Identification of a novel protein kinase that affects on the chronological lifespan in fission yeast

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Running head: chronological lifespan of fission yeast

Abstract

Chronological lifespan is defined by how long a cell can survive in a non-dividing state. In yeast, it is measured by viability after entry into the stationary phase. To understand the regulatory mechanisms of chronological lifespan in *Schizosaccharomyces pombe*, it is necessary to identify and characterize novel factors involved in the regulation of chronological lifespan. To this end, we have screened for a long-lived mutant and identified that novel gene *nnk1*$^+$ that encodes an essential protein kinase is the determinant of chronological lifespan. We showed that the expression of major glucose transporter gene, *ght5*$^+$ is decreased in the isolated *nnk1-35* mutant, suggesting Nnk1 protein is involved in the regulation of *ght5*$^+$. The consumption of glucose in the growth medium after saturated growth was lower in the *nnk1-35* mutant than that in wild type cell. The isolated *ght5* deletion mutant showed long-lived phenotype. Based on these results, we propose that Nnk1 regulates chronological lifespan through the regulation of *ght5*$^+$. Nnk1 might coordinate glucose availability and lifespan in fission yeast.

Key words

Lifespan, *Schizosaccharomyces pombe*, aging, glucose transporter, Nnk1, yeast

Introduction

In the natural environment, most microorganisms exhibit only brief periods of rapid growth. Nutrient starvation is the most common natural situation, so the ability to adapt to nutrient limitation is crucial for microorganisms. Cells respond to starvation by ceasing growth and entering the stationary phase to maintain viability for extended periods in the absence of nutrients (Werner-Washburne *et al.*,1993). In yeast, the period in which the cells keep their viability after entry into the stationary phase is recognized as the chronological lifespan (Roux *et al.*, 2010).

In *S. cerevisiae*, two of the major pathways that control chronological lifespan have been identified: the Ras / PKA / Msn2/4 pathway and the Sch9 pathway (Fabrizio and Longo, 2003; Fabrizio *et al.*, 2001). The down-regulation of either pathway promotes lifespan extension. Importantly,

similar pathways (insulin/IGF-I-like) regulate longevity in higher eukaryotes, suggesting a common evolutionary origin for the life span-regulatory mechanisms (Longo and Fabrizio, 2002; Longo and Kennedy, 2006).

In *S. pombe*, the Pka1 and Sck2 are regulators of chronological lifespan; each mutant shows a long-lived phenotype, and the *pka1* and *sck2* double mutant displays an additive effect on chronological lifespan extension, suggesting that these two factors regulate related but independent pathways (Roux *et al.*, 2006). We determined previously that the mutant of *pma1*$^{+}$, which encodes a P-type H^{+}-ATPase involved in the generation of proton gradient across the cytoplasmic membrane, showed long-lived phenotype after entry into the stationary phase, and suggested that defect of glucose uptake results in the extension of chronological lifespan in the mutant (Ito *et al.*, 2010; Naito *et al.*, 2014).

Because lifespan is supposed to be regulated by many factors complicatedly and important factors for lifespan regulation were conserved in eukaryote, it is necessary to identify new factors affecting chronological lifespan for understanding lifespan regulation (Smith *et al.*, 2007). To accomplish this, we have screened and identified a mutant that extends chronological lifespan of *S. pombe*.

Materials and Methods

Strain and media- *S. pombe* strain JY333 (*h*$^{-}$ *leu1-32 ade6-M216*) was used for mutant screening. Strains were grown in SD medium (0.67 % yeast nitrogen base without amino acids [Difco], 2 % glucose) or YE medium (0.5 % yeast extract [Difco], 3 % glucose) supplemented with necessary growth requirements in standard amounts at 30 ˚C. The analysis of viability was carried out as described previously (Ohtsuka *et al.*, 2008).

Library construction and whole genome resequencing- Fragment libraries were constructed using

purified genomic DNA of *Schizosaccharomyces pombe* strain JY333 and its derived mutant strain L35. All genomes were sequenced using the SOLiD 5500xl NGS platform (Life Technologies). Briefly, DNA fragments with mean size of about 125-bp were generated by the sonicator Covaris S2 system (Covaris Inc.). The DNA fragments were blunted by T4 DNA polymerase and were phosphorylated by T4 kinase. Two different SOLiD P1 and P2-barcoded adapters were ligated to each fragment end by T4 DNA ligase. These adaptor-ligated DNA fragments were nick-translated and amplified by PCR. The PCR products were separated by E-gel SizeSelect gel (2% agarose gel; Invitrogen Corp.) and 150 to 200 bp DNA fragments were collected. After all enzymatic process above mentioned, the DNA fragments were purified using Pure Link PCR purification Kit (Invitrogen Corp.). Concentrations of each DNA library were determined by quantitative RT-PCR method using SOLiD Library TaqMan Quantitation Kit (Life Technologies).

