Sulfur depletion induces autophagy through Ecl1 family genes in fission yeast

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Abstract

Autophagy is an intracellular degradation system widely conserved among various species. Autophagy is induced by the depletion of various nutrients, and this degradation mechanism is essential for adaptation to such conditions. In this study, we demonstrated that sulfur depletion induces autophagy in the fission yeast *Schizosaccharomyces pombe*. Based on the finding that autophagy induced by sulfur depletion was completely abolished in a mutant in which the *ecl1*, *ecl2*, and *ecl3* genes were deleted (Δ*ecls*), we report that these three genes are essential for the induction of autophagy by sulfur depletion. Furthermore, autophagy-defective mutant cells exhibited poor growth and short lifespan (compared with wild-type cells) under the sulfur-depleted condition. These results indicated that the mechanism of autophagy is necessary for the appropriate adaptation to sulfur depletion.

Introduction

Autophagy comprises one of the intracellular degradation systems conserved from yeasts to humans. This system plays a vital role in the degradation of intracellular components such as proteins and organelles in the processes of their exclusion and

turnover. Autophagy is also necessary for adaptation to environmental changes, including depletion of various nutrients. Till date, several autophagy-related genes and mechanisms have been identified, wherein the budding yeast *Saccharomyces cerevisiae* has significantly contributed to this identification (Xie and Klionsky, 2007, Mizushima *et al*., 2011, Ohsumi, 2014). Although autophagy is widely conserved, there exist some differences in the conditions that induce autophagy and its related factors among species. For instance, autophagy is induced by carbon depletion in *S. cerevisiae*, but not in *Schizosaccharomyces pombe* (Mukaiyama *et al*., 2009). Moreover, autophagy factors are partially different between these two species (Mukaiyama *et al*., 2010).

Yeasts have also contributed to lifespan studies, and a chronological lifespan is defined as the survival period after entry into the stationary phase (Fabrizio and Longo, 2003). Earlier, the Ecl1 family genes (*ecl1*$^+$, *ecl2*$^+$, and *ecl3*$^+$, respectively) were identified whose overexpression was found to cause the extension of the chronological lifespan in *S. pombe* (Ohtsuka *et al*., 2008, 2009). These genes encode small proteins consist of about 80 amino acids respectively, but their molecular functions have been unclear. However, phenotypic analysis revealed that Ecl1 family genes are also essential for the appropriate cellular response to sulfur depletion (Ohtsuka *et al*., 2017). Sulfur is one of the essential nutrients and is present in sulfur compounds such as biotin, thiamine, coenzyme A, lipoic acid, and sulfur amino acid. Sulfur depletion induces cellular responses such as lifespan extension, morphological changes, and cell cycle arrest in *S. pombe*, and these phenomena are almost completely dependent on the Ecl1 family genes (Ohtsuka *et al*., 2017).

In the present study, we demonstrated that the Ecl1 family genes are essential for the execution of autophagy induced by sulfur depletion. In addition, autophagy was required for lifespan extension under the sulfur-depleted condition. These results suggested that the Ecl1 family genes mediate sulfur-depleted signaling to induce autophagy for the appropriate adaptation to such conditions.

Results

【Sulfur depletion induced autophagy in fission yeast】

In *S. pombe*, it has been already demonstrated that nitrogen depletion induces autophagy (Mukaiyama *et al*., 2009), but it was unclear whether sulfur depletion induces autophagy. In the budding yeast, a previous study reported that sulfur depletion induced

