

Analyses of the Physical Interactions of Yeast Alg5p with Dolichyl-Phosphate-Related Enzymes in the Dolichol Cycle

by

SAMANTHA^{*1}, Nanami ADACHI^{*2} and Tetsuo TAKAHASHI^{*3}

(Received on Mar. 17, 2025 and accepted on Jun. 20, 2025)

Abstract

In the dolichol cycle for assembling dolichol-linked oligosaccharide (DLO), yeast dolichyl-phosphate-glucose synthase (DPGS), Alg5p, produces dolichyl-phosphate-glucose (DPG). Previously we investigated the physical interaction of Alg5p and demonstrated that it physically interacts with Dpm1p, Sec59p, Cwh8p and Alg7p, all of which are involved in the dolichol cycle. In this study, we further investigated the physical interactions of Alg5p with Dpm1p, Sec59p and Cwh8p in detail and successfully refined regions essential for each interaction on Alg5p. Our results strongly suggest that the physical interactions of Alg5p might occur on both *N*- and *C*-terminal portions. Namely, it was considered that the *N*-terminal portion of Alg5 is crucial for the interaction with Dpm1p, while the *C*-terminal portion is critical for the interaction with Sec59p and Cwh8p in addition to Alg7p and Alg5p itself.

Keywords: Dolichyl-phosphate-glucose synthase, Physical interaction, Split-ubiquitin system

1. Introduction

The assembly of dolichol-linked oligosaccharides (DLOs) on the rough endoplasmic reticulum (rER) membrane is conducted by a biosynthetic pathway known as dolichol cycle, which is essential for the protein *N*-glycosylation in yeast¹⁻⁵). In the cycle, dolichyl-phosphate-glucose (DPG) is required for three luminal glucosyltransferases (Alg6p, Alg8p and Alg10p) as a donor substrate, and provided by DPG synthase (DPGS)⁶). Yeast Alg5p protein is the DPGS that was firstly identified and characterized in eukaryotes⁷⁾⁸).

The yeast split-ubiquitin system (YSUS) is a technique which was specially devised for detection of physical interaction between two membrane proteins⁹⁻¹²). By using the YSUS, many interactions of membrane proteins have been successfully uncovered so far¹³⁻²¹). By using it, we have previously investigated the membrane topology of Alg5p and demonstrated that it had two transmembrane domains with both termini oriented toward the cytoplasmic side of the rER membrane^{22),23}). We also investigated the physical interaction of Alg5p and detected its interaction with itself and four dolichyl-phosphate (Dol-P)-related enzymes (Dpm1p, Sec59p, Cwh8p and Alg7p) in the dolichol cycle^{22),23}). Dpm1p is dolichyl-phosphate-mannose (DPM) synthase (DPMS), which shares Dol-P with Alg5p as an acceptor substrate²⁴⁾²⁵). Alg7p is GlcNAc-1-P transferase (GPT), which also utilizes Dol-P as an acceptor substrate²⁶). Sec59p is dolichol kinase (DK), which *de novo* produces Dol-P²⁷). Cwh8p is dolichyl-

pyrophosphate phosphatase (DPP), which also produces Dol-P^{28),29}). In addition, we refined the regions which are required for the self-interaction of Alg5p and interaction with Alg7p by using several truncated Alg5p baits in the previous reserach^{22),23}). Therefore, using novel truncated baits additionally, we also tried to refine regions on Alg5p, which are important for the interactions with Dpm1p, Sec59p and Cwh8p.

