarkable and gradual reduction in the autophagy and autophagy flux, evidenced upon identification of Pho8 activity (Fig 5A), GFP accumulation from GFP-Atg8 (Fig 5B), GFP-Atg8 localisation (Fig 5C), and both Atg13HA and Atg1HA phosphorylation (Fig 5D). Moreover, iron addition to wt cultures caused the activation of TORC1 through the induction of Atg13 phosphorylation (Fig 5D) and the delocalisation of Sfp1 to the nucleus (Fig 5E). In addition, when we tested mutants in several signalling pathways related to nutritional sensing tor1, snf1, ypk1, ras2, gcn2 and (not shown), only tor1 and snf1 showed a clear impairment in signalling the autophagy down regulation upon iron refeeding given that we did not observe the descent in the autophagy flux, GFP-Atg8 translocation to the cytoplasm nor Atg13 phosphorylation as it did occurred in wt cultures (Fig 5B to E).

Given that Aft1 is involved in iron homeostasis and that is responsive to iron availability, we decided to investigate the potential contribution of the transcription factor in the detection and transmission of the iron availability and the transmission to the autophagy machinery. The absence of Aft1 prevented the down regulation of autophagy when iron was added to the culture medium (Fig 5B to E). These results suggest that Snf1, Aft1 and Tor1 are involved in the down regulation of autophagy upon iron refeeding.

We rule out the possibility that Snf1 or Aft1 act as Tor1 functional regulators upon iron refeeding since Sfp1 translocated to the nucleus in both snf1 and aft1 mutant as occurred in wt cells (Fig 5E), suggesting that the signal of iron repletion flows from Snf1 to Aft1 and Tor1 only to specific substrates such is the case of Atg13.
In order to ascertain whether Snf1 kinase activity is responsive to iron signalling, we chose wt and aft1 posdiauxic cultures because upon 2 days of growth in SD minimum medium, glucose levels are undetectable (data not shown). At the same time, Snf1 reached a maximum kinase activity (Fig 5F). Addition of iron to the culture medium provoked a fast inhibition of autophagy flux in wt cells growing in the presence or absence of Fe (Fig 5G). However, neither snf1 nor aft1 mutants experienced any reduction in autophagy flux neither in SD nor in SD-Fe media (Fig 5G), on despite that the Snf1 kinase activity was high and equivalent in both aft1 or wt cultures growing in SD (Fig 5F and G). As expected, iron repletion did not repress Snf1 activity neither in wt nor in aft1 cultures (Fig 5F). In conclusion, since in a situation in which Snf1 is active, iron addition is not capable to inactivate autophagy in the absence of Aft1, we speculate that Snf1 might signal to Aft1 to inhibit autophagy upon Atg13 phosphorylation through Tor1.

Taking together our results we conclude that iron excess actively represses autophagy through Snf1, Aft1 and Tor1-Atg13 signalling. Iron availability is a determinant factor to regulate autophagy and autophagy flux in optimal nutrient conditions when cells are not exposed to any other nutritional stress.

Iron scarcity promotes trehalose accumulation and resistance to several stresses resembling a quiescent state that is independent of bulk autophagy.

Iron deprivation caused an early entrance in diauxic shift and stationary phase (Fig 6A) already reported by [64]. Our data are in agreement with these observations but, in addition, we also observed a cell cycle blockade in G0, as evidenced by the accumulation of unbudded rounded and big cells, suggestive of quiescence (Fig 6B). To check this we determined in cells depleted or not for iron, both the accumulation of trehalose (Fig 6C) and the resistance to different stresses in stationary cells (Fig 6D) since quiescent cells are more resistant to different environmental stresses [65]. Iron deprivation induced trehalose accumulation and certain increase in the resistance to oxidative stress, high temperature and osmolarity during stationary phase (Fig 6C and D). The mutants atg7 and atg17 also accumulated trehalose as a response to iron deprivation during exponential phase, (Fig 6C). Calculation of the index of respiratory competence (IRC) indicated that iron deprivation did not reduce the respiratory capacity of the cells during exponential or stationary phase (Fig 6E). In addition,
mitochondria mutation frequency was null in cells growing in iron depleted media during 15 days of observation. However in control cultures growing in SD minimum medium, non-depleted for iron upon 6 days of growth some colonies erythromycin resistant were detected, and the number was sequentially increasing until the end of the experiment, indicating that mitochondrial mutations accumulate during aging when iron is not limited in the growth medium (Fig 6F). Taking altogether these results, our hypothesis is that iron depletion causes a premature entrance into a quiescent like state that has a positive effect in the mitochondrial function.