autophagy (Ohsumi, 2014). Sulfur is an essential nutrient, and sulfur depletion causes cellular responses such as cell cycle arrest, morphological changes, and lifespan extension as well as nitrogen depletion in *S. pombe* (Ohtsuka *et al*., 2017). Therefore, we anticipated that sulfur depletion also induces autophagy in the fission yeast. To confirm our assumption, we prepared strains harboring the pTN54-Atg8 plasmid. This plasmid encodes the GFP-Atg8 protein regulated by the P41-nmt1 promotor (Mukaiyama *et al*., 2009). Atg8 is a ubiquitin-like protein that is essential for autophagosome formation (Nakatogawa *et al*., 2007, Xie *et al*., 2008). Upon the induction of autophagy, the GFP-Atg8 protein is cleaved into GFP, and this degradation is used as an indicator of autophagy (Shintani and Klionsky, 2004, Mukaiyama *et al*., 2009). To confirm that sulfur depletion induces autophagy, the wild-type strain harboring the pTN54-Atg8 plasmid was exposed to a sulfur-free medium. After sulfur depletion, there was robust degradation of GFP-Atg8 and the accumulation of free GFP reached the maximum after 24 h (Fig. 1A). To confirm whether this degradation depends on autophagy, the same experiment was performed using the Δ*atg1* mutant. Previous researches showed that as Atg1 is the kinase that is essential for autophagy, autophagy is abolished in the Δ*atg1* mutant (Mukaiyama *et al*., 2009). As anticipated, there was no degradation of the GFP-Atg8 protein by sulfur depletion in the Δ*atg1* mutant (Fig. 1B). Furthermore, fluorescence microscopy observation was performed to confirm the GFP-Atg8 localization under sulfur-depleted condition. Under this condition, the GFP-Atg8 localized in vacuole (Fig. 1C). We also confirmed that the degradation of the cytoplasmic protein Tdh1 (Tdh1-GFP) was enhanced by sulfur depletion (data not shown). Based on these results, we concluded that sulfur depletion induces autophagy in *S. pombe*.

【Ecl1 family genes are essential for autophagy induced by sulfur depletion】

We had earlier reported that the Ecl1 family genes (namely $ecl1^{+}$, $ecl2^{+}$, and $ecl3^{+}$) are essential for the response to sulfur depletion in *S. pombe* (Ohtsuka *et al*., 2017). Therefore, we anticipated that the induction of autophagy by sulfur depletion depends on the Ecl1 family genes. As expected, sulfur depletion did not induce autophagy in the Δ*ecls* mutant (Fig. 2A). Interestingly, however, nitrogen depletion induced autophagy in this mutant (Fig. 2A). These results indicated that the Ecl1 family genes are essential for the induction of autophagy specifically by sulfur depletion.

In *S. cerevisiae*, it has been reported that almost half of autophagy genes are upregulated under an autophagy-inducing condition (Jin *et al*., 2014). Therefore, we next analyzed the expression levels of several Atg genes comparing between wild-type and Δ*ecls* mutant strains under the sulfur-depleted condition. We observed that in the wild-type strain, the expression of these Atg genes increased by approximately four-fold after 3 h from sulfur depletion (Fig. 2B). In contrast, these inductions were obviously declined in the Δ*ecls* mutant compared with the wild-type strain (Fig. 2B). These results are consistent with the results of our previous RNA sequence analysis (Ohtsuka *et al*., 2017).

【Autophagy is important for the maintenance of viability under sulfur-depleted condition】

A previous study reported that autophagy is necessary for the maintenance of viability under the nitrogen-depleted condition (Kohda *et al*., 2007). Because sulfur depletion extends chronological lifespan in *S. pombe*, we assumed that autophagy is also required for lifespan extension under sulfur depletion. To confirm this assumption, we measured the viability of the Δ*atg1* mutant under the sulfur-depleted condition. Our results showed that under normal growth condition (i.e., in SD medium), there were no obvious differences in both growth and viability between the wild-type and Δ*atg1* mutant strains. However, the Δ*atg1* mutant exhibited poor growth and low viability under the sulfur-depleted condition compared with wild-type cells (Fig. 3A and 3B). These results indicated that autophagy is required for lifespan extension under the sulfur-depleted condition, similar to that under the nitrogen-depleted condition.

Discussion

In the present study, we have demonstrated that sulfur depletion induces autophagy in *S. pombe*, and this phenomenon was dependent on the Ecl1 family genes. As autophagy was induced by nitrogen depletion in the absence of Ecl1 family genes, it can be stated that these genes are not required for the autophagy machinery itself. Since the Ecl1 family genes are essential for the response to sulfur depletion, it is considered that disruption of these genes failed to induce autophagy caused by sulfur depletion.