A 500 pM aliquot was prepared from a stock DNA Library and titrated 0.8-1.0 pM for input into ABI 1.0 M scale emulsion reactions (Emulsion PCR Kit; Life Technologies). Emulsion PCR was conducted using ABI GeneAmp PCR system 9700 for 40 cycles of amplification. After emulsion breaking and subsequent washing, enrichment for template beads was conducted using the SOLiD capture beads with P2 affinity. Beads lacking a template or a P2 adaptor were filtered out via gradient centrifugation with glycerol. The P2-enriched beads were isolated from the upper glycerol layer, modified with a 3′ amino group for surface attachment, and prepared for deposit on a glass Flow Chip slide. After 5bp barcode sequencing, the 75-base sequences were obtained by SOLiD 5500xl sequencer.

Data analysis- After base calling, mapping to the reference sequence of *Schizosaccharomyces pombe* (ASM294 v2.20, ftp://ftp.ebi.ac.uk/pub/databases/pombase) and SNPs calling was conducted using the ABI Bioscope v. 1.2 (Applied Biosystems), with default parameter settings for targeted resequencing. Called SNPs data were tabulated in Excel file and visually confirmed by IGV 1.5 viewer (Robinson *et al*., 2011). DNA sequence data were deposited in the DNA Data Bank of Japan

(http://www.ddbj.nig.ac.jp/). Accession number is DRA005170.

Construction of nnk1-35::kan^R mutation on the chromosome - To construct the *nnk1-35::kan^R* mutation on the chromosome, Km-resistant marker was inserted at just downstream of the designed Glu-744 to termination codon in *nnk1$^+$* gene by the methods described (Krawchuk and Wahls, 1999). For this construction, both the upstream and downstream regions of the desired *nnk1$^+$* region were PCR-amplified by using F1 and F2 primers and R1 and R2 primers, respectively, and both fragments were purified. After mixing both DNA fragments with pFA6a-kanMX6, a PCR reaction was performed with the F1 and R1 primers. JY333 (*h$^-$ leu1-32 ade6-M216*) was transformed with the amplified DNA fragment, and stable G418-resisitant transformants were selected. Then, the construct on the chromosome was confirmed by PCR using appropriate primers and sequencing. The primers used were: F1, TGCTGATATGTGGTCTACCG; F2, TTAATTAACCCGGGGATCCGTTATTGGACAAAGGGAAGATTTAAAGC; R1, CTAGCAAAGGGGCAACAAAC; and R2, GTTTAAACGAGCTCGAATTCCTGTCGATGATTGTACATGC.

Assay of glucose concentration- Cells were grown in SD medium. In addition to monitoring cell growth, 20 µl of culture was sampled to determine the remaining glucose concentrations in medium using a Glucose CII-test kit (Wako Co., Ltd).

Results and Discussion

Identification of a long-lived mutant that has extended chronological lifespan. We previously screened for some long-lived mutant *S. pombe* candidates (Ito *et al.*, 2010). In this study, we analyzed one uncharacterized mutant, named L35 among them. First we analyzed the long-lived phenotype of L35 (Fig.1A). Wild type cells decreased its viability to 1/1000 within 3 days incubation in SD medium. On the other hand, the L35 mutant kept its viability for a long period compared to wild type cell. It

should be noted that the calculated doubling time at logarithmic growth phase of L35 and JY333 were almost identical, that is 197 min and 194 min respectively, under growth condition we used.

Next to identify the causative mutation in L35 mutant, whole genome sequences of the L35 mutant and parental wild type strain, JY333 were determined. We identified one nonsense (GAA to TAA) mutation that causes a Glu-744 to termination codon change in the predicted Nnk1 protein (Fig. 2A). Because Nnk1 is as an essential serine/threonine protein kinase that encoded by *SPCC70.05c* gene, the *nnk1-35* mutation might be a partial loss of function mutation. The orthologous protein, Nnk1p in *Saccharomyces cerevisiae* is described as that implicated in proteasome function, interacts with TORC1, Ure2 and Gdh2, and overexpression leads to the hypersensitivity to rapamycin and nuclear accumulation on Gln3 (in *Saccharomyces* genome database: http://www.yeastgenome.org/). However, no functional characterization of Nnk1 was carried out to date in *S. pombe*.

Next, we confirmed that the identified mutation (designated as *nnk1-35* allele) was the causative mutation for the long-lived phenotype of the L35 mutant, as follows. First, after crossing the L35 mutant with a wild type strain, progenies were obtained. From them we randomly selected both long-lived cells (n = 5) and non-long-lived cells (n = 5) as described (Ito *et al.*, 2010), and sequenced their chromosomal regions corresponding to the *nnk1-35* mutation. We confirmed that all long-lived cells and non-long-lived cells had the *nnk1-35* mutation and wild type *nnk1$^+$* allele, respectively. Representative sequencing data of each cell were shown in Fig. 1B. Second, the identified GAA to TAA mutation that caused a Glu-744 to termination codon change was introduced into the wild type *nnk1$^+$* gene on the chromosome. The constructed *nnk1-35::kan^R* mutant showed the same long-lived phenotype as found in the original L35 mutant (Fig. 2A and 2B). Based on these results we concluded that *nnk1-35* is the causative mutation for long-lived phenotype in L35 mutant.