2. Experimental Methods

2.1 Construction of recombinant plasmids for expression of truncated Alg5p baits

In our previous study, we prepared five bait recombinant plasmids (bait constructs) for expression of truncated Alg5p²³). One of them, pBT-C-ALG5HR2 was again used in this study (Fig. 1). In addition, six novel bait constructs were prepared as follows. PCRs were performed using forward primer AG5_fw and each of reverse primers (AG5HR1_rv, AG5LR2_rv, AG5HR2_rv and AG5LR3_rv) by standard method³⁰) and then amplified DNA fragments were cloned into the pBT-N vector to generate pBT-N-ALG5HR1, pBT-N-ALG5LR2, pBT-N-ALG5HR2 and pBT-N-ALG5LR2, respectively (Fig. 1). In addition, PCRs were performed using forward primer (AG5ΔN10_BPfw or AG5ΔN10_BPf) and reverse primer AG5_rv by standard method³⁰) and then amplified DNA fragments were cloned into the pBT-C vector to generate pBT-C-ALG5ΔN10 and pBT-C-ALG5ΔN33 bait constructs, respectively (Fig. 1). PCR primers are listed in Table 1 and DNA cloning was conducted according to the same procedure as previously described^{31),32}).

*1 Graduate Student, Course of Applied Science and Chemistry

*2 Undergraduate Student, Department of Bioengineering

*3 Associate Professor, Department of Bioengineering

Table I The PCR primers used in this study. Additional sequences containing a *Sfi* I- cleavage site are shown in lowercase letters.

Primer name	Nucleotide sequence	Purpose
AG5_fw ²²⁾	5'-tgtaatggccattacggccATGAGAGCGTTGAGATTCCTGATTG-3'	Cloning of C-terminally truncated <i>ALG5</i>
AG5_rv ²²⁾	5'-gcctttggccgagggcgccCATTTCTTATTATCTCTATATATCCCTAA-3'	Cloning of N-terminally truncated <i>ALG5</i>
AG5HR1_rv ²³⁾	5'-gcctttggccgagggcgccCGAAAATAAATAGACCAATAAATACAGTG-3'	Cloning of <i>ALG5</i> from N-terminus to HR1
AG5LR2_rv ²³⁾	5'-gcctttggccgagggcgccgaGTTTCTTATCATTGATCTCTTTATGACG-3'	Cloning of <i>ALG5</i> from N-terminus to LR2
AG5HR2_rv ²³⁾	5'-gcctttggccgagggcgccgaTGTGTCTTTGATAGACCTGATGCCG-3'	Cloning of <i>ALG5</i> from N-terminus to HR2
AG5LR3_rv ²³⁾	5'-gcctttggccgagggcgccGCCATCTTAGAGCCATCAACCTC-3'	Cloning of <i>ALG5</i> from N-terminus to LR3
AG5ΔN10_BPfw	5'-tgtaatggccattacggccatgAGAAACACTGTCTTTTTTACGCTCTTAG-3'	Cloning of <i>ALG5</i> from HR1 to C-terminus
AG5ΔN33_BPfw	5'-tgtaatggccattacggccatgTCGCATACACCAAGGCCGCCATATC-3'	Cloning of <i>ALG5</i> from LR2 to C-terminus

