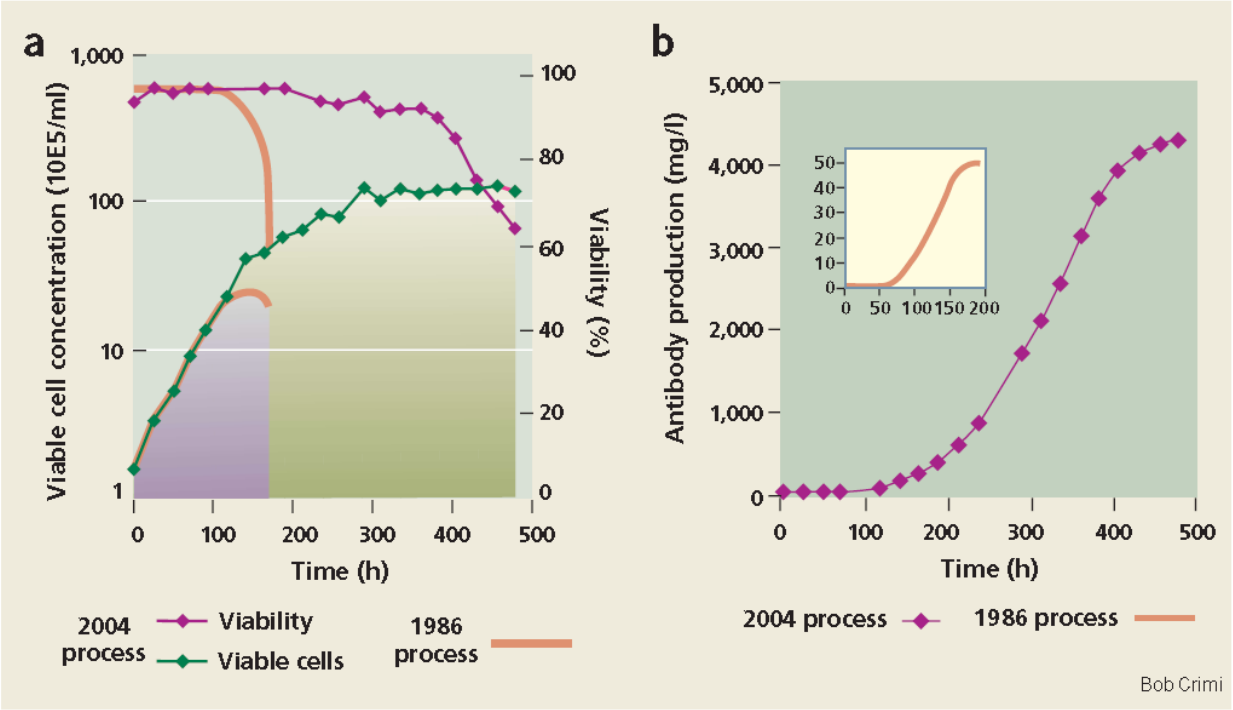
Engineering cell factories: Eukaryotes some general observations

Lecture 6

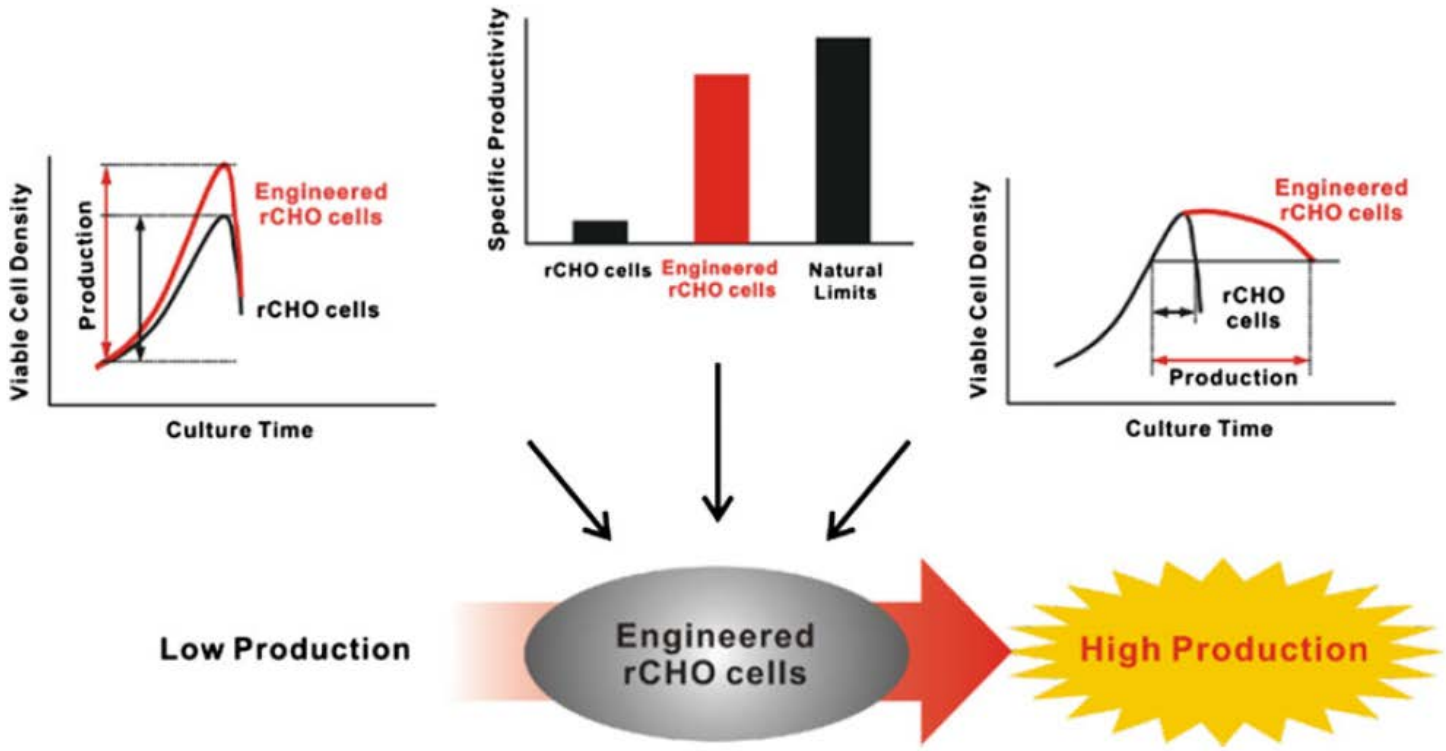
Improvement of productivity over a 20 year period