Wild type *nnk1$^+$* gene was cloned on plasmid and resultant plasmid, pNnk1, was introduced into *nnk1-35::kan^R* mutant to confirm the phenotype. As shown in Fig. 2C, the long-lived phenotype of *nnk1-35::kan^R* mutant was complemented by the introduction of pNnk1. This indicated that the *nnk1-35* mutation is recessive.

Phenotypic characterization of nnk1-35 mutant. To find any other phenotypes in *nnk1-35* mutant, hereafter we used the *nnk1-35::kan*^{R} mutant and analyzed its growth on several conditions as follows. In yeast, it has been reported that chronological long-lived mutants exhibit oxidative stress-resistant phenotype (Fabrizio and Longo, 2003; Roux *et al*., 2006; Ito *et al.*, 2010). Therefore, we first analyzed the phenotype. The *nnk1-35::kan*^{R} mutant cells were more resistant to oxidative stress (1mM or 3mM H_2O_2) than wild type cells (Fig. 3A). The phenotype was also found in Δ*ctt1* mutant background lacking catalase. That is, the Δ*ctt1 nnk1-35::kan*^{R} double mutant was more sensitive to hydrogen peroxide than *nnk1-35::kan*^{R} mutant. These results suggested that *nnk1-35::kan*^{R} mutation render cells hydrogen peroxide resistant in the *ctt1*$^+$ independent manner. Second, the *nnk1-35::kan*^{R} mutant showed caffeine sensitive phenotype (Fig. 3B). Third, the mutant showed NaCl resistant phenotype (Fig. 3C). At present we do not know the reason of these phenotype, however change in sensitivity to caffeine and salt is often associated with defects in the protein kinase C (PKC)-mitogen activated protein kinase pathway or TOR pathway in *S. cerevisiae and S. pombe* (Weisman and Choder, 2001; Reinke *et al*., 2006; Takarhara and Maeda, 2012). Nnk1 might be involved in these pathway and such analysis is waiting in the future.

The expression of ght5*$^+$*, gene encoding a major glucose transporter was decreased in nnk1-35 mutant. To investigate how the *nnk1-35::kan*^{R} mutant shows long-lived phenotype, we analyzed cell viability in a glucose-limiting medium. The analysis of viability in a glucose-limiting medium is known as a calorie-restriction experiment, and calorie restriction is a condition that extends lifespan in a variety of species (Guarente and Picard, 2005; North and Sinclair, 2007; Roux *et al*., 2009; Azuma *et al*., 2012; Takuma *et al.*, 2013). As shown in Fig. 4A, calorie restriction extended the chronological lifespan of wild type cells but not *nnk1-35::kan*^{R} mutant. This suggested that the *nnk1-35::kan*^{R} mutant has any defects in glucose utilization. Saitoh *et al*. (2015) analyzed eight hexose transporter and showed evidence indicating that the Ght5 is a major glucose transporter in *S. pombe*. Then we

analyzed the expression of *ght5*$^+$ gene in the *nnk1-35::kan*^{R} mutant. As shown in Fig. 4B, the expression of *ght5*$^+$ was low, about one fifth of wild type cell, in the *nnk1-35::kan*^{R} mutant. This suggested that the glucose uptake was impaired in the mutant, so we compared the consumption of glucose along cell growth between wild-type and *nnk1-35::kan*^{R} mutant (Fig. 4C). Although both wild-type and *nnk1-35::kan*^{R} mutant showed the essentially same growth in SD medium, the *nnk1-35::kan*^{R} mutant consumed less glucose compared with wild type cells. The difference in glucose consumption is likely to be caused by the difference in the expression of *ght5*$^+$ gene.

Based on these results, we propose the following scenario explaining the long-lived phenotype of *nnk1-35::kan*^{R} mutant. In *nnk1-35::kan*^{R} mutant, the reduced expression of *ght5*$^+$ mRNA causes any defect in glucose uptake. This might cause physiological changes that are equivalent to the changes caused by calorie restriction. To clarify this assumption, we made Δ*ght5* mutant and it lifespan was analyzed (Fig. 4D). The mutant showed long-lived phenotype that was nearly equal to the lifespan of wild type cells under caloric restriction (Fig. 4D). This supports the idea mentioned above.

Saito *et al.* (2015) have reported eight hexose transporters including Ght5, and mentioned that Ght1, Ght2, Ght5, and Ght8 might be involved in glucose transport. Then we analyzed the expression of other transporter genes in *nnk1-35::kan*^{R} mutant (supplemental data). The expressions of *ght1*$^+$, *ght6*$^+$, and *ght8*$^+$ genes were decreased in the *nnk1-35::kan*^{R} mutant indicating these genes were also under the control of *nnk1*$^+$ gene. Because we have not yet characterized the phenotype of these mutants, we cannot exclude that possibility that these hexose transporters might be involved in the phenotype of *nnk1-35::kan*^{R} mutant. Nnk1 could have several targets and thus its mutation causes pleiotropic effects, including a reduction in glucose consumption.