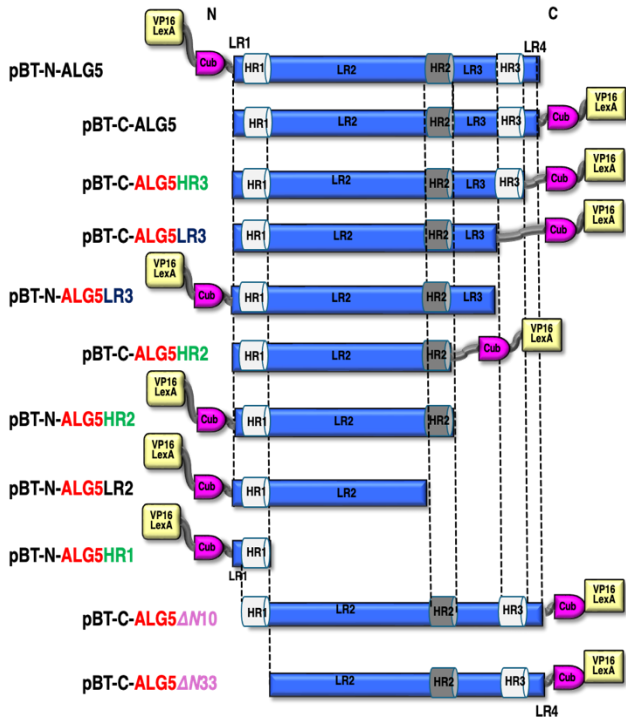


Fig. 1 Bait constructs used in previous and present studies. In structure of expressed Alg5p, bars and boxes indicate loop regions (LRs) and hydrophobic regions (HRs), respectively. Transcription factor VP16/LexA and Cub (C-terminal half of ubiquitin) are added at either N- or C- terminus of Alg5p.

2.2 Assays for the physical interaction of Alg5p

In our previous study, three prey recombinant plasmids (prey constructs), pPR-N-DPM1, pPR-N-SEC59 and pPR-N-CWH8, were prepared for expression of full-length Dpm1p, Sec59p and Cwh8p, respectively²³⁾.

Each of the six novel bait constructs mentioned above was combined with each of the three prey constructs, then they were used for co-transformation of yeast NMY51 strain³³⁾. After the co-transformation, the co-transformants grown on the synthetic dextrose (SD) medium lacking leucine and tryptophan (SD-LW) were subject to growth examination on the SD medium lacking leucine, tryptophan and histidine

(SD-LWH) and SD medium lacking leucine, tryptophan, histidine and adenine (SD-LWHA) according to the manual supplied by Dualsystems Biotech (www.dualsystems.com).

3. Results and Discussion

3.1 The physical interaction of truncated Alg5p with Sec59p and Cwh8p

First, we examined growth of co-transformants with pBT-N-ALG5LR3 bait construct (Fig. 1), which would express N-terminally tagged Alg5/LR3p lacking the third hydrophobic region (HR3) and the fourth loop region (LR4). In our previous analysis, it has been demonstrated that C-terminally tagged Alg5/LR3p could physically interact with Dpm1p, Sec59p and Cwh8p preys²³⁾. As shown in Fig. 2A, co-transformants expressing Dpm1p and Cwh8p normally grew on two selective media (two red frames), but those expressing Sec59p displayed reduced growth on two selective media (a green frame). This result indicated that HR3 of Alg5p was at least partially related to the physical interaction with Sec59p.

Next, we examined growth of co-transformants with pBT-C-ALG5HR2 bait construct (Fig. 1), which would express C-terminally tagged Alg5/HR2p lacking the third loop region (LR3), HR3 and LR4. As shown by a red frame in Fig. 2B, co-transformants expressing Dpm1p were able to grow on two selective media (a red frame), but those expressing Sec59p or Cwh8p were not (a blue frame). Subsequently we examined growth of co-transformants with pBT-N-ALG5HR2 bait construct (Fig. 1), which would express N-terminally tagged Alg5/HR2p. As a result, co-transformants expressing Dpm1p or Cwhp1p were able to grow on two selective media (two red frames in Fig. 2C), while those expressing Sec59p were not (a blue frame in Fig. 2C). These observations clearly indicated that LR3 of Alg5p was critical for the interaction with Sec59p and partially contributed to the interaction with Cwh8p.

Co-transformants expressing Cwh8p with N-terminally tagged Alg5HR2p survived on the selective media (a lower red frame in Fig. 2C), but those with N-terminally tagged Alg5LR2p lacking HR2 seldom survived (a blue frame in Fig.