- 1986: cells density $2x10^6$ cells/ml, product titer 50 mg/l
- 2004: cells density $10x10^6$ cells/ml, product titer 4.7 g/l



Wurm, 2004, Nature Biotechnol. 22:1939-1398

Effect of cell engineering on improving culture characteristics



- Cell engineering leads to enhancement of
  - cell growth
  - specific productivity
- which leads to increased product titer

Cell engineering strategies in yeast for improving production of biopharmaceuticals

Table 1. Examples of improving biopharmaceutical production by engineering of *Saccharomyces cerevisiae*.

Protein	Category	Size (AA)	Disulfide bonds/ N-glycosylation	Production level	Host modification targets#	Production pathway affected	Ref.
Hirudin	Anticoagulants	65	3/0	1.1 g/l 36 mg/l	*PDI1+*, *ERO1+* BiP+	Disulfide bond formation Regulate unfolded protein response	[50] [49]
Human serum albumin	Blood factors	585	17/0	6 g/l	*SIL1+*, *LHS1+*, *JEM1+* and *SCJ1+*	Regulate the ATPase cycle of Kar2p	[19]
Human transferrins	Blood factors	679	19/2	2.25 g/l	*PDI1+*, *YPS1-*, *HSP150-*	Disulfide bond formation, Reduce protein degradation	[69,70]
Insulin precursor	Hormone	51	3/0	85 mg/l 19 mg/l	*SLY1+*, *SEC1+* Mutant *HSF1+*	ER to Golgi transport, exocytosis Activate heat shock response	[61] [53]
Human parathyroid hormone	Hormone	84	0/0	350 mg/l	*YPS1-*, *YPS2-*, *YPS3-*, *YPS6-*, *YPS7-*	Reduce protein degradation	[66]
Glucagon	Hormone	29	0/0		*YPS1-*	Reduce protein degradation	[63]
sHBsAg	Antigen	226	–	74.4 mg/l	*PDI1+*	Disulfide bond formation	[51]
PDGF	Human Growth Factor	241	5/1	1.5 mg/l	*PDI1+*	Disulfide bond formation	[52]

-: Deletion or down regulation; +: Overexpression or up regulation; AA: Amino acid; sHBsAg: S domain of hepatitis B virus surface antigen.

→ Protein folding

→ Protein folding

→ Protein folding & degradation

→ transport

→ Protein degradation

→ Protein degradation

→ Protein folding

→ Protein folding

Cell engineering strategies in mammalian cells for improving production of biopharmaceuticals

Gene	Mechanism of action	Effects	Reference(s)
BiP	Facilitates folding and assembly of proteins in ER	Impeded secretion of Factor VIII, von Willebrand Factor and IgG in CHO cells	[43,44]
PDI	Catalyses formation of disulfide bonds	Improved IgG secretion in CHO cells but no effect in hybridoma cells,	[36,44,45]
ERp57	Catalyses formation of disulfide bonds	Improved TPO secretion in CHO cells	[46]
CNX, CRT	Glycoprotein quality control	Improved TPO secretion in CHO cells	[48]
XBP-1	Global regulator of UPR	Varying effects on productivity of secretion of recombinant proteins in mammalian host cells. Improvement was most pronounced where secretory bottlenecks were demonstrated to have existed	[35,52–55]
Sly1/Munc18c	Regulation of membrane fusion	Improved IgG secretion in CHO cells. Synergistic effects with XBP-1S over-expression	[58]

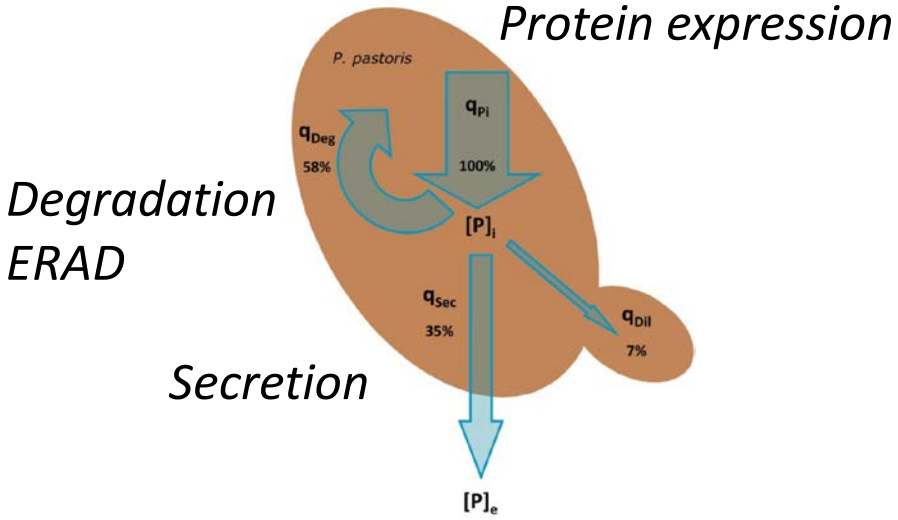
→ Protein folding & transport

Lim et al., 2010 Biotechnol. Appl. Biochem. 55:175–189

Protein folding & quality control & secretion

Production - Folding – Degradation - Secretion

- The balance between intracellular protein formation, degradation and secretion defines the major bottleneck of the production system.