In summary, we identified uncharacterized kinase Nnk1 as a regulator of a major glucose transporter gene *ght5*$^+$ and chronological lifespan in *S. pombe*. This is the first evidence showing a link between Nnk1 and lifespan in *S. pombe*. Identification of the target of the Nnk1 kinase and the regulation of Nnk1 await future investigations.

Acknowledgements

We thank H. Murakami (Chuo University) for helpful discussions, K. Kato (our laboratory) for technical assistance, and S. Saitoh (Kurume University) for materials. This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan. Part of this work was also supported by Institute for Fermentation, Osaka and The Asahi Glass Foundation [to HA]. Authors have no conflict of interest to declare.

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Figure Legends

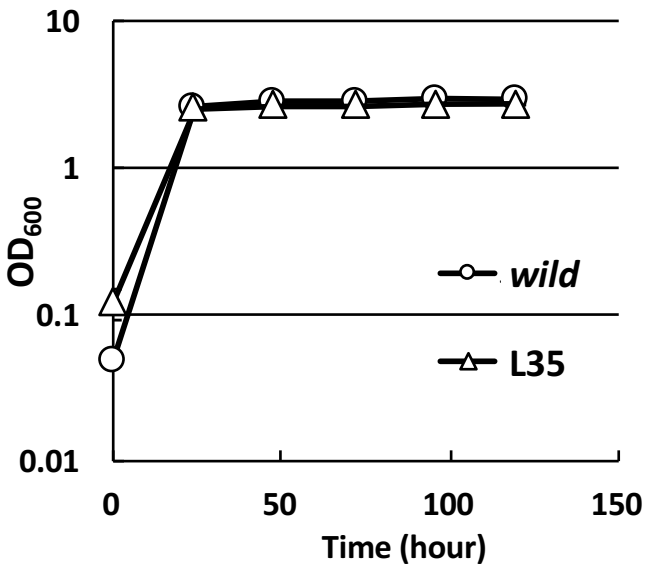
Fig. 1. Long-lived phenotype of L35 mutant that increases cell viability after entry into the stationary phase. A. Cell growth (left panel) and cell viability (right panel) of wild type cells (open circles) and L35 mutant (open triangles) in SD medium were monitored. Data shown represent the average of three independent experiments ±s.d.. B. Sequencing data corresponding to the identified mutation locus in *nnk1*$^{+}$ gene. The representative data for a parental strain, a long-lived progeny, and a normal-lived progeny after crossing were shown. The region corresponding to the *nnk1-35* mutation site was shown with shade. Note that the original GAA to TAA mutation that caused a Glu-744 to termination codon in Nnk1 protein was shown as C to A mutation in this data for complementary strand sequencing.

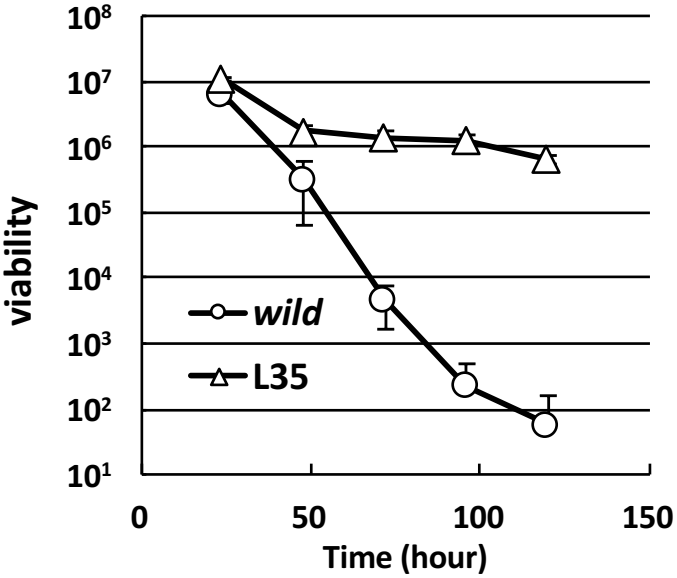
Fig. 2. *nnk1-35* mutation found in L35 mutant causes a long-lived phenotype. A. *nnk1-35* mutation found in L35 mutant and constructed *nnk1-35::kan*^{R} mutant are schematically shown. B. Cell growth (left panel) and cell viability (right panel) of wild type cells (open circles), L35 mutant (open triangles), and *nnk1-35::kan*^{R} mutant (open rectangles) in SD medium were monitored. Data shown represent the average of three independent experiments ±s.d.. C. Wild type (JY333) and *nnk1-35::kan*^{R} mutant cells were transformed with vector plasmid (pLB-Dblet) or pNnk1 plasmid that carries *nnk1*$^{+}$ gene. The cells were cultured in SD liquid medium for one day (Day1) and 3 days (Day3) and then spotted on YE plate after serial dilutions to analyze their viability. The plates were incubated for 4 days at 30 °C and photographed.