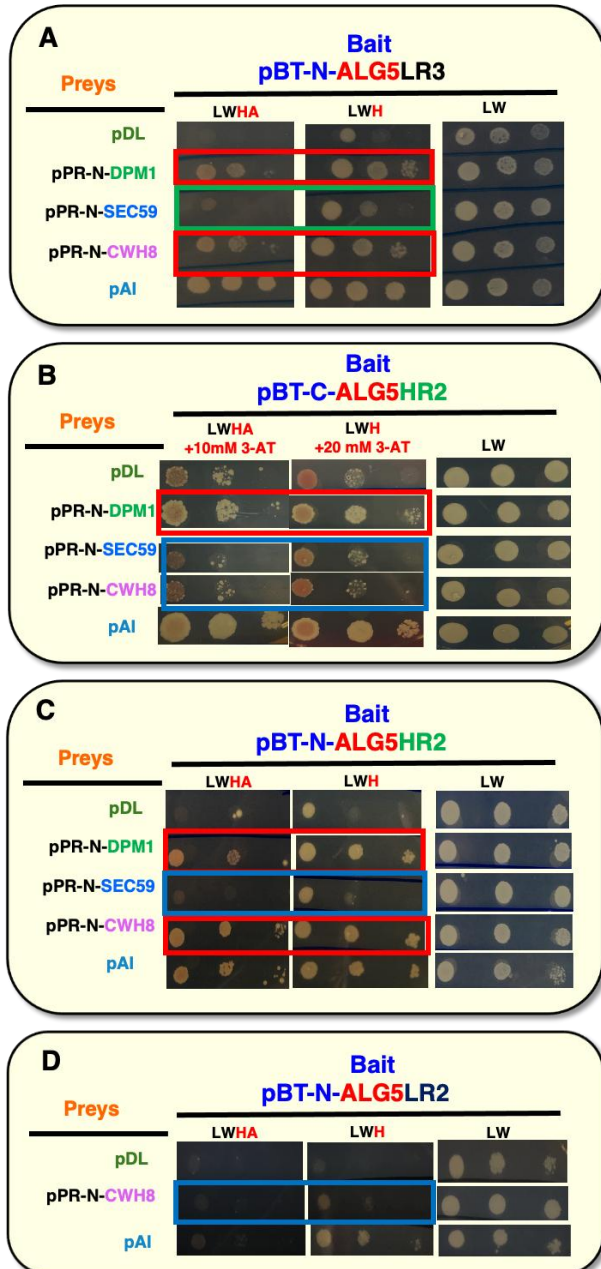


Fig. 2 Growth examination of co-transformants using pBT-N-ALG5LR3 (A), pBT-C-ALG5HR2 (B), pBT-N-ALG5HR2 (C) and pBT-N-ALG5LR2 (D) bait constructs. After the selection of colonies derived from the co-transformants on the SD-LW medium, their suspensions were diluted with sterilized water and adjusted to the OD₆₀₀ values of 1.0, 0.1 and 0.01 (from left to right). These diluents were orderly spotted on the SD-LWH and SD-LWHA media for reporter detection, and SD-LW media for growth control, and then incubated at 30 °C for 2–4 days.

Alg5LR2p lacking HR2 seldom survived (a blue frame in Fig.2D). This result means that HR2 of Alg5p would be also required for the interaction with Cwh8p, in addition to LR3. In contrast, LR3 should make no contribution to the interaction with Dpm1p (Fig. 2A and 2B).