Similar to nitrogen depletion, it was observed that autophagy is required for lifespan extension under the sulfur-depleted condition. The Δ*atg1* mutant exhibited poor growth and lifespan extension after sulfur depletion compared with the wild-type strain.

However, the Δ*atg1* mutant appeared to possess the ability to sustain its viability from the midway of sulfur depletion, i.e., from 50 h approximately (Fig. 3B). This phenomenon was observed reproducibly, which can be explained based on certain possibilities. One reason is that there might be other cellular responses that occurred to sustain the viability. Some previous studies have reported that autophagy and proteasome activity were cross-talked complementarily (Iwata *et al*., 2005, Pandey *et al*., 2007). Thus, a compensatory function such as proteasome activity might contribute to the maintenance of the Δ*atg1* mutant viability under the sulfur-depleted condition. Another possibility is that the Δ*atg1* mutant merely exhibited regrowth under such condition. Adaptive regrowth occurs due to the regrowth of surviving cells through the utilization of nutrients released from dead cells (Fabrizio *et al*., 2004). Regardless of these possibilities, these results suggested that autophagy is necessary for the response to acute sulfur depletion.

This study demonstrated that autophagy is required for the maximal lifespan extension induced by sulfur depletion. We had previously reported that lifespan extension induced by sulfur depletion is due to the downregulation of ribosomal activity as well as the depletion of nitrogen and carbon sources (Ohtsuka *et al*., 2017). Based on electron microscopy observations, it was observed that large amounts of ribosome were contained in the autophagosome under nitrogen- and carbon-depleted conditions in *S. cerevisiae* (Takeshige *et al*., 1992), and thereafter, ribosome-specific autophagy (generally known as ribophagy) was identified (Kraft *et al*., 2008). Therefore, autophagy induced by sulfur depletion might be required for the downregulation of ribosomal activity to extend the lifespan. It is necessary to verify these possibilities in the future.

Materials and Methods

Strain and Media

S. pombe strains used in this study are listed in Table S1 (supplementary material). The Δ*atg1* mutant (JY333 background) was generated from the ARC039 Δ*atg1* mutant (a kind gift from Dr. Kaoru Takegawa) by mating. The constituents of EMM and SD medium have been described previously (Ohtsuka *et al*., 2017). In sulfur-free and nitrogen-free EMM, Na_2SO_4, and NH_4Cl were omitted, respectively. In sulfur-free SD

medium, $MgSO_4$ and $(NH_4)_2SO_4$ were omitted and $MgCl_2$ and NH_4Cl were supplied alternatively. Cells were cultured in the respective medium at 30°C with shaking.

Measurement of viability

The viability of each strain was measured as described previously (Ohtsuka *et al*., 2019). OD_{600nm} was measured using a Bactmonitor (BACT-550) equipped with a 600-nm filter (Nissho Denki Co.)

Real-time PCR analysis

Real-time PCR analysis was performed as described previously (Ohtsuka *et al*., 2019). The oligonucleotides used in this experiment are listed in Table S2 (supplementary material).

Western blot analysis

Western blot analysis was performed as described previously (Hibi *et al*., 2018). GFP antibody (Roche) and α-tubulin antibody (Sigma-Aldrich) were used for the primary antibody reaction, and mouse IgG (Jackson Immune Research) was used for the secondary antibody reaction.

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Figure legends

Figure 1

A: Wild-type strain (JY333) carrying the pTN54-Atg8 plasmid was cultured in EMM to OD_{600nm} = 0.5, and then the cells were washed and transferred into sulfur-free EMM. Thereafter, the cells were harvested at each time point, and Western blot analysis was performed. The amount of α-tubulin was used as loading control. B: JY333 (WT) and Δ*atg1* strains harboring pTN54-Atg8 were cultured in EMM to OD_{600nm} = 0.5, and then the cells were washed and transferred into EMM (control) and sulfur-free EMM (-S), respectively. Cells were harvested at each time point, and Western blot analysis was performed. C: Wild-type strain (JY333) carrying the pTN54-Atg8 plasmid was cultured in EMM to OD_{600nm} = 0.5, and then the cells were washed and transferred into sulfur-free EMM. After 24 h, cells were subjected to fluorescence microscopy observation. FM4-64 was employed for vacuole staining.