Fig. 3. Other phenotypes found in *nnk1-35::kan*^{R} mutant. A. The cells indicated were spotted on SD plate or SD plate containing 1 mM or 3 mM H_2O_2 with serial dilution. B. Wild type cells, L35 mutant, and independently isolated *nnk1-35::kan*^{R} mutants were spotted on YE plate or YE plate containing 10 mM caffeine. C. Wild type cells and *nnk1-35::kan*^{R} mutant were spotted on YE plate or YE plate containing 0.3 M NaCl with serial dilution. The plates were incubated for several days at 30 °C and photographed.

Fig. 4. *nnk1-35::kan*^{R} mutant has defect in both the expression of *ght5*$^{+}$ gene and glucose consumption. A. Cell growth (left panel) and cell viability (right panel) of the wild type cells grown in SD medium (open circles) and SD low glucose medium (open triangles), and *nnk1-35::kan*^{R} mutant grown in SD medium (closed circles) and SD low glucose medium (closed triangles) were shown. B. The amount of *ght5*$^{+}$ mRNA expressed in wild type cells or *nnk1-35::kan*^{R} mutant were analyzed by real time PCR analysis. Data shown represent the average of three independent experiments ±s.d. Statistical analyses were done with the Student's t-test (indicated as $P < 0.01$). C. Wild type (open circles) and *nnk1-35::kan*^{R} mutant (open triangles) cells were grown in SD medium at 30°C and cell growth (upper panel) and the glucose concentrations in the medium (lower panel) were analyzed. D. Cell growth (upper panel) and cell viability (lower panel) of the wild type cells grown in SD medium (open circles) and SD low glucose medium (open rectangles), and *ght5*Δ mutant grown in SD medium (open triangles) were shown. Data shown in panels A, C and D represent the average of three independent experiments ±s.d.. The standard deviations are too small to note on these graphs.

A





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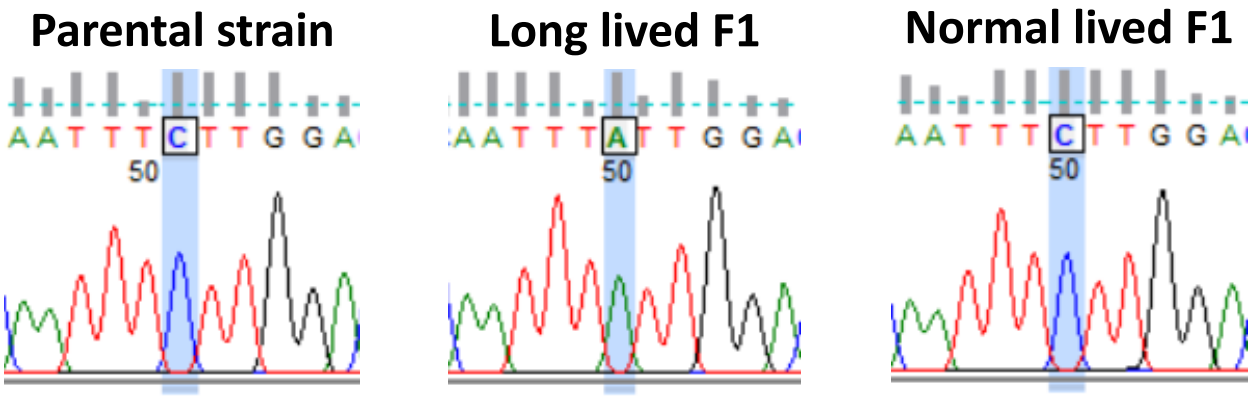
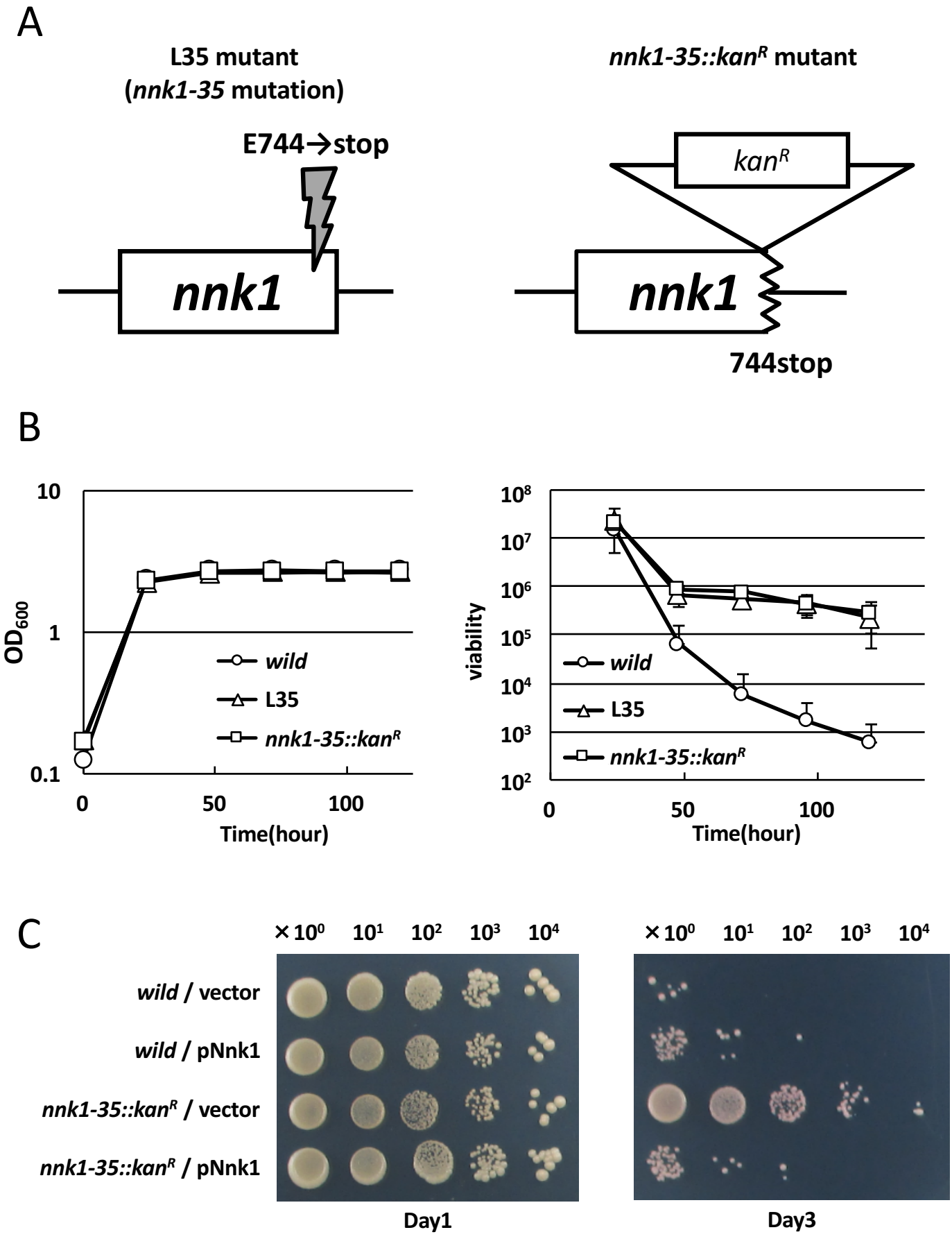