Overview of the intracellular fluxes of the recombinant protein in P. pastoris.

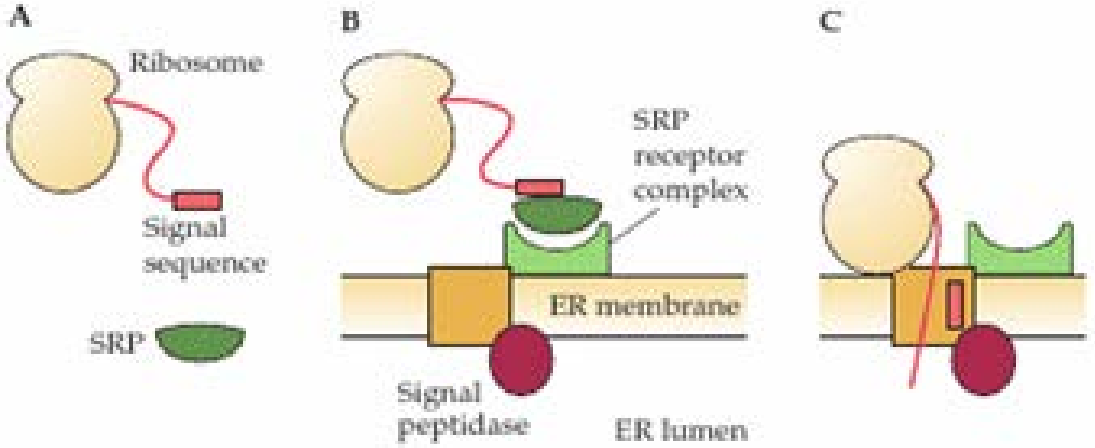
The amount of intracellular protein (Pi) depends on the fluxes of intracellular protein formation (q_{Pi}), protein secretion (q_{Sec}), intracellular protein degradation (q_{Deg}) and protein dilution into the daughter cells (q_{Dil})

Many different processes are lumped into "*protein expression or secretion" !*

Pfeffer et al. 2011 Microb Cell Fact. 10:47.

Recap: translocation process in eukaryotes

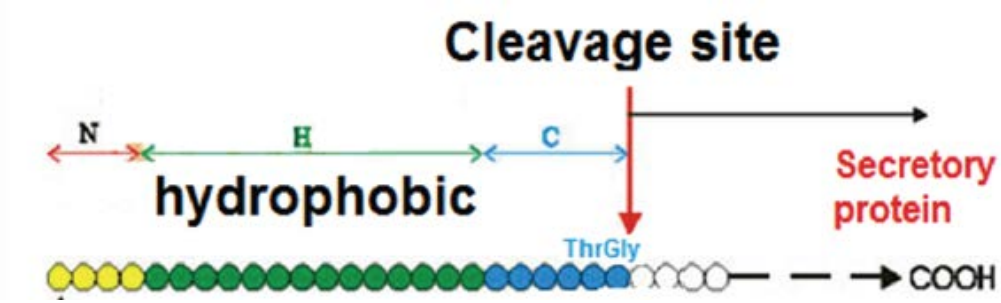
A. A signal recognition particle (SRP) binds to the signal sequence of a secretory protein.
B. The SRP attaches to an SRP receptor on the endoplasmic reticulum (ER) membrane.
C. The secretory protein is translocated into the lumen of the ER, and a signal peptidase removes the signal sequence.



Molecular Biotechnology: Principles and Applications of Recombinant DNA, Fourth Edition
Bernard R. Glick, Jack J. Pasternak, and Cheryl L. Patten

Signal sequences guide proteins to ER

Import into ER	$^{+}H_3N$-Met-Met-Ser-Phe-Val-Ser-Leu-Leu-Leu-Val-Gly-Ile-Leu-Phe-Trp-Ala-Thr-Glu-Ala-Glu-Gln-Leu-Thr-Lys-Cys-Glu-Val-Phe-Gln-



- Signal peptides do not consist of a strict consensus sequence but have a three-region design consisting of a positively charged N-terminal region (N-region), a hydrophobic central region (H-region) and a neutral, polar C-terminal region (C-region)
- Signal sequences (SS) vary but have a segment of 8 or 9 hydrophobic amino acids at their center