3.2 The physical interaction of truncated Dpm1p

In order to further refine a region interacting with Dpm1p,

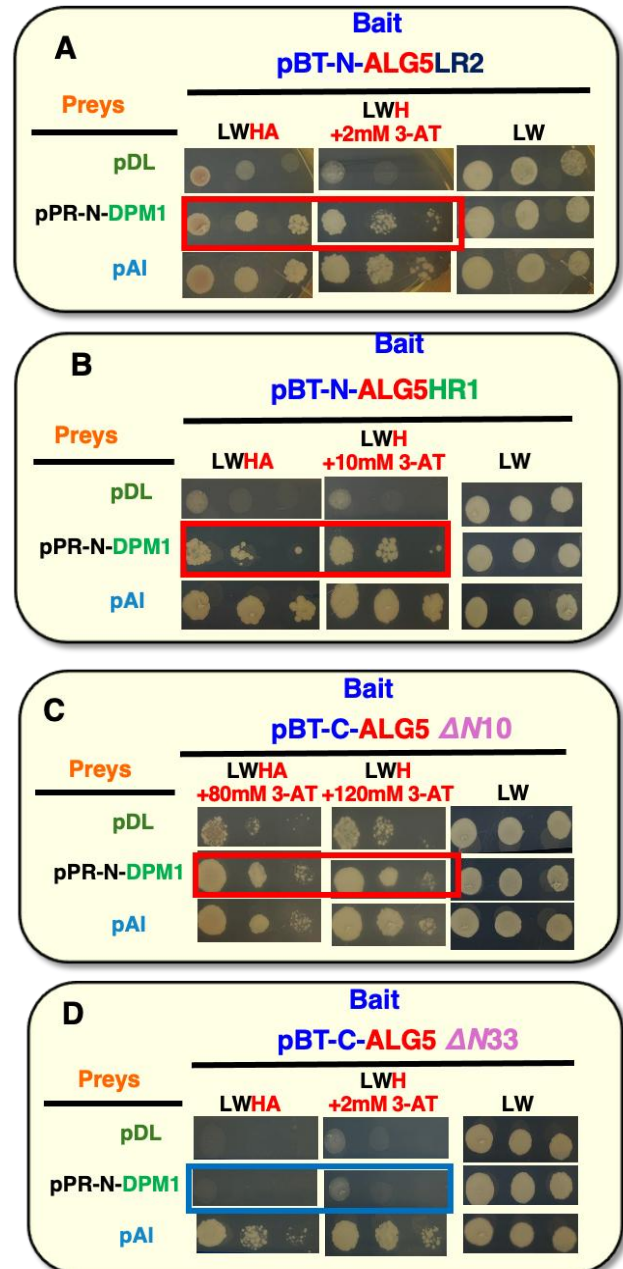


Fig. 3 Growth examination of co-transformants using pBT-N-ALG5LR2 (A), pBT-N-ALG5HR1 (B), pBT-C-ALG5ΔN10 (C) and pBT-C-ALG5ΔN33 (D). The experiments were carried out according to the same procedure as described in the legend of Fig. 2.

we examined growth of co-transformants with novel bait constructs (pBT-N-ALG5LR2 and pBT-N-ALG5HR1) which would express the more truncated Alg5p. For analyzing the membrane topology of Alg5p, we have previously prepared pBT-C-ALG5LR2 and pBT-C-ALG5HR1 bait constructs which would express C-terminally tagged Alg5/LR2p ranging from N-terminus to the second loop region (LR2) and C-terminally tagged Alg5/HR1p ranging from N-terminus to the first hydrophobic region (HR1), respectively²³. However, as it was demonstrated that LexA/VP16-Cub tag in these truncated Alg5p proteins was localized within the rER lumen²³, these bait constructs were proved to be unavailable

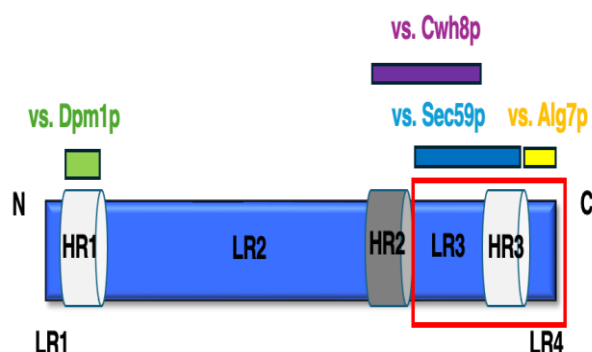


Fig. 4 Summary of interacting regions of Alg5p. A red frame indicates a region that are critical of self-interaction of Alg5p. The interacting regions with Alg5p itself and Alg7p are based on the results of Ref. 23.

for analysis of the physical interaction. Therefore, we newly prepared and used pBT-N-ALG5LR2 and pBT-N-ALG5HR1 bait constructs (Fig. 1) which would express *N*-terminally tagged Alg5/LR2p and *N*-terminally tagged Alg5/HR1p, where LexA/VP16-Cub tags are oriented toward the cytosolic side, respectively. These co-transformants expressing Dpm1p were still able to grow on two selective media (red frames in Fig. 3A and 3B), suggesting that Alg5p should physically interact with Dpm1p via an *N*-terminal portion.