Iron depletion contributes to extend life conditioned to autophagy flux activation whereas iron overload shortens CLS

In view of the clear involvement that autophagy flux and activation of quiescence have in the response to iron depletion, we decided to analyse the possible biological role that iron starvation and the concomitant activation of autophagy flux could be playing in the chronological life span. First, we carried out experiments of chronological life span (CLS) up to 15 days in 3 different wt backgrounds (Fig 7A). Iron depletion caused a significant life extension in the three backgrounds tested as compared to their corresponding control cultures not starved for iron (Fig 7A). This result demonstrates that iron starvation causes a genuine effect expanding chronological life. We observed that cells starved for iron presented a clear tendency to become rounded unbudded and presented a healthier aspect during all the CLS experiment than wt cells growing in SD medium, consequence of the entrance in quiescent state, as described in the previous section (Fig 6B). Iron deprivation did not preclude mitochondrial function since cell survival was equivalent in glucose and in glycerol during the CLS experiment (Fig 7B). Mutants in different stages of autophagy: atg7, atg13 and atg17 already experienced a significant reduction in their CLS as previously reported for atg7 in [17]. Interestingly, iron deprivation cultures experienced an additional reduction in their CLSs (Fig 7C). In conclusion, our results demonstrate that in S. cerevisiae, iron homeostasis is particularly relevant in aging associated and conditioned to the regulation of autophagy and autophagy flux.

Discussion
Results shown here indicate that iron depletion is itself a signal to activate autophagy when it is the only limiting nutrient in the culture medium. The observation that this response also occurs in rho0 strain suggest that the reservoir of iron in the mitochondria is not the source of the main signal to activate the autophagy machinery. Some authors have reported that activation of the autophagy when iron is scarce could be a response to overcome the deleterious effects in the cells in human cells, however the molecular mechanism is poorly understood [8, 66]. According to the elegant studies of [21, 22, 23, 57] our results could indicate that an intact TOR2 function might be required to maintain a correct mitochondrial function in order to activate autophagy flux when iron is the only limiting nutrient. Or alternatively that Tor2 would induce autophagy through Msn2/Msn4 [50, 21, 23]. However, the observation that a rho0 mutant induced autophagy upon iron starvation suggested that mitochondrial function turned out to be dispensable for this response in the conditions established in this study. In addition, the observation that Msn2 did not translocate to the nucleus in response to iron deprivation also ruled out the possibility that Ypk1 would be signalling to the activation of this transcription factor. Iron scarcity reveals the requirement for a TOR2 activity as a positive regulator of autophagy through YPK1 when any other nutrient is limiting. Our results suggest that iron starvation induces TOR1 inactivation to dephosphorylate Atg13 and consequently to induce the autophagy machinery only when TOR2/YPK1 is active, meaning that these proteins are relevant to sense iron scarcity and to transmit the signal to the autophagy machinery for the adaptive response to iron deprivation specifically (Fig 8). Iron deficient cells exhibit alterations in the properties and functions of membranes [67]. A recent review by [68] updates the evidences that connect TOR2 to membrane homeostasis. Therefore we could speculate that TOR2 might play a role as membrane vigilant when iron is scarce and that one of the mechanisms to overcome this deficiency would be the activation of autophagy, although this hypothesis requires further research. Intriguingly, the transmission of the signal of iron deficiently from Ypk1 to TORC1 only occurs to certain substrates such is the case of Atg13 and Atg1. At the same time, the signal of iron deprivation influences TORC1 regulation on Sfp1 and Rtg1 substrates independently of Tor2/Ypk1 (Fig 8). Further investigation in this mechanism will be required. Nevertheless, other nutritional limitations such as nitrogen [69] or even metals such as zinc [70] have been reported to also provoke the inactivation of TORC1. Nitrogen starvation, in particular signals only to specific TORC1 substrates, such as
Sfp1 coincidentally, leading to autophagy activation. An elevated autophagy flux ensures iron availability for essential functions such as mitochondrial functioning and synthesis of iron-sulphur clusters, DNA repair, amino acid synthesis and others. In line with this, our results suggest that in conditions of serious iron limitation the requirements for a high autophagy flux to efficiently recycle the metal will preserve life extension.

Respiratory function is essential for standard CLS [71]. Iron depletion not only did not curtail CLS but notably extended it, respiration capacity was not negatively affected by low iron levels, meaning that mitochondrial function was potentially optimal. In accordance with other authors, our results support the hypothesis that iron limitation during aging requires an optimal mitochondrial function that is relevant for the modulation of autophagy flux [58] and life extension [72].

Iron hormesis is another possible explanation to the notable increase in life extension that cells experience in conditions of iron deprivation since the beginning of the experiment. This limitation forces cells to recycle iron from cellular components through autophagy and, in addition, it avoids an excess of iron circulation in the cell, provided that unnecessary vacuolar accumulation of the metal as a result of Aft1 and Aft2 activation, would be prone to cause oxidative stress.