RESEARCH | OPEN ACCESS

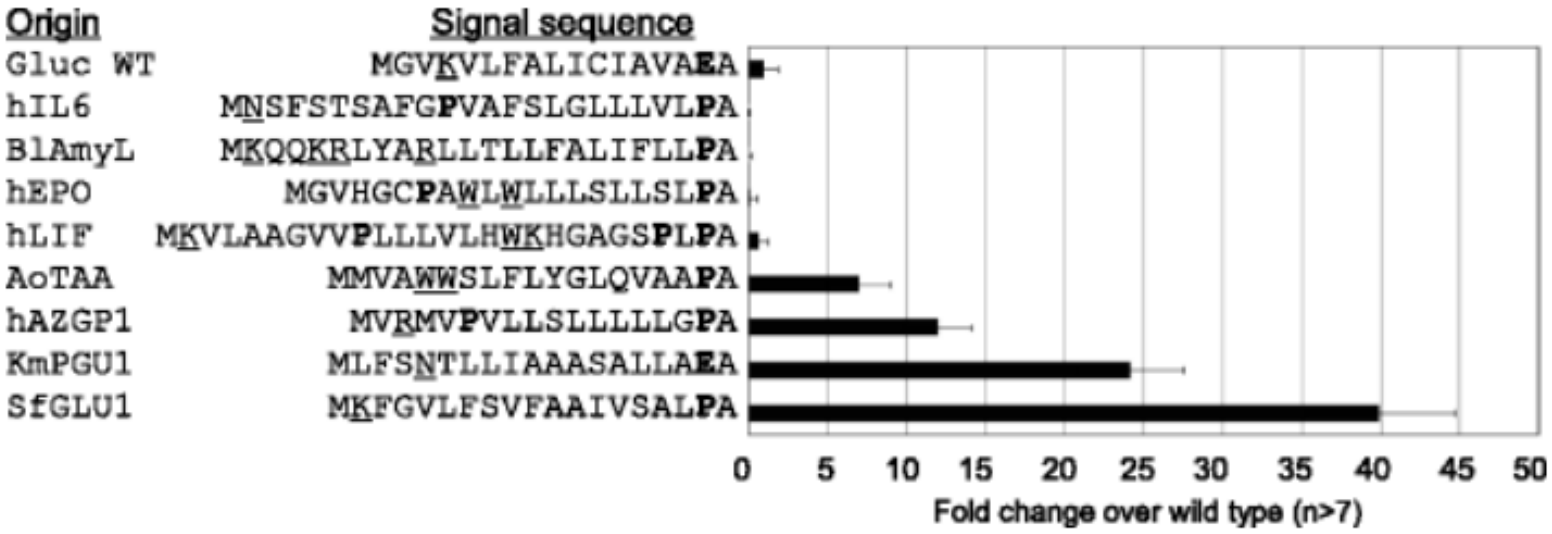
Synthetic signal sequences that enable efficient secretory protein production in the yeast *Kluyveromyces marxianus*

Tohru Yarimizu, Mikiko Nakamura, Hisashi Hoshida ✉ and Rinji Akada

Microbial Cell Factories 2015 **14**:20 | **DOI:** 10.1186/s12934-015-0203-y

Received: 19 September 2014 | **Accepted:** 27 January 2015 | **Published:** 14 February 2015

Signal sequences strongly affect translocation process



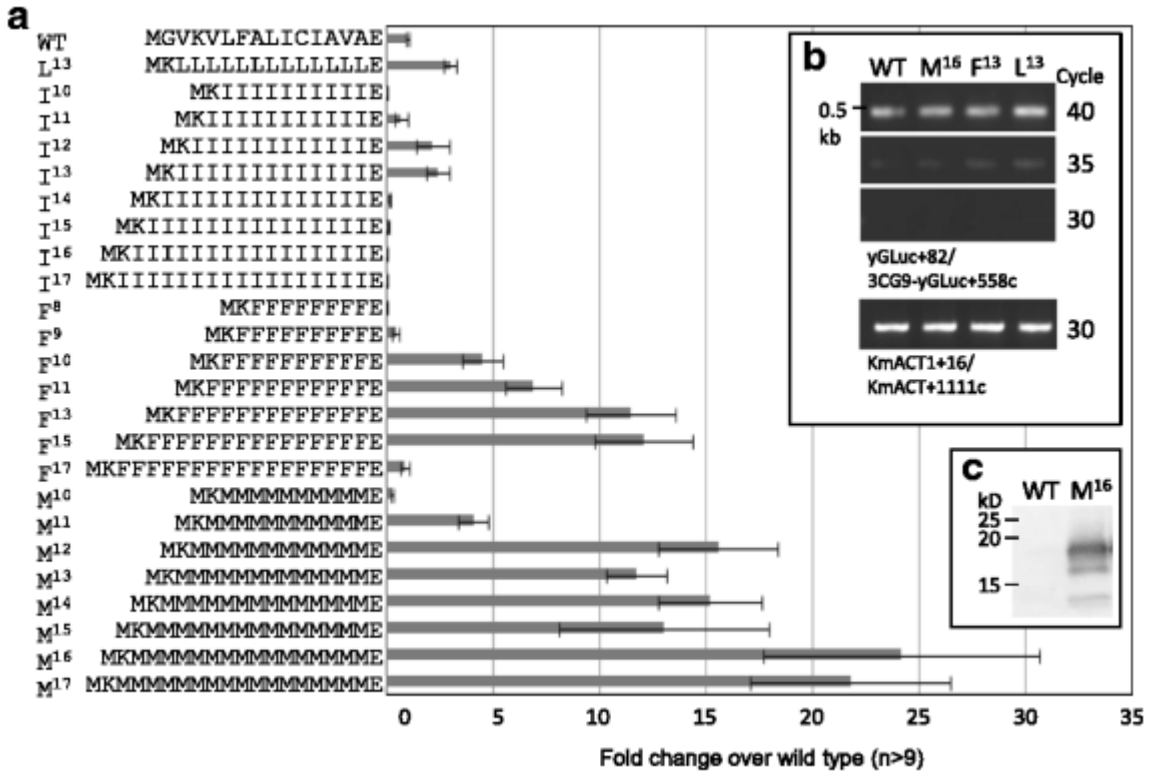
Relative secreted luciferase activity

- By exchange of signal peptide translocation efficiency can be strongly improved
- Your target protein can be fused to any desired signal sequence

Yarimizu et al., 2015 Microbial Cell Factories 14:20

Effect of repeat number of the single amino acid between K and E on secretory activities