Hence, we tried two additional bait constructs (pBT-C-ALG5 Δ N10 and pBT-C-ALG5 Δ N33) which would express *N*-terminally truncated Alg5p lacking the first 10 and 33 amino acids, respectively (Fig. 1). Because the first loop region (LR1) consists of the first 10 amino acids, and HR1 comprises the following 23 amino acids. As a result of growth examination, co-transformants with pBT-C-ALG5 Δ N10 could still grow on two selective media (a red frame in Fig. 3C), but those with pBT-C-ALG5 Δ N33 could not (a blue frame in Fig. 3D). These findings obviously mean that LR1 of Alg5p is not required for the interaction and that HR1 is necessary and sufficient.

4. Conclusion

In previous study, we detected the physical interactions of Alg5p with Alg5p itself, Dpm1p, Sec59p, Cwh8p. Moreover, we refined Alg5p regions interacting with Alg5p and Alg7p, but unsuccessfully refined those with Sec59p, Cwh8p and Dpm1p. Here, we could achieve the refinement of regions interacting with these three enzymes. Our data obtained from previous and present studies are summarized in Fig. 4.

In the late stages of DLO assembly, both DPM and DPG, which are provided by Dpm1p and Alg5p respectively, are essential. For efficient progression of DLO assembly, these two enzymes might physically interact with each other, and share the common substrate Dol-P in a balanced fashion. Our result that Alg5p interacts with Dpm1p via HR1 (Fig. 4) well agrees with this purpose. By our previous topological analysis, it was revealed that HR1 of Alg5p serves as a transmembrane domain²³). Therefore it is estimated that the interaction between Alg5p and Dpm1p would be rigid than

that via non-transmembrane region.

On the other hand, in order to effectively obtain Dol-P from two providing enzymes (Sec59p and Cwh8p), Alg5p might need to simultaneously interact with them via a different region other than HR1. Interestingly, our data strongly suggest that Alg5p interacts with both via overlapping LR3, which is distal to HR1 (Fig. 4). Perhaps the interactions of LR3 on Alg5p with them would occur in a reciprocal manner. In contrast, the interacting region of Alg5p with Alg7p is another LR4²³), which would not interfere with Dpm1p and Sec59p/Cwh8p (Fig. 4).

More interestingly, a self-interacting region of Alg5p extends from LR3 to LR4, which also overlaps with interacting region with Sec59p, Cwh8p and Alg7p (Fig. 4). This fact raises a possibility that complex formation would be competing among Alg5p, Sec59p, Cwh8p and Alg7p, on the rER membrane. It is estimated that Alg5p/Alg5p complex preferentially forms rather than other complexes, because the interacting region with itself is longer than others. To prove out the possibility, stoichiometric analysis of Alg5p-containing complexes would be required.

We are now progressing analysis of physical interactions between Alg5p and the four enzymes by using prey constructs which would express truncated Dpm1, Sec59p, Cwh8p or Alg7p, to refine regions on them, which are critical for interacting with Alg5p bait. Together with those results, our research should be very significant for elucidation of multiple complexes centering Alg5p on the rER membrane.