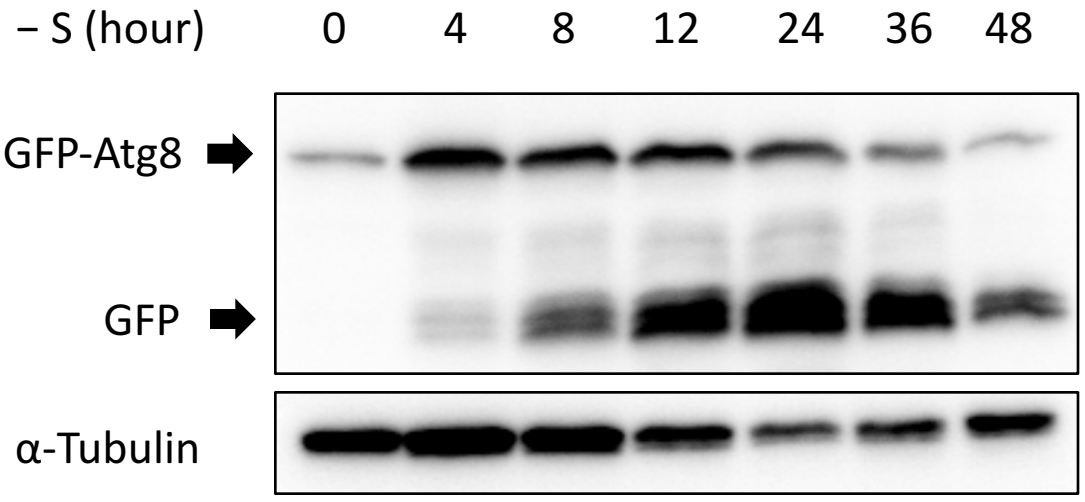
Figure 2

A: JY333 (WT) and Δ*ecl1.2.3* (Δ*ecls*) strains were cultured in EMM to OD_{600nm} = 0.5, and then the cells were washed and transferred into EMM (control), sulfur-free (-S), and nitrogen-free (-N) EMM, respectively. Cells were harvested at each time point, and then Western blot analysis was performed. B: JY333 (WT) and Δ*ecl1.2.3* (Δ*ecls*) strains were cultured in EMM to OD_{600nm} = 0.5, and then the cells were washed and transferred into EMM and sulfur-free EMM, respectively. Cells were harvested at each time point, and then real-time PCR analysis was performed. The expression of $cdc2^{+}$ was used as the quantitative control. The results are represented as the mean of three independent experiments with standard deviation.