Several studies have shown that quiescence [14] and autophagy contribute to life extension [74, 19, 73]. Cells deprived of iron presented quiescent characteristics such as an increase in stress resistance, increase in the percentage of unbudded cells and trehalose accumulation. Some studies have demonstrated that trehalose accumulation as a consequence of nitrogen limitation was related to autophagy induction [75, 76], we do not rule out this possibility in the case of iron limitation. It has been recently published that iron starvation, in conditions of glucose limitation is associated to trehalose accumulation and life extension in a manner dependent on the autophagy [77]. Here we report that iron deprivation can induce trehalose production even in the presence of high glucose concentrations. Under these nutritional conditions and in accordance to [77], our results support the hypothesis that activation of the quiescent program is not sufficient to prolong life without the participation of the autophagy machinery. Iron limitation is another example of treatment intervention to extend life that requires the autophagy machinery since the CLS of *atg1* and *atg7* mutants were severely impaired, as compared to cultures not starved for iron, on despite of having activated the quiescent program.
The observation that iron refeeding can provoke a dramatic descent in CLS and autophagy flux reinforce the idea that the entry in this quiescent state accompanied of autophagy flux induction is essential to extend life when iron is scarce, being any additional metabolic activity detrimental.

In our study we observed that iron limitation caused a mild shift in Snf1 kinase activity as reported in rat esqueletal muscle [78] apparently because in this metabolic condition there is a higher dependence on the glucose metabolism [67]. However, Snf1 is not directly involved in signalling autophagy induction, on the opposite its role is involved in down regulating autophagy flux once iron is refeeded and it does so through Aft1 (Fig 8). The Aft1 transcription factor, whose known function is to regulate iron homeostasis in S. cerevisiae is not required to induce autophagy in conditions of iron deficiency, as previously reported by [64]. However, and interestingly, Aft1 participates in the transmission of the iron repletion signal to inactivate autophagy through Tor1 and Atg13 phosphorylation (Fig 8). This is not the first time in which an association between Snf1 and Aft1 has been reported [11] already described that both proteins were related in order to induce certain genes from the iron regulon during diauxic shift. Although autophagy is important for maintaining cellular viability during nutrient stress, excessive autophagy can be deleterious to cells and lead to apoptosis or necrosis [79]. As such, autophagy paradoxically serves as a mechanism for the suppression as well as the proliferation and survival of tumour cells [80]. Iron limitation in the time requires a fine tuning in nutrient recycling and metabolism and signalling activation, a further increase in any of these mechanisms disables the hormetic mechanism leading to early aging and cell death.
Acknowledgements

We want to thank Dr D. Abeliovitch (The Hebrew University of Jerusalem Cell Biology, Freiburg, Germany) for kindly sending us the plasmid pGFP-Atg8 and to Dr T. Powers (Department of Molecular and Cellular Biology, College of Biological Sciences, UC Davis, USA) for sending us the plasmid pAMS363 and to Dr M. Cyerts (Department of Biology, Stanford, California, USA) for her permission. We want to acknowledge Ms Inmaculada Montoliu for her technical support. The research described in this publication was partly supported by the Plan Nacional de I+D+I of the Spanish Ministry of Economy, Industry and Competitiveness (BIO2017-87828-C2-2-P). Sandra Montella is funded by a fellowship from the Catalan Government (Spain).

Conflict of Interests

The authors declare no conflict of interests

Data Availability Statement:

Data sharing is not applicable since supporting data is included within the main article or supplementary material.

Our article does not contain information nor results that require any of the mandatory datasets listed below:

- Structural/crystallographic data for both macromolecular structures and small molecules
- Protein and nucleic acid sequence data (this includes RNA Seq data)
- Functional genomics and molecular interactions/proteomics/metabolomics data
- Computational models
- Genetics data (genetic polymorphisms; genotype data)
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Legends to Figures

Figure 1. Iron deprivation as the only nutrient restriction induces bulk autophagy and increases the accumulation of both Atg8 and Atg13 foci. A) wt, atg7 and atg1 cultures in which the fusion protein GFP-Atg8 was integrated (strains GSL197, 226 and 325, respectively), were grown to log phase (OD₆₀₀: 0.6) in SD and SD-Fe medium at 30°C. Aliquots were collected for total protein extraction and western blot. GFP-Atg8 was monitored using an anti-GFP antibody. As loading control we used anti-Pgk1 to detect Pgk1. B) Autophagic flux was calculated as the ratio between free GFP and total GFP-Atg8 detected in A. Total GFP-Atg8 was determined as the addition of the high mobility band corresponding to the form GFP-Atg8 and the slow mobility band corresponding to GFP, as a result of Atg8 vacuolar degradation. C) Total Atg8 was determined as the ratio between total GFP-Atg8 and Pgk1 expression. D) In vivo observation in the fluorescence microscope of a sample obtained from the experiment described in A. E) Percentage of Atg8 foci quantified in the experiment described in D was calculated upon microscopic observation of 1,000 cells. F) wt, atg7 and atg1 strains were transformed with plasmid pATG13-GFP. Culture conditions were identical as those described in A. Atg13 foci were counted from a total of 1,000 cells observed in the fluorescence microscopy. G) Autophagy activity was measured in exponential cultures limited or nor for iron by using the alkaline phosphatase assay in the strain BY471Δpho8Δ expressing a plasmid with the inactive Pho8 proenzyme targeted to the cytosol.