- K (lysine) and E (glutamic acid) mark the start and stop of the hydrophobic core of the original (wt) signal sequence



Relative secreted luciferase activity

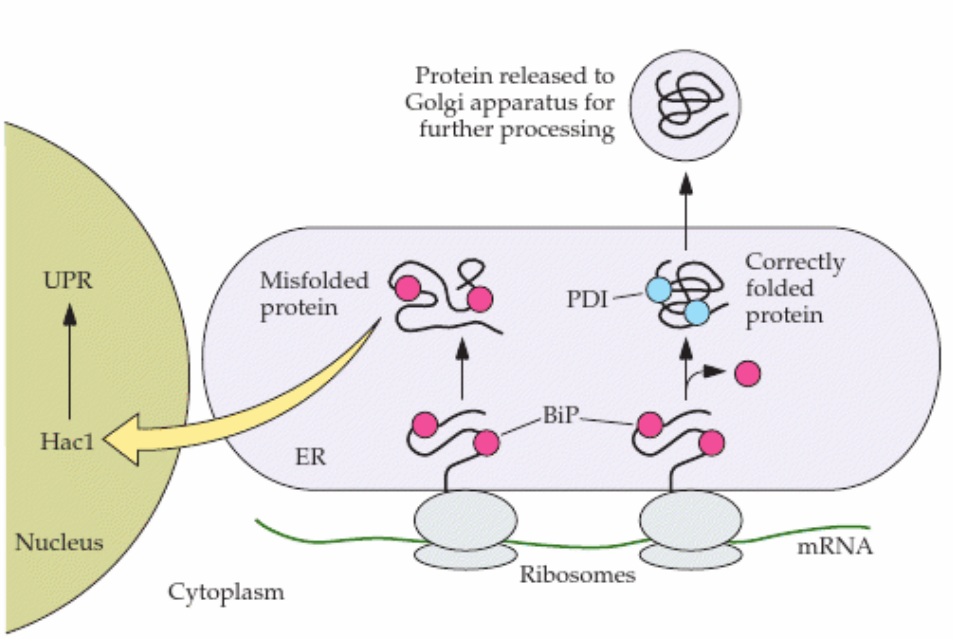
B) RT-PCR of the sequenced clones for WT, M^{16}, F^{13} and L^{13} to determine mRNA levels

C) Western blotting analysis of the supernatants from and M^{16} using anti-GLuc antibody

Yarimizu et al., 2015 Microbial Cell Factories 14:20

Protein folding in the endoplasmic reticulum

- Nascent proteins are bound by the chaperone BiP and calnexin
- Protein disulfide isomerase (PDI) catalyzes the formation & isomerization of disulfide bonds between cysteine amino acids
- Quality control systems ensure that only correctly folded proteins are released from the ER -> ERAD
- Prolonged binding of BiP to misfolded proteins leads to activation unfolded-protein response (UPR)
- Proteins released from the ER are transported to the Golgi apparatus for further processing



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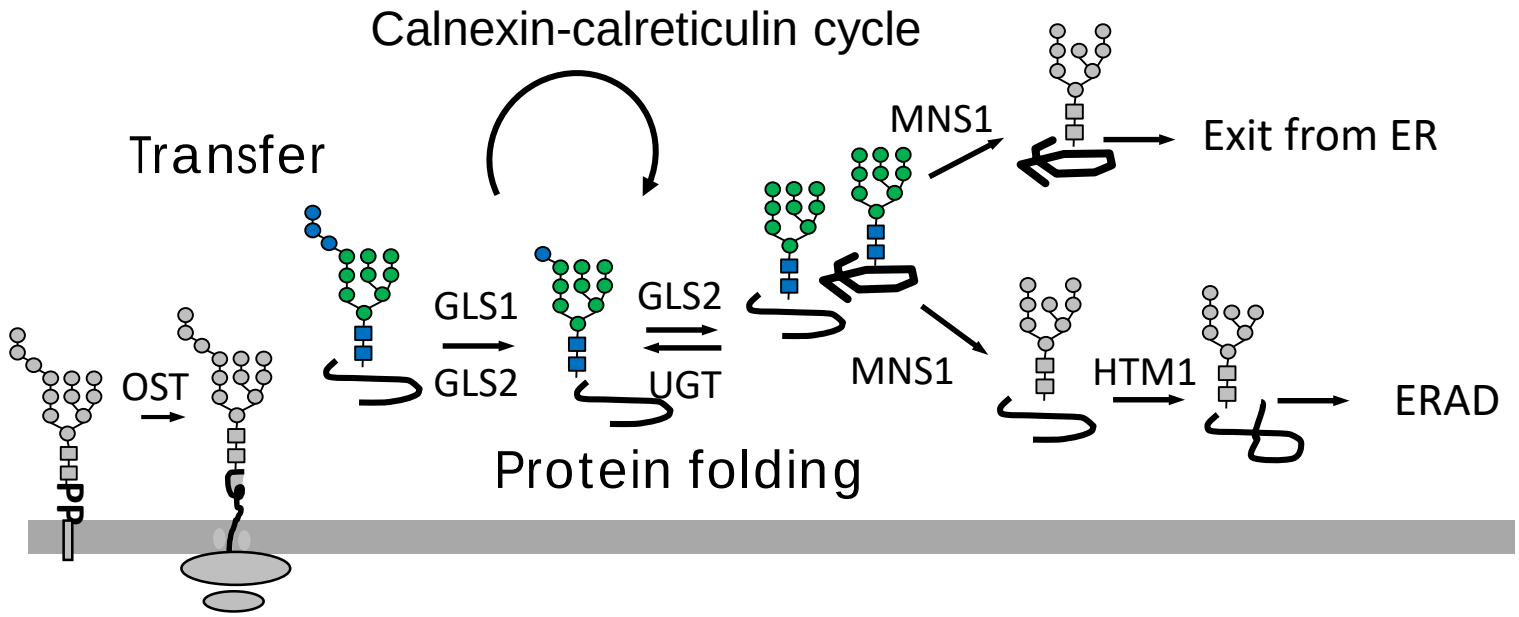
Functions the N-glycans in protein folding calnexin/calreticulin cycle