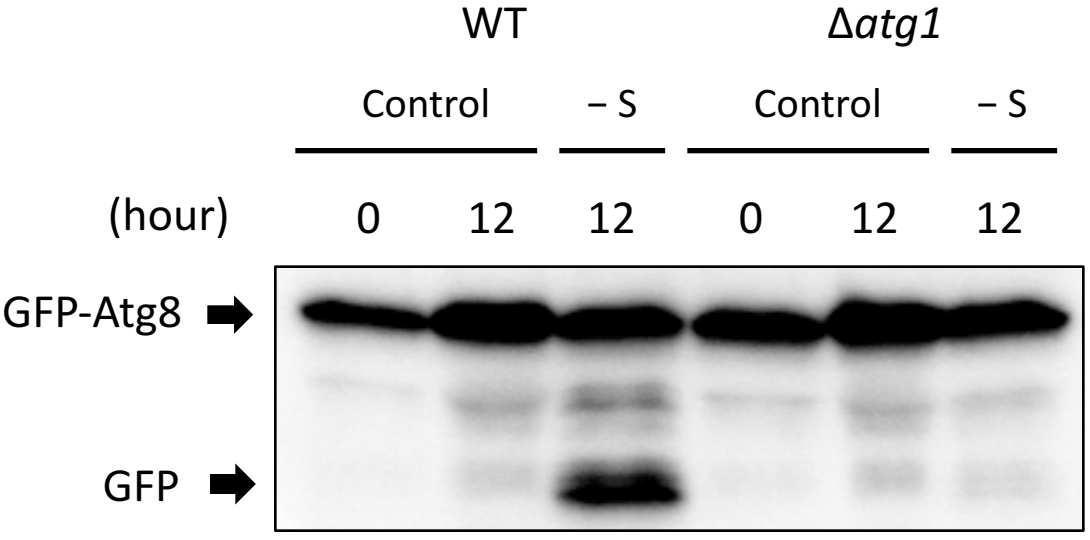
Figure 3

JY333 (WT) and Δ*atg1* strains were cultured in SD medium to OD_{600nm} = 1.5, and then the cells were washed and transferred into SD and sulfur-free SD medium, respectively. A: Growth of each strain was monitored at each time point. B: Viability of each strain was measured at each time point. The results are represented as the mean of three independent experiments with standard deviation.