Kurauchi *et al*. Fig. 1



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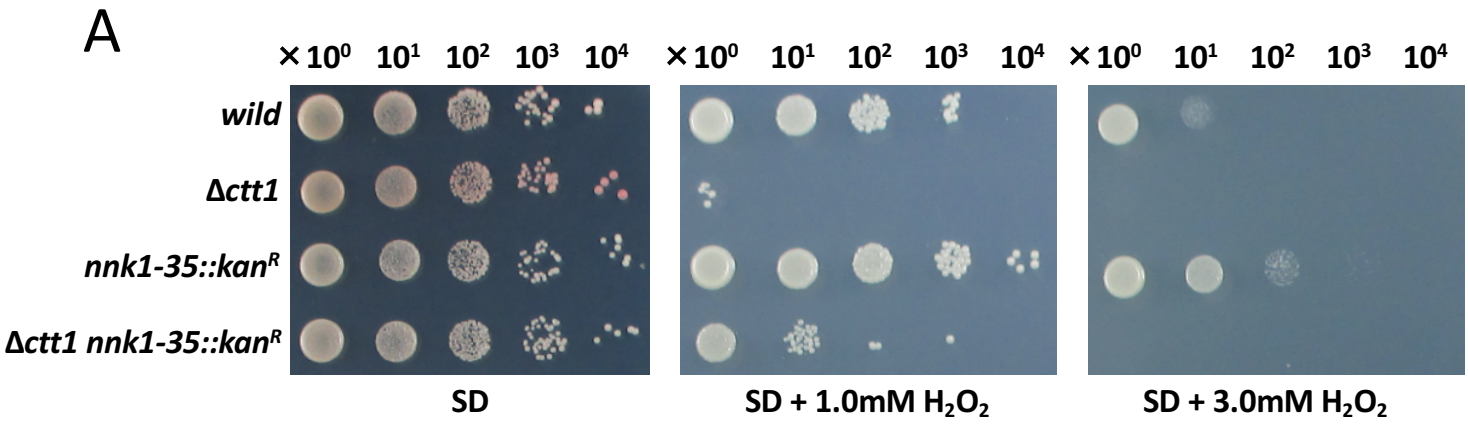