For all the figures: We used anti-Pgk1 to detect Pgk1, selected as a loading control in all the western blots shown in this study. For western blot and microscopy images in this paper, we have selected representative samples. Error bars in the histograms represent the standard deviation (SD) calculated from 3 independent experiments. Significance of the data was determined by P-values from a Student unpaired t-test denoted as follows: *0.05>P>0.01; **0.01>P>0.001; ***0.001>P>0.0001; ****P>0.0001

Figure 2. Tor1 inhibition to specific substrates mediates the induction of bulk autophagy in response to iron deprivation. A) Strains wt and atg7 were each transformed with the plasmids Sfp1GFP, Rtg1GFP or Msn2GFP, respectively, to be subsequently exponentially grown at 30°C in SD and SD-Fe media. Aliquots were collected for in vivo observation in the fluorescence microscope. Histograms represent percentages of
**in vivo** nuclear or cytoplasmic localization out of 1000 cells. B) Cultures of wt and atg7 strains were transformed with a plasmid bearing Rtg1GFP to be subsequently grown at 30°C to log phase in both SD and SD-Fe media, cultures were treated with N-Acetyl cysteine (NAC) 5mM for 2 hours. C) Strains wt and atg7 were exponentially grown in both SD and SD-Fe and samples were collected for total protein extraction and western blot analysis. The phosphorylated form of eIF2α was detected using anti-eIF2α-P antibody. A 2 days culture of wt strain in SD medium, was included as a control to detect eIF2α phosphorylation. Histogram represents the values of phosphorylated eIF2α normalized with respect to values determined with the loading control. D) wt, atg7 and atg1 strains were transformed with plasmids bearing Atg13HA or Atg1HA, respectively. Cultures were exponentially grown in either SD or SD-Fe. Values of Atg13 or Atg1 proteins were determined upon western blot analysis by adding anti-HA antibody. Samples were collected and each of them was split into two parts, one part was treated 1h at 37°C with alkaline phosphatase and the other remained untreated. E) wt and atg7 bearing GFP-Atg8 were exponentially grown at 30°C in SD and SD-Fe media. Rapamycin was added to the cultures at 200 ng/ml and samples were collected upon 2 hours of exposure to the drug for total protein extraction and western blot. GFP-Atg8 monitoring in microscope images and histograms were performed as described in Fig 1F. F) Autophagic activity was determined through the alkaline phosphatase assay as in Fig 1G. Rapamycin treatment conditions were as described in Fig 2E.

**Figure 3.** TOR2/Ypk1 activity is required to transmit the iron starvation signal to the autophagy machinery. A) wt and ypk1 strains in which the fusion protein GFP-Atg8 was integrated, were grown and GFP-Atg8 or GFP were detected as in Fig 1A. Autophagy flux and total Atg8 were determined as in Fig 1B and C, respectively. B) Microscopic observation of GFP-Atg8 was carried out as in Fig 1D. C) wt and ypk1 strains were transformed with the plasmids Sfp1GFP, Rtg1GFP or Msn2GFP to be subsequently grown at 30°C in SD and SD-Fe, respectively, until OD_{600}: 0.6. Aliquots were collected to be in vivo observed in the fluorescence microscope as in Fig 2A. Histograms represent percentages of **in vivo** nuclear or cytoplasmic localization. D) Atg13HA and Atg1HA phosphorylation and treatment with alkaline phosphatase were determined in wt and ypk1 strains as described in Fig 2D. E) Autophagy determination in wt and rho0 strains was performed as in A. F) Microscope images from samples collected in E.
Figure 4. Atg13 dephosphorylation dependent of TORC1 inactivation, requires a previous signalling by TOR2/Ypk1 upon iron starvation in order to initiate bulk autophagy. A) wt and ypk1 expressing either GFP-Atg8, Atg13HA or Atg1HA were exponentially grown at 30°C in SD and SD-Fe media to OD$_{600}$: 0.6. Rapamycin was added to half of the cultures at 200ng/ml and samples were taken upon 2 hours for western blot analysis. Protein detection, autophagy flux, total Atg8 and in vivo observation in the fluorescence microscope was performed as described in Fig1A-D and 2D. B) wt and ypk1 strains transformed with the plasmids Rtg1GFP, Sfp1GFP and Msn2GFP were treated as in A. Aliquots were collected for in vivo observation in the fluorescence microscope. C) wt and ypk1 strains were exponentially grown in SD and SD-Fe media at 30°C. Samples were collected for total protein extraction and western blot to analyse the phosphorylated form of eIF2α as in Fig 2C. D) wt and ypk1 cells containing plasmid pAMS363 expressing a 2xCDRE: lacZ fusion were at 30°C. Samples were harvested at OD$_{600}$: 0.6 to determine β-galactosidase activity as described in Material and Methods. E) wt, tor1, ras2, gcn2 and snf1 strains expressing GFP-Atg8, Atg13HA or Atg1HA, respectively, were grown at 30°C in SD and SD-Fe media. Protein detection, treatment with alkaline phosphatase and in vivo observation in the fluorescence microscope was performed as described in Fig1A-D and 2D. F) wt and aft1 transformed with GFP-Atg8, Atg13HA and Atg1HA, respectively were exponentially grown at 30°C in SD and SD-Fe conditions. Protein detection, autophagy flux, total Atg8 calculations and in vivo observation in the fluorescence microscope was performed as described in Fig 1A-D and 2D.