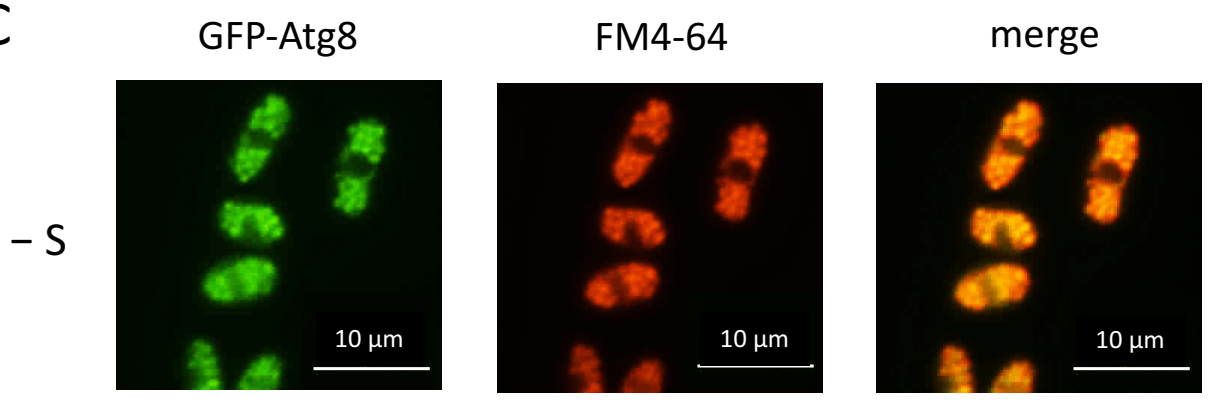
A


– S (hour)
0
4
8
12
24
36
48
GFP-Atg8
GFP
α-Tubulin

B


WT
Δatg1
Control
– S
Control
– S
(hour)
0
12
12
0
12
12
GFP-Atg8
GFP

C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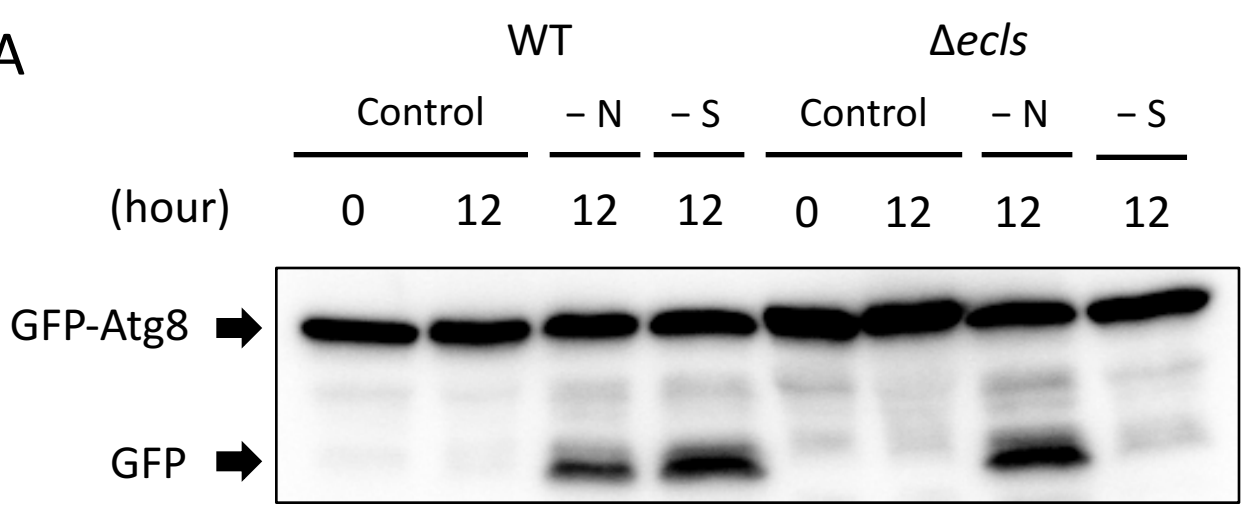
FM4-64
merge
– S
10 μm
10 μm
10 μm

Fig.1

A


WT
Δecls
Control
– N
– S
Control
– N
– S
(hour)
0
12
12
12
0
12
12
12
GFP-Atg8
GFP