References

- 1) M. Aebl, *N*-linked protein glycosylation in the ER, Biochim. Biophys. Acta Vol.1833, pp.2430-2437 (2013).
- 2) A. D. Elbein, Inhibitors of the biosynthesis and processing of *N*-linked oligosaccharides, CRC Crit.Rev. Biochem. Vol.16, pp.21-49 (1984).
- 3) A. Herscovics and P. Orlean, Glycoprotein biosynthesis in yeast, FASEB J. Vol.7, pp.540-550 (1993).
- 4) M. A. Kukuruzinska, M. L. E. Bergh and B. J. Jackson, Protein glycosylation in yeast, Ann. Rev. Biochem. Vol.56, pp.915-944 (1987).
- 5) C. B. Hirschberg and M. D. Snider, Topography of glycosylation in the rough endoplasmic reticulum and Golgi apparatus, Annu. Rev. Biochem. Vol.56, pp.63-87 (1987).
- 6) T. Takahashi, Dolichyl-phosphate beta-glucosyltransferase (ALG5), Handbook of glycosyltransferases and related genes, 2nd edition, Springer, chapter146, pp.1649-1655 (2014).
- 7) G. Palamarczyk, R. Drake, B. Haley and W. J. Lennarz, Evidence that the synthesis of glucosylphosphodolichol in yeast involves a 35-kDa membrane protein, Proc. Natl. Acad. Sci. USA Vol.87, No.7, pp.2666-2670 (1990).
- 8) S. te Heesen, L. Lehle, A. Weissmann and M. Aebl, Isolation of the *ALG5* locus encoding the UDP-glucose: dolichyl-phosphate glucosyltransferase from *Saccharomyces cerevisiae*. Eur. J. Biochem. Vol.224, pp.71-79 (1994).