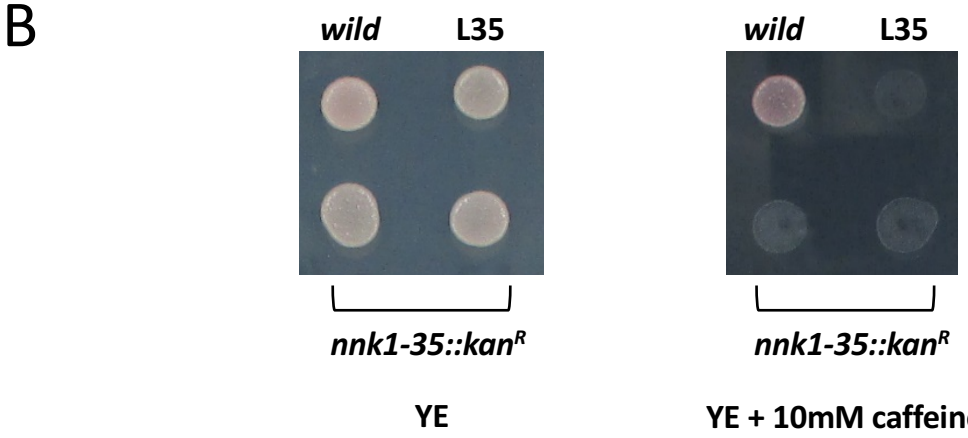

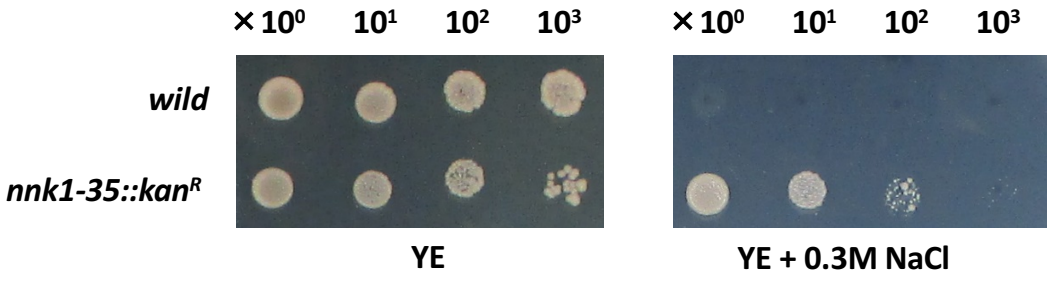

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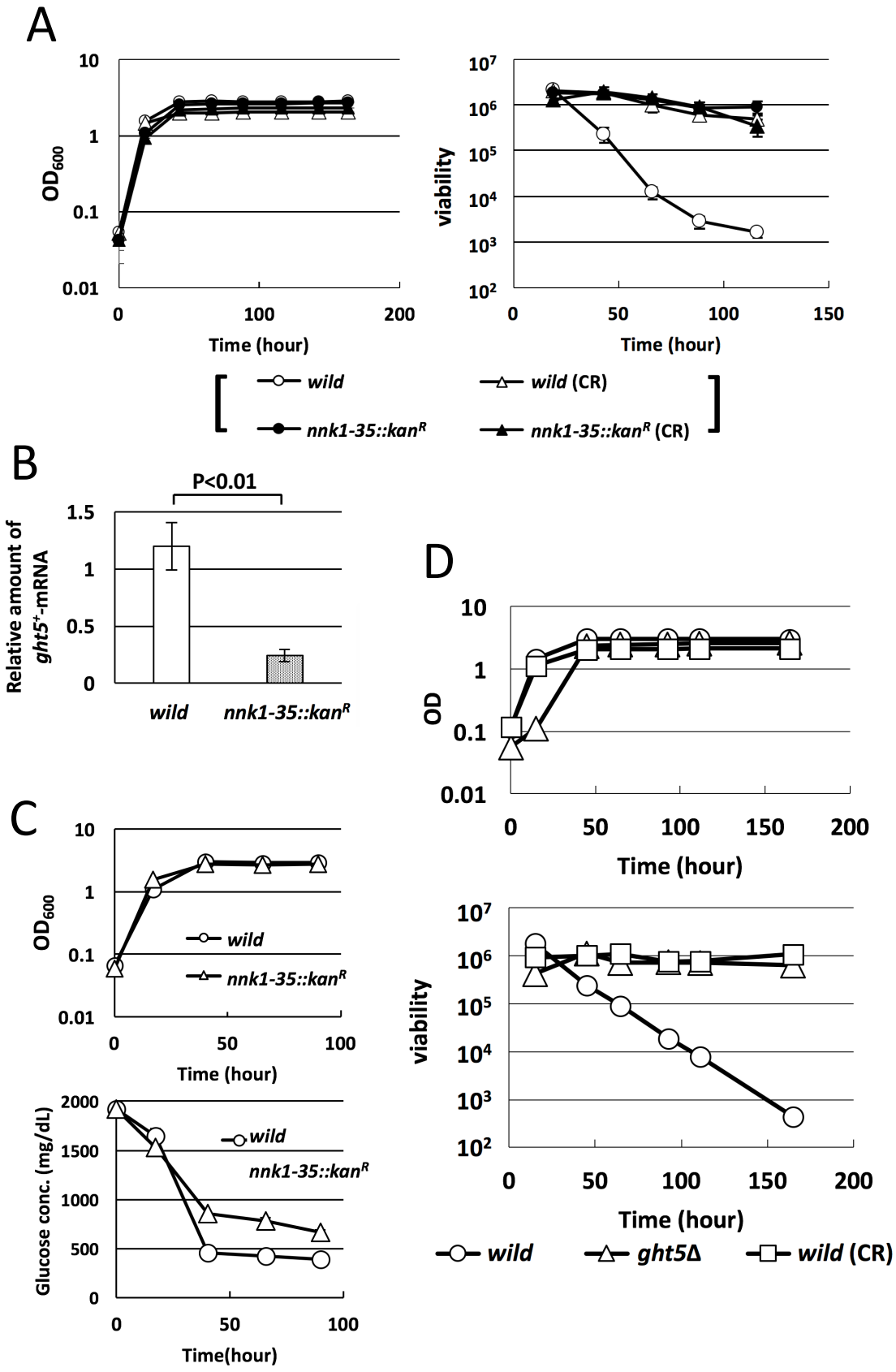