Figure 5. Snf1, Aft1 and Tor1 are required to inactivate autophagy upon iron refeeding. A) Autophagy activity was measured by means of the alkaline phosphatase assay as explained in Fig 1G. Iron was added to wt cultures at OD$_{600}$: 0.6, and samples were taken upon 2 hours of addition. B) Strains wt, tor1, aft1 and snf1 bearing GFP-Atg8 in the genome, were grown to OD$_{600}$: 0.6 at 30°C in SD and SD-Fe media. Iron was added to the cultures as in A, and samples were collected upon 2 and 6 hours to detect GFP-Atg8 and C) cellular localization of GFP-Atg8. Autophagy flux and GFP-Atg8 total expression were determined as in Fig1. D) wt, tor1, aft1 and snf1 transformed either with Atg13HA or Atg1HA plasmids and subsequently treated as in B. Samples were processed for western blot assay by using anti-HA antibody. E) Localisation of Sfp1
was determined microscopically upon transformation of the strains wt, tor1, aft1 and snf1 with plasmid bearing the protein fused to GFP as described in the former figures. Cultures were carried out as described in B). F) wt and aft1 strains were cultured in SD and SD-Fe medium during 2 days, iron was added and samples were collected upon the times indicated in the Figure for western blot analysis. AMPK1 was detected using an anti-AMPK1-P. G) wt, aft1 and snf1 expressing the fusion protein GFP-Atg8, were cultured in SD and SD-Fe medium during 2 days. Iron was added to the cultures and aliquots were collected as indicated in the Figure for protein extraction and western blot analysis and for microscopic observation to detect GFP-Atg8 protein. Autophagic flux and total Atg8 expression were determined as determined in Fig 1.

Figure 6. Iron deprivation induces an early entrance into quiescence independent of bulk autophagy. A) wt cells were grown in each SD or SD-Fe media at 30°C for 15 days in continuous shacking. Samples were taken daily to monitor the absorbance at 600nm to build the growth curves. Average values from three independent experiments were represented along with the corresponding error bars. B) The number of budded or unbudded cells was counted in a sample of 1,000 cells collected from wt cultures grown at OD600: 0.6 in both SD or in SD-Fe. C) Trehalose concentration [µg/µL] was referred to the total protein determined in cultures from wt, atg7 and atg17 strains growing exponentially in either SD and SD-Fe media at 30°C. D) Samples from wt cultures growing exponentially in SD or SD-Fe media were collected and the same number of cells was plated onto YPD plates to be incubated at 30°C, 39°C or 41°C or alternative, cells were plated onto media containing H2O2 or sorbitol. Cell survival was determined as the ratio between the number of colonies isolated from the treatment plates and the number of colonies isolated in control YPD plates incubated at 30°C. E) The index of respiratory competence (IRC) in wt cells and F) mitochondria mutation frequency, were both determined as described in Materials and Methods.

Figure 7. Iron starvation prolongs life span in a manner dependent of the activation of bulk autophagy. Cultures were exponentially grown either in SD, SD-Fe, SGly (containing glycerol as unique carbon source) or SGly-Fe media plus amino acids at 30°C. Samples were taken at the indicated times to determine CLS, as described in Materials and Methods. Numerical data regarding maximum life span (the day when
cultures reach 10% survival) and average life span (the day at which 50% survival was recorded) for each strain is depicted.

Chronological life span curves for A) wt CML128, wt SEY6210 and wt FY250 in SD and SD-Fe media, respectively. B) wt cells growing in SD, SGly, SD-Fe and SGly-Fe media. C) wt, atg7, atg13 and atg17 strains cultured in SD and SD-Fe, respectively.

Figure 8. Working model.