- After addition of the glycan to polypeptide chain by OST:
  - Outermost glucose residues is removed by glucosidase I (GLS1)
  - Glucosidase II (GLS2) removes the middle glucose
  - $Glc_1Man_9GlcNAc_2$ is recognition motif for calnexin/calreticulin

Recap: Calnexin-calreticulin cycle

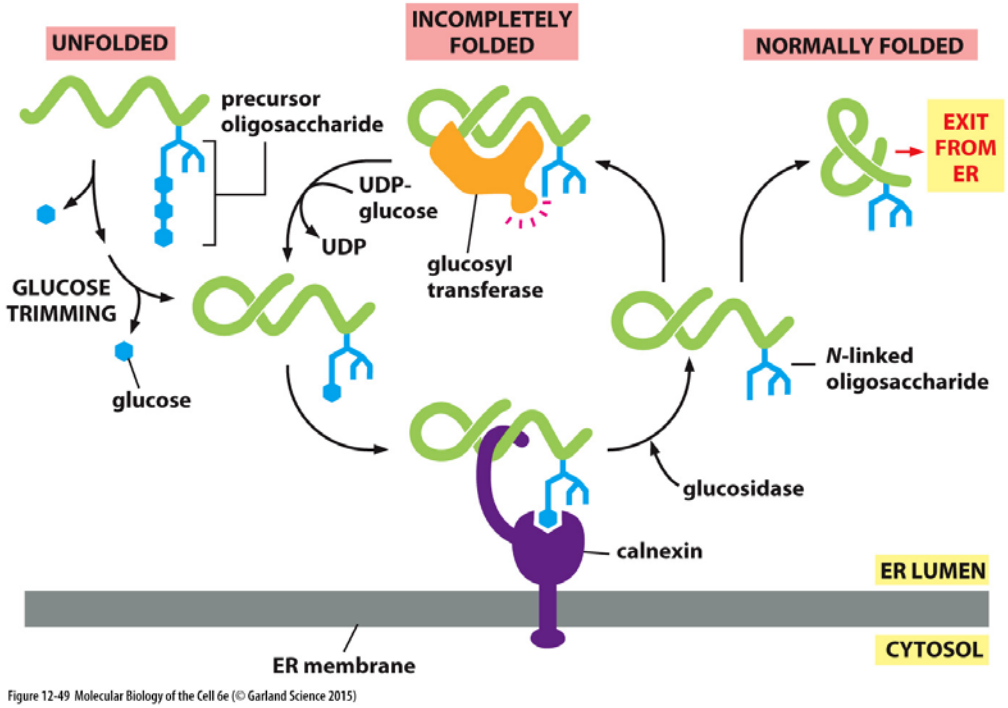


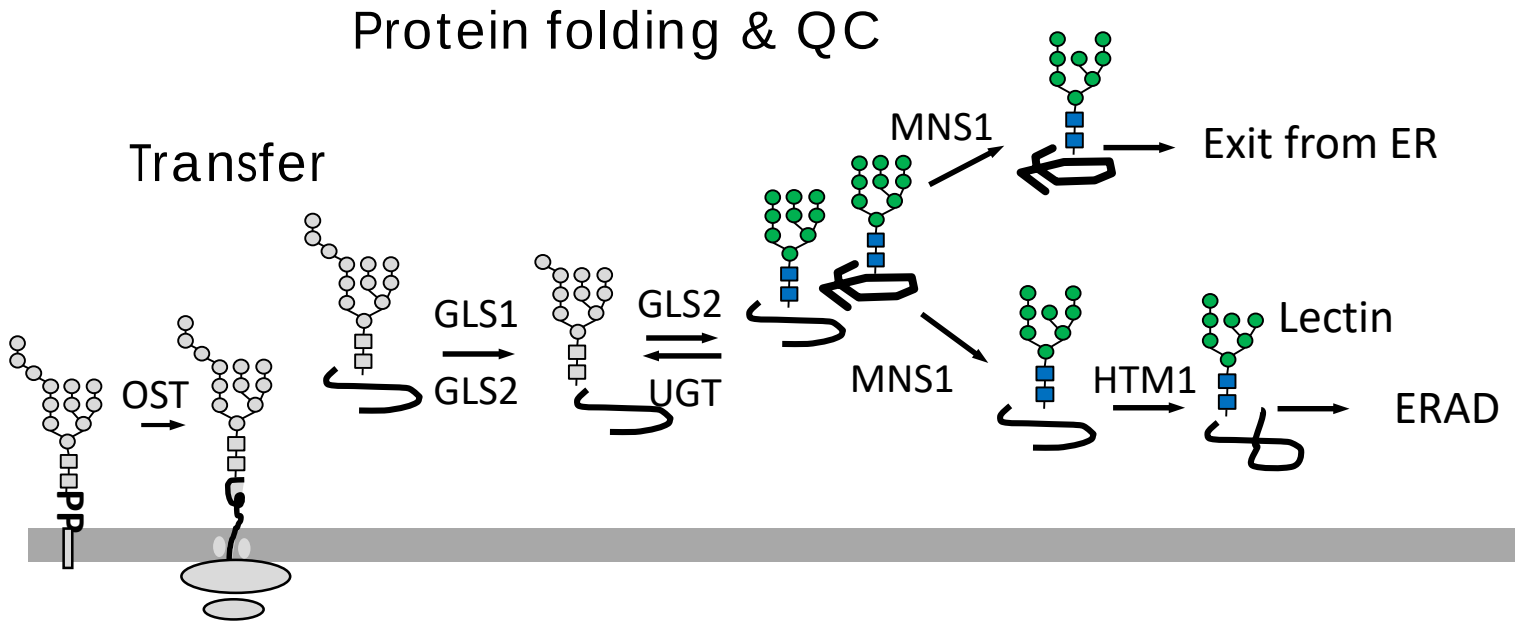
Figure 12-49 Molecular Biology of the Cell 6e (© Garland Science 2015)