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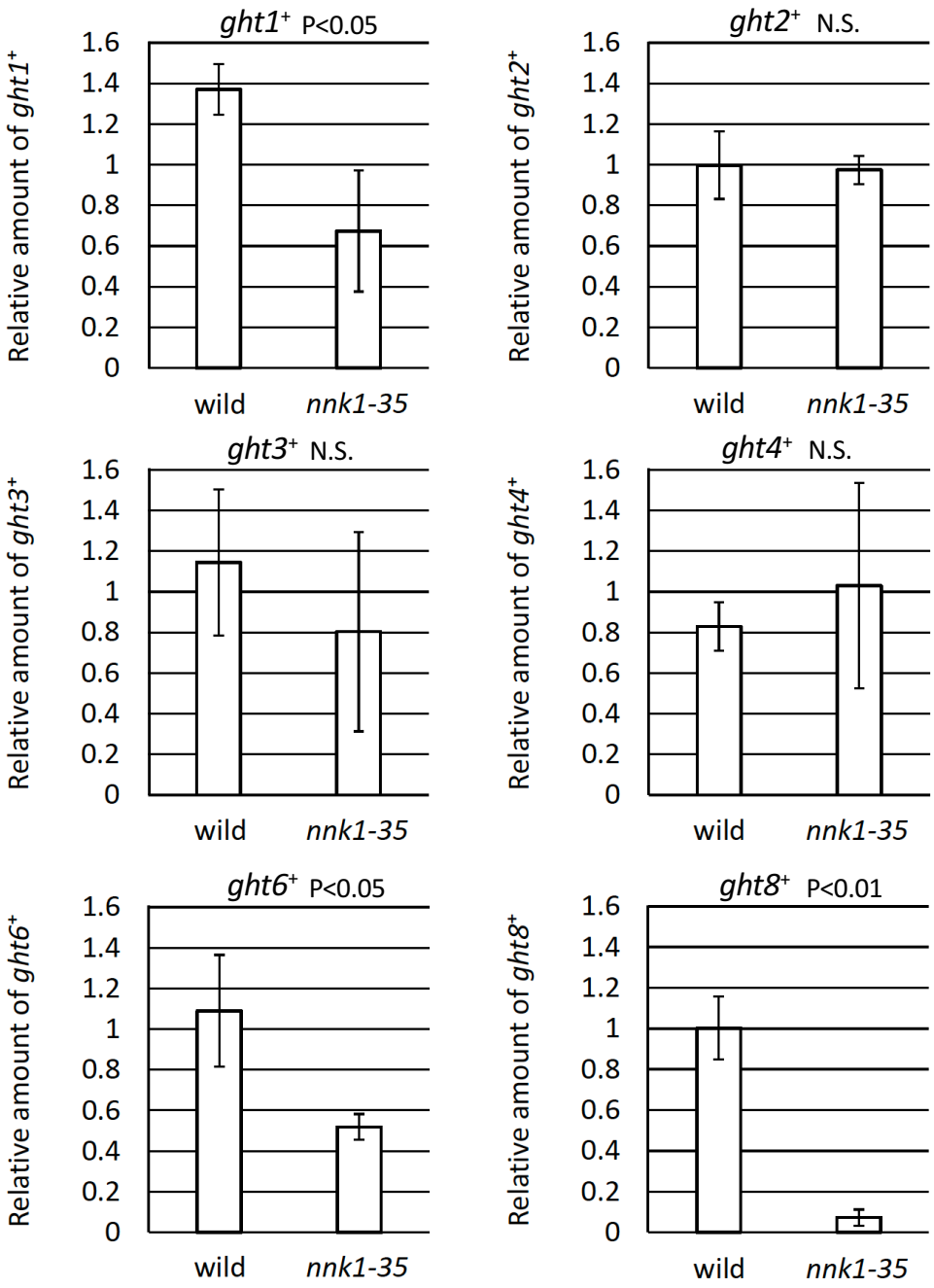

Supplemental Data. The amount of hexose transporter (*ght1*$^+$ to *ght8*$^+$) mRNAs expressed in wild type cells and *nnk1-35::kan*^{R} mutant were analyzed by real time PCR analysis. Data shown represent the average of three independent experiments ±s.d. Statistical analyses were done with the Student's t-test (indicated as P<0.05, P < 0.01, or nonsignificant (N.S.)). The expression of *ght7*$^+$ mRNA was not detected probably for its low expression (Saitoh *et al*. 2015)