- 9) N. Johnsson and A. Varshavsky, Split ubiquitin as a sensor of protein interactions *in vivo*, Proc. Natl. Acad. Sci. USA Vol.91, No.22, pp.10340-10344 (1994).
- 10) I. Stagljar, C. Korostensky, N. Johnsson and S. te Heesen, A genetic system based on split-ubiquitin for the analysis of interactions between membrane proteins *in vivo*, Proc. Natl. Acad. Sci. USA Vol.95, pp.5187-5192 (1998).
- 11) M. Fetchko and I. Stagljar, Application of the split-ubiquitin membrane yeast two-hybrid system to investigate membrane protein interactions, Methods Vol.32, pp.349-362 (2004).
- 12) S. Thaminy, J. Miller and I. Stagljar, The split-ubiquitin membrane-based yeast two-hybrid system, Methods. Mol. Biol. Vol.261, pp.297-312 (2004).
- 13) M. Dünwald, A. Varshavsky and N. Johnsson, Detection of transient *in vivo* interaction between substrate and transporter during protein translocation into the endoplasmic reticulum, Mol. Biol. Cell Vol.10, pp.329-344 (1999).
- 14) M. J. Massaad and A. Herscovics, Interaction of the endoplasmic reticulum alpha 1,2-mannosidase Mns1p with Rer1p using the split-ubiquitin system. J. Cell Sci. Vol.114, pp.4629-4635 (2001).
- 15) W. Scheper, S. Thaminy, S. Kais, I. Stagljar and K. Römisch, Coordination of *N*-glycosylation and protein translocation across the endoplasmic reticulum membrane by Sss1 protein, J. Biol. Chem. Vol.278, No.39, pp.37998-38003 (2003).
- 16) A. Yan and W. J. Lennarz, Studies on yeast oligosaccharyl transferase subunits using the split-ubiquitin system: Topological features and *in vivo* interactions, Proc. Natl. Acad. Sci. USA Vol.102, No.20, pp.7121-7126 (2005).
- 17) T. Takahashi and X. -D. Gao, Physical interactions among human glycosyltransferases involved in dolichol-linked oligosaccharide biosynthesis, Trends in Glycoscience and Glycotechnology Vol.24, No.136, pp.65-77 (2012).
- 18) T. Takahashi, N. Yamada and N. Kurimoto, Analyses on the physical interactions of the human dolichyl-phosphate mannanose synthase, Proc. Schl. Eng. Tokai Univ., Ser. J. Vol.57, No.1, pp.5-10 (2017).
- 19) T. Takahashi and T. Takeuchi, Membrane topological characterization of the human Alg14 protein, Proc. Schl. Eng. Tokai Univ., Ser. E Vol.44, pp.1-6 (2019).
- 20) T. Takahashi, K. Nishimura, N. Maeda and R. Oshiro, Characterization of the membrane topology and physical interaction of human *N*-acetylglucosamine-1-phosphate transferase, Proc. Schl. Eng. Tokai Univ., Ser. E Vol.46, pp.1-6 (2021).
- 21) T. Takahashi, N. Yamada and R. Oshiro, Characterization of the membrane topology and physical interaction of human dolichol-phosphate-glucose synthase, Proc. Schl. Eng. Tokai Univ., Ser. E Vol.47, pp.1-6 (2022).
- 22) Samantha, M. Ismail, A. Takeshima and T. Takahashi, Construction of a novel set of control preys for the yeast split-ubiquitin system, Eng. Tokai Univ., Ser. E Vol.48, pp.1-5 (2023).
- 23) Samantha and T. Takahashi, Analyses of the membrane topology and physical interactions of yeast dolichyl-phosphate glucose synthase Alg5p, Proc. Schl. Eng. Tokai Univ., Ser. E Vol.49, pp.19-25 (2024).
- 24) P. Orlean, Dolichol phosphate mannanose synthase is required *in vivo* for glycosyl phosphatidylinositol membrane anchoring, *O*-mannosylation, and *N*-glycosylation of protein in *Saccharomyces cerevisiae*, Mol. Cell. Biol. Vol.10, pp.5796-5805 (1990).
- 25) T. Takahashi, Dolichyl-phosphate mannosyltransferase polypeptide (DPM1-3), Handbook of glycosyltransferases and related genes, 2nd edition, Springer, chapter146, pp.1637-1647 (2014).
- 26) G. Barnes, W. J. Hansen, C. L. Holcomb and J. Rine, Asparagine-linked glycosylation in *Saccharomyces cerevisiae*: genetic analysis of an early step, Mol. Cell. Biol. Vol.4, pp.2381-2388 (1984).
- 27) L. Heller, P. Orlean and W. L. Adair Jr., *Saccharomyces cerevisiae sec59* cells are deficient in dolichol kinase activity, Proc. Natl. Acad. Sci. USA Vol.89, pp.7013-7016 (1992).
- 28) M. A. A. van Berkel, M. Rieger, S. te Heesen, A. F. J. Ram, H. van den Ende, M. Aebi and F. M. Klis, The *Saccharomyces cerevisiae CWH8* gene is required for full levels of dolichol-linked oligosaccharides in the endoplasmic reticulum and efficient *N*-glycosylation, Glycobiology Vol.9, No.3, pp.243-253 (1999).
- 29) F. Fernandez, J. S. Rush, D. A. Toke, G.-soo Han, J. E. Quinn, G. M. Carman, J.-Y. Choi, D. R. Voelker, M. Aebi and C. J. Waechter, The *CWH8* gene encodes a dolichyl pyrophosphate phosphatase with a lumenally oriented active site in the endoplasmic reticulum of *Saccharomyces cerevisiae*, J. Biol. Chem. Vol.276, No.44, pp.41455-41464 (2001).
- 30) H. A. Erlich, PCR Technology, Stockton Press, pp.61-70 (1989).
- 31) H. Inoue, H. Nojima and H. Okayama, High efficiency transformation of *Escherichia coli* with plasmids, Gene Vol.96, pp.23-28 (1990).
- 32) L. Sambrook, E. F. Fritsch and T. Maniatis, Molecular Cloning: A Laboratory Manual. Second edition, Cold Spring Harbor Laboratory Press, NY, (1989).
- 33) H. Ito, Y. Fukuda, K. Murata and A. Kimura, Transformation of intact yeast cells treated with alkali cations, J. Bacteriol. Vol.153, No.1, pp.163-168 (1983).