- Calnexin-calreticulin cycle keeps protein in the folding cycle protecting it from protein degradation
- Terminal glucose is essential for recognition of glycoproteins by Calnexin/Calreticulin
- When glucosidase II removes the remaining glucose (I), the glycoprotein dissociates from calnexin and calreticulin

Recap: The calnexin/calreticulin cycle

- After release from calnexin/calreticulin, the protein encounters one of three possible fates.
  - If properly folded, it is free to leave the ER.
  - If incompletely folded, Glucosyltransferase reglucosylates the glycans located in improperly folded regions. Through these glycans, the glycoprotein rebinds to calnexin and calreticulin.
  - The third fate is ER-associated protein degradation (ERAD) of the misfolded glycoprotein

Functions the N-glycans in protein folding ER-associated protein degradation (ERAD)



- ERAD of glycoproteins occurs when they have stayed in the ER lumen for some time not reaching their native state
- A slow acting mannosidase (HTM1) removes a terminal mannose creating a $Man_7GlcNAc_2$ glycan

Recap: ER associated protein degrdation

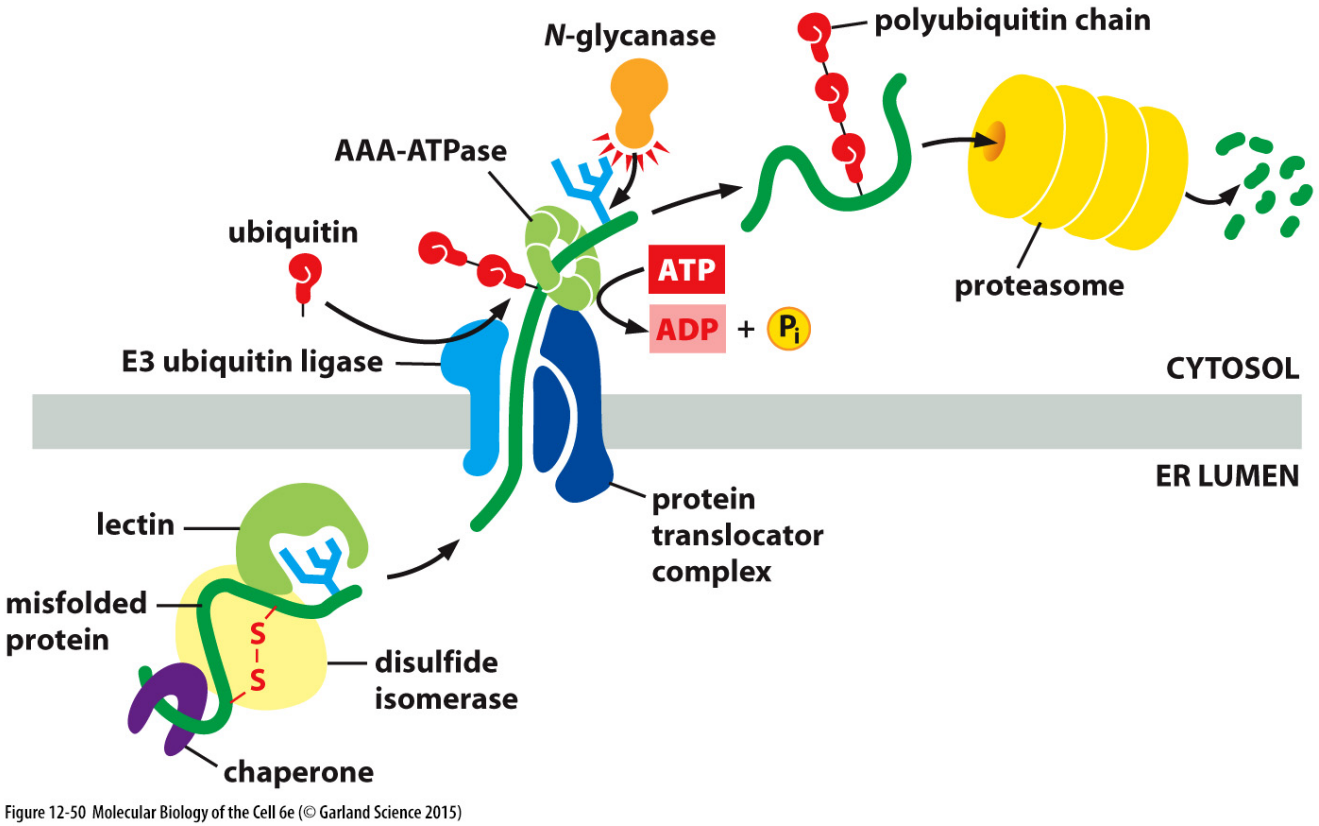


Figure 12-50 Molecular Biology of the Cell 6e (© Garland Science 2015)

- Glycan signal is recognized by a lectin
- Proteins are retrotranslocated into the cytoplasm, ubiquitinylated and degraded by the proteasome