Our results suggest that iron starvation induces TOR1 inactivation to dephosphorylate Atg13 and consequently to induce the autophagy machinery, only when TOR2/YPK1 is active. In addition, reduction of TOR1 activity also signals to the readouts Sfp1 and Rtg1/Rtg3, independently of Tor2/Ypk1. Once iron is refeeded, both Snf1 and Aft1 down regulate autophagy flux through Tor1 and the phosphorylation of Atg13.
Table 1. Yeast strains used in this study

| Strain  | Genotype                                                                 | Source       |
|---------|---------------------------------------------------------------------------|--------------|
| CML128  | MATa leu2-3,112, ura3-52, trp1, his4                                       | [39]         |
| GSL034  | MATa tor1::KanMx4                                                         | [40]         |
| GSL053  | MATa ras2::Leu2MX5                                                        | [40]         |
| GSL197  | MATa leu2-3,112, ura3-52, trp1, his4 GFP-ATG8::URA3                       | This work    |
| GSL199  | MATa tor1::KanMx4 GFP-ATG8::URA3                                         | This work    |
| GSL201  | MATa ras2::Leu2MX5 GFP-ATG8::URA3                                         | This work    |
| GSL218  | MATa atg7::NatMx4                                                         | This work    |
| GSL222  | MATa atg13::NatMx4                                                        | This work    |
| GSL226  | MATa atg7::NatMx4 GFP-ATG8::URA3                                         | This work    |
| GSL238  | MATa atg17::NatMx4                                                        | This work    |
| GSL284  | MATa aft1::KanMx4                                                         | [41]         |
| GSL293  | MATa atg11::NatMx4                                                        | This work    |
| GSL297  | MATa atg11::NatMx4 GFP-ATG8::URA3                                         | This work    |
| GSL313  | MATa aft1::KanMx4 GFP-ATG8::URA3                                         | This work    |
| GSL324  | MATa atg1::NatMx4                                                         | This work    |
| GSL325  | MATa atg1::NatMx4 GFP-ATG8::URA3                                         | This work    |
| GSL350  | MATa gcn2::KanMx4                                                         | [42]         |
| GSL352  | MATa gcn2::KanMx4 GFP-ATG8::URA3                                         | This work    |
| GSL364  | MATa atg32::KanMx4 GFP-ATG8::URA3                                         | This work    |
| GSL370  | MATa rho0 GFP-ATG8::URA3                                                 | This work    |
| GSL371  | MATa atg32::KanMx4 GFP-ATG8::URA3                                         | This work    |
| GSL372  | MATa leu2-3,112, ura3-52, trp1, his4 ATG1-HA::LEU2                        | This work    |
| GSL374  | MATa ypk1::KanMx4 GFP-                                                   | This work    |
ATG8::URA3

GSL382  MATa snf1::KanMx4 GFP-ATG8::URA3  This work

GSL384  MATa ypk1::KanMx4  This work

GSL389  MATa tor1::KanMx4 ATG1-HA::LEU2  This work

GSL390  MATa aft1::KanMx4 ATG1-HA::LEU2  This work

GSL393  MATa ypk1::KanMx4 ATG1-HA::LEU2  This work

GSL394  MATa snf1::KanMx4  This work

GSL395  MATa snf1::KanMx4 ATG1-HA::LEU2  This work

GSL398  MATa atg1::NatMx4 ATG1-HA::LEU2  This work

GSL399  MATa atg7::NatMx4 ATG1-HA::LEU2  This work

GSL401  MATa gcn2::NatMx4 ATG1-HA::LEU2  This work

BY4741 pho8Δ  MATa pho8 his3D1, leu2D0, met15D0, ura3D0  [43]

FY250  MATa his3-200, leu2-1, trp1-63, ura3-52  [44]

SEY6210  MATa his3-200, leu2-3, lys2-801, trp1-901, ura3-52, suc2-9 GAL  [45]
Table 2. Plasmids used in this study

| Plasmid                  | Restriction sites to clone the ORF | Marker | Promoter | Epitope | Source       |
|-------------------------|-----------------------------------|--------|----------|---------|--------------|
| pSfp1-GFP               | SalI, SmaI                        | URA3   | MET25    | GFP     | This work    |
| pGFP-Atg8               | EcoRI, XhoI                       | URA3   | ATG8     | GFP     | [46]         |
| pAtg13-HA               | NotI, PstI                        | URA3   | ADH1     | HA      | This work    |
| pYX242-cytPho8          | AvrII, MluI                       | LEU2   | PHO8     |          | [43]         |
| pAdh1-Msn2-GFP          | KspI, SalI                        | LEU2   | ADH1     | GFP     | [47]         |
| pAtg13-GFP              | XbaI, SalI                        | URA3   | MET25    | GFP     | This work    |
| pMM351                  | PstI, HindIII                     | LEU2   | ADH1     | HA      | [48]         |
| pAtg1-HA                | PmeI, PstI                        | LEU2   | ADH1     | HA      | This work    |
| pRtg1-GFP               | XhoI, EcoRI                       | URA3   | RTG1     | GFP     | [49]         |
| pAMS363                 | XhoI, SalI                        | URA3   | 2xCDRE: lacZ | [50]     |
Figure 1

A) 

|       | wt | atg7 | atg1 |
|-------|----|------|------|
| SD    | S  | S    | S    |
| SD-Fe | S  | S    | S    |

GFP-Atg8
GFP
anti-PGK1

B)

Autophagic flux

SD
SD-Fe

C)