B

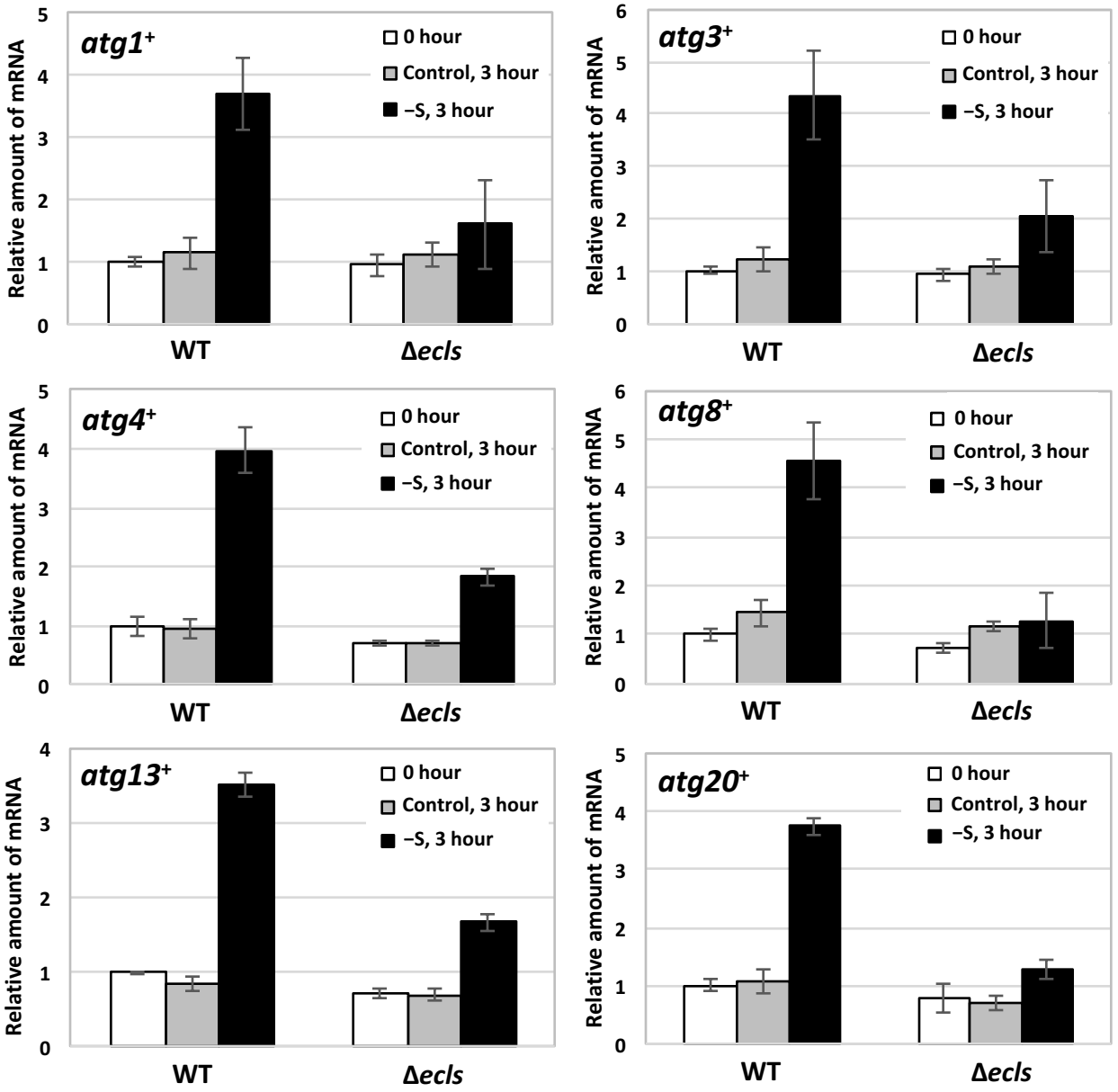
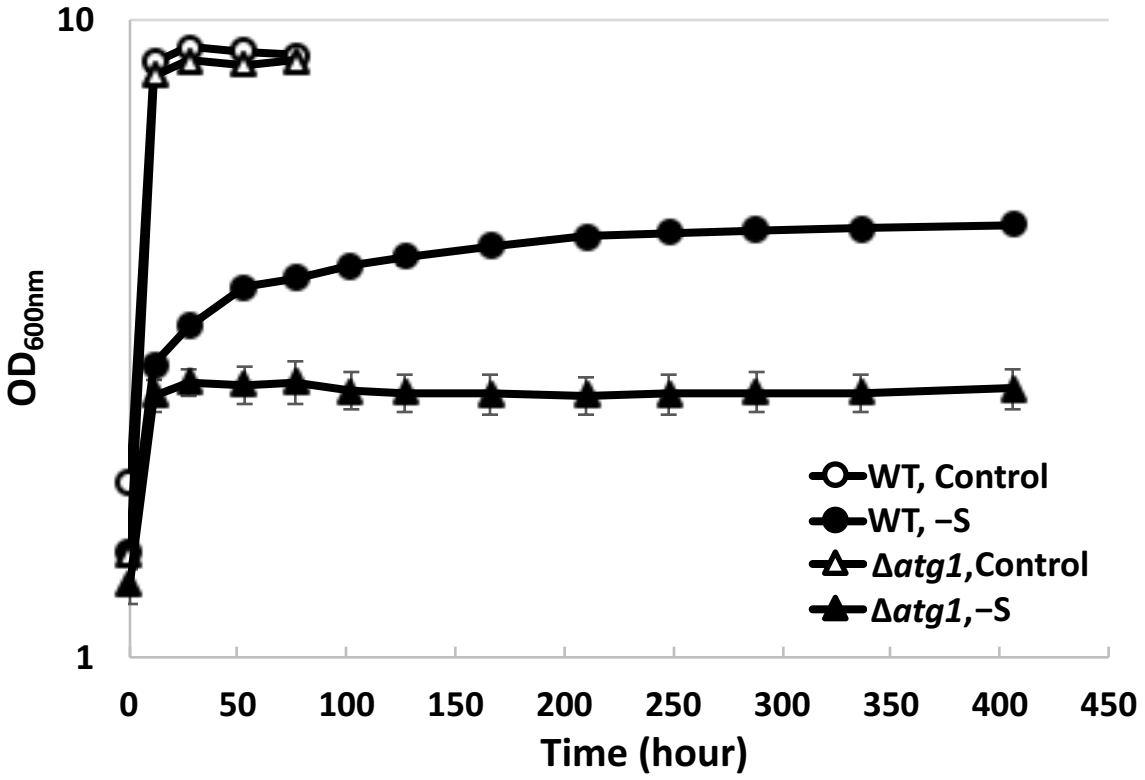

atg1+
atg3+
atg4+
atg8+
atg13+
atg20+
0 hour
Control, 3 hour
–S, 3 hour
Relative amount of mRNA
WT
Δecls