Atg8 expression

SD
SD-Fe
Figure 2

A) Sfp1 subcellular localization (%)

|          | Nomarski | Sfp1GFP | Nomarski | Sfp1GFP |
|----------|----------|---------|----------|---------|
| **wt**   | ![Image](https://example.com) | ![Image](https://example.com) | ![Image](https://example.com) | ![Image](https://example.com) |
| **atg7** | ![Image](https://example.com) | ![Image](https://example.com) | ![Image](https://example.com) | ![Image](https://example.com) |

B) Rtg1 subcellular localization (%)

|          | Nomarski | Rtg1GFP | Nomarski | Rtg1GFP |
|----------|----------|---------|----------|---------|
| **wt**   | ![Image](https://example.com) | ![Image](https://example.com) | ![Image](https://example.com) | ![Image](https://example.com) |
| **atg7** | ![Image](https://example.com) | ![Image](https://example.com) | ![Image](https://example.com) | ![Image](https://example.com) |

C) Msn2 subcellular localization (%)

|          | Nomarski | Msn2GFP | Nomarski | Msn2GFP |
|----------|----------|---------|----------|---------|
| **wt**   | ![Image](https://example.com) | ![Image](https://example.com) | ![Image](https://example.com) | ![Image](https://example.com) |
| **atg7** | ![Image](https://example.com) | ![Image](https://example.com) | ![Image](https://example.com) | ![Image](https://example.com) |
D) 

\[\begin{array}{c|cc|cc}
\text{wt} & \text{atg7} & \text{atg1} \\
\hline
- & - & - & + & + & + \\
\text{SD} & \text{SD} & \text{SD} & \text{SD} & \text{SD} & \text{SD} \\
\text{SD-Fe} & \text{SD-Fe} & \text{SD-Fe} & \text{SD-Fe} & \text{SD-Fe} & \text{SD-Fe} \\
\end{array}\]

alkaline phosphatase

- anti-HA
- anti-PGK1
- anti-HA
- anti-PGK1
- anti-HA
- anti-PGK1

E) 

\[\begin{array}{c|cc|cc}
\text{SD} & \text{SD-Fe} \\
\hline
\text{wt} & \text{atg7} & \text{wt} & \text{atg7} & \text{wt} & \text{atg7} & \text{wt} & \text{atg7} \\
- & - & - & + & + & - & - & + \\
\text{Rapamycin} & \text{Rapamycin} & \text{Rapamycin} & \text{Rapamycin} & \text{Rapamycin} & \text{Rapamycin} & \text{Rapamycin} & \text{Rapamycin} \\
\text{GFP-Atg8} & \text{GFP-Atg8} & \text{GFP-Atg8} & \text{GFP-Atg8} & \text{GFP-Atg8} & \text{GFP-Atg8} & \text{GFP-Atg8} & \text{GFP-Atg8} \\
\text{GFP} & \text{GFP} & \text{GFP} & \text{GFP} & \text{GFP} & \text{GFP} & \text{GFP} & \text{GFP} \\
\text{anti-PGK1} & \text{anti-PGK1} & \text{anti-PGK1} & \text{anti-PGK1} & \text{anti-PGK1} & \text{anti-PGK1} & \text{anti-PGK1} & \text{anti-PGK1} \\
\end{array}\]
Figure 3

A) 

|       | wt | ypk1 |
|-------|----|------|
| SD    |    |      |
| SD-Fe |    |      |

GFP-Atg8
GFP
anti-PGK1

B) 

|       | SD       | SD-Fe     |
|-------|----------|-----------|
| Nomarski | wt | ypk1     |
|         | 20.0 μm | 20.0 μm   |
| Nomarski |    |          |
|         |      |          |
| GFP     |    |          |
|         |      |          |

** Autophagic flux **

** Atg8 expression **

* SD
* SD-Fe

** SD
** SD-Fe

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C) **Sfp1 subcellular localization (%)**

- **wt SD**
- **wt SD-Fe**
- **ypk1 SD**
- **ypk1 SD-Fe**

- **Nucleus**
- **Cytoplasm**

- **Rig1 subcellular localization (%)**

- **wt SD**
- **wt SD-Fe**
- **ypk1 SD**
- **ypk1 SD-Fe**

- **Nucleus**
- **Cytoplasm**

D) **Msn2 subcellular localization (%)**

- **wt SD**
- **wt SD-Fe**
- **ypk1 SD**
- **ypk1 SD-Fe**

- **Nucleus**
- **Cytoplasm**

**D)**

|         | **wt** |         | **ypk1** |
|---------|--------|---------|----------|
|         | **SD** | **SD-Fe** | **SD** | **SD-Fe** |
| **-**   | **-**  | **+**   | **-**  | **+**   |
| **+**   | **+**  | **-**   | **+**  | **-**   |

**alkaline phosphatase**

- **anti-HA**
- **anti-PGK1**

**Atg13**

- **anti-HA**
- **anti-PGK1**

**Atg1**
Figure 4

A)

|        | wt | ypk1 |
|--------|----|------|
| SD     | -  | -    |
| SD-Fe  | +  | +    |
| Rapamycin | -  | +    |