Fig.2

A


10
1
OD_{600nm}
0
50
100
150
200
250
300
350
400
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Time (hour)
WT, Control
WT, −S
Δatg1,Control
Δatg1,−S

B

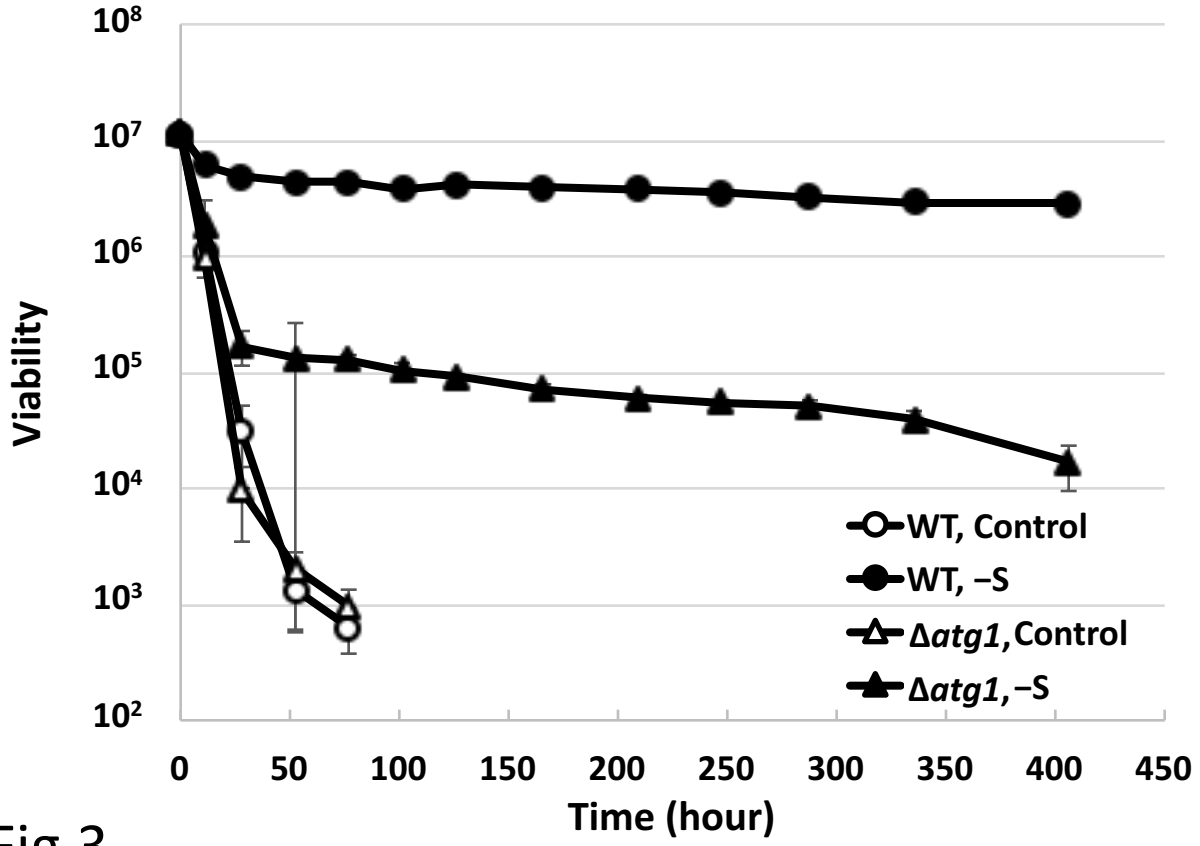
10^8
10^7
10^6
10^5
10^4
10^3
10^2
Viability
0
50
100
150
200
250
300
350
400
450
Time (hour)
WT, Control
WT, −S
Δatg1,Control
Δatg1,−S

Fig.3