GFP-Atg8
GFP

anti-PGK1

![Autophagic flux graph](image)

![Atg8 expression graph](image)

![Nomarski and GFP images](image)
Figure 5

A) Specific cytPho8 activity

B) GFP-Atg8

anti-PGK1

GFP

anti-PGK1

Autophagic flux

Alg8 expression

log

2h

6h

2h +Fe

6h +Fe
C) SD-Fe  
|       | SD-Fe +Fe 6h |
|-------|-------------|
| wt    |             |
| tor1  |             |
| aft1  |             |
| snf1  |             |

D) wt SD-Fe  
|       | tor1 SD-Fe |
|-------|------------|
| Atg13 | anti-HA    |
|       | anti-PGK1  |
| Atg1  | anti-HA    |
|       | anti-PGK1  |
G)

|          | wt          | aft1        | snf1        |
|----------|-------------|-------------|-------------|
|          | SD          | SD-Fe       | SD          | SD-Fe       |
| days     | 0 2 2 2 3   | 0 2 2 3     | 0 2 2 2 3   | 0 2 2 3     |
| hours +Fe| 2 6 24      | 2 6 24      | 2 6 24      | 2 6 24      |

GFP-Atg8
GFP
anti-PGK1

**Figure Legend:**

- **GFP-Atg8**: Green Fluorescent Protein-Atg8
- **GFP**: Green Fluorescent Protein
- **anti-PGK1**: Anti-Phosphoglycerate Kinase 1

**Graph:**

- **Autophagic flux**
- **X-axis**: Days and Hours + Fe
- **Y-axis**: 0.0 to 0.9
- **Conditions**: 0 days w/o Fe, 2 days w/o Fe, 2 days 2h Fe, 2 days 6h Fe, 2 days 24h Fe
The bar graph and images illustrate the expression level of Atg8 in different strains over time with and without Fe. The bar graph shows the expression levels at 0 days, 2 days, 2 days with 2h Fe, 2 days with 6h Fe, and 2 days with 24h Fe. The images below the graph depict the microscopic view of cells under different conditions, demonstrating the GFP expression in each strain and time point.

- **wt** and **aft1**: Both strains show GFP expression at different time points with and without Fe.
- **snf1**: The strain displays GFP expression in a distinct manner compared to the other two strains, particularly at 2 days with 2h Fe and 2 days with 6h Fe.

The graphs and images together provide a comprehensive view of how iron deficiency affects Atg8 expression in these strains.
Figure 6

A) Absorbance at 600nm over time (days)

- ○ wt SD
- ● wt SD-Fe

B) % of cells

- SD
- SD-Fe

- 0 hours
- 8 hours

C) Trehalose (µg/µl) / Total protein

- SD
- SD-Fe

D) Cell Survival%

- H₂O₂
- 39°C
- 41°C
- Sorbitol

- SD
- SD-Fe

- Log
- 8 hours

Nomarski

DHE

DAPI

Merge
Figure 7

A) Cell Survival (%) vs Time (days)

| Strains            | Maximum lifespan | SD    | Average lifespan |
|--------------------|------------------|-------|------------------|
| wt CML128 SD       | 18.5             | ±0.82 | 9.3              |
| wt SEY6210 SD      | 19.1             | ±0.53 | 9.7              |
| wt FY250 SD        | 18.1             | ±0.41 | 9.1              |
| wt CML128 SD-Fe    | 23.7             | ±0.82 | 10.9             |
| wt SEY6210 SD-Fe   | 24.1             | ±0.65 | 10.7             |
| wt FY250 SD-Fe     | 24.4             | ±0.70 | 10.8             |
**B)**

![Graph showing cell survival over time for different strains](image)

| Strains   | Maximum lifespan | SD   | Average lifespan |
|-----------|------------------|------|------------------|
| wt SD     | 18.3             | ±0.24| 7.9              |
| wt SGly   | 18.0             | ±0.36| 7.2              |
| wt SD-Fe  | 23.0             | ±0.21| 11.2             |
| wt SGly-Fe| 22.8             | ±0.53| 10.9             |
C) Cell Survival (%) over time for different strains:

**Graph and Table:**

| Strains       | Maximum lifespan | SD  | Average lifespan |
|---------------|------------------|-----|-----------------|
| wt SD         | 17.5             | ±0.21 | 8.2            |
| atg7 SD       | 7.0              | ±0.56 | 1.5            |
| atg13 SD      | 10.0             | ±0.70 | 1.6            |
| atg17 SD      | 9.0              | ±0.14 | 1.9            |
| wt SD-Fe      | 22.8             | ±0.35 | 11.2           |
| atg7 SD-Fe    | 5.4              | ±0.28 | 0.7            |
| atg13 SD-Fe   | 6.3              | ±0.56 | 1.2            |
| atg17 SD-Fe   | 7.4              | ±0.72 | 1.4            |
Figure 8