Molecular chaperones and folding factors in the ER

- Heat shock protein (HSP) family members
  - Family includes chaperones and co-chaperones
  - HSP90 (GRP94)
  - HSP70 (BiP)
  - HSP70-Nucleotide exchange factor (GRP170)
  - HSP90-Nucleotide exchange factor (SIL1)

- Protein disulfide isomerases (PDI/Ero/Erv) family members
  - PDI (PDI)
  - Ero1alpha, Ero1beta

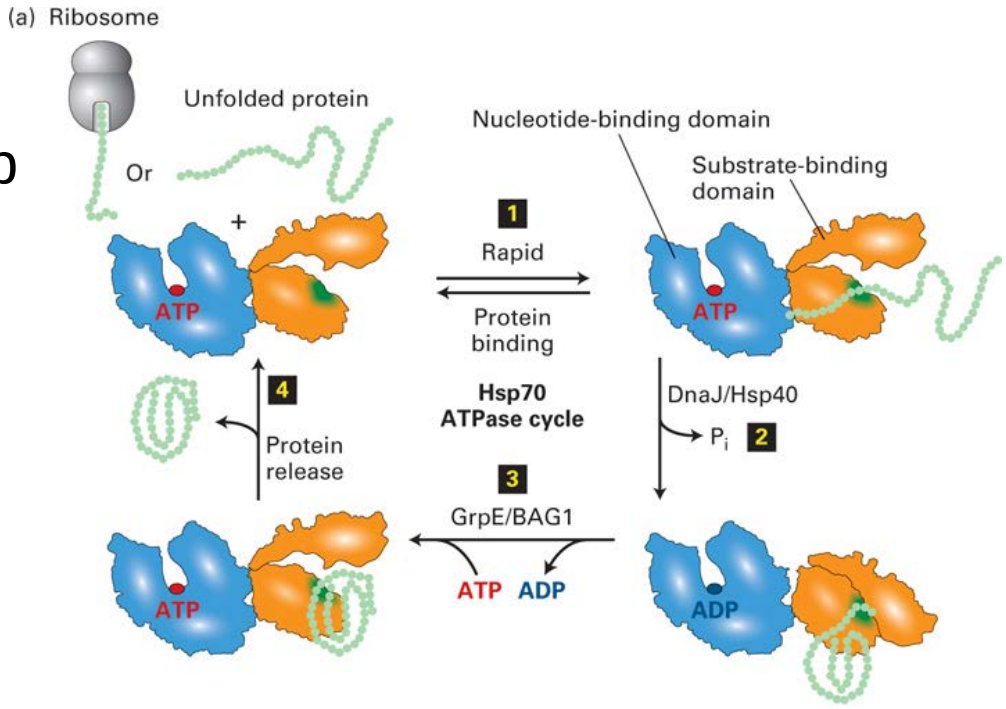
- Peptidyl-prolyl isomerases (PPIases) family members

Chaperone-assisted protein folding

- The folding of many proteins, particularly large ones, is kinetically slow and is assisted in vivo by folding agents known as chaperones
- Chaperones prevent proteins to veer of their folding path
- Chaperones assist in
  - folding of nascent polypeptides made by translation
  - re-folding of proteins denatured by environmental damage, such as heat shock

Chaperone-assisted protein folding

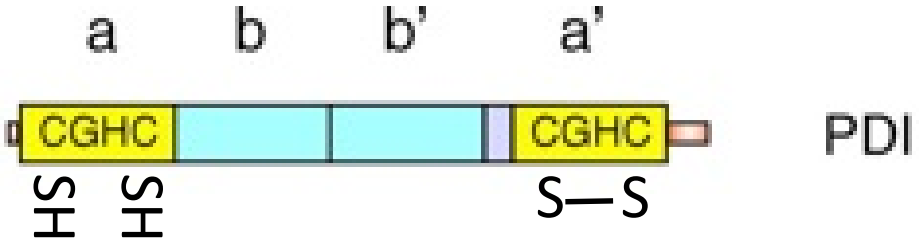
- The hydrolysis of ATP by the chaperone drives conformational changes that prevent aggregation and help driving protein folding
- Accessory proteins (Co-chaperone) participate in the process, e.g. nucleotide exchange factors
- Eukaryotic molecular chaperones such as Hsp 70 (cytosol & mitochondial matrix) and BiP (ER) are related to the bacterial protein DnaK



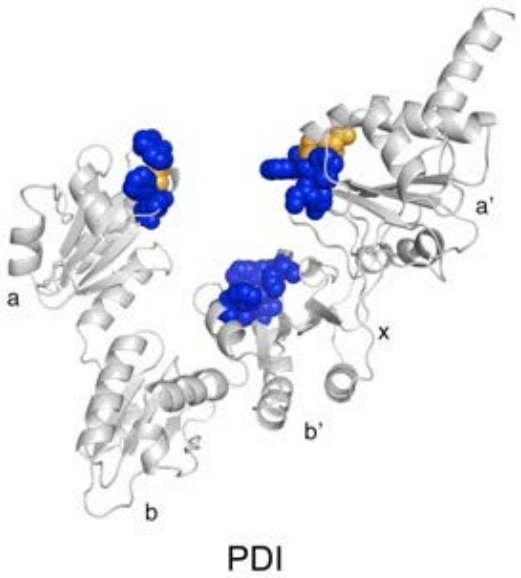
Disulfide bond formation

- Disulfide bond formation occurs spontaneously, but takes hours
- Most specialized cells produce 1000 IgM pentamers per second, each containing approx. 100 S-S bonds

Protein disulfide isomerases



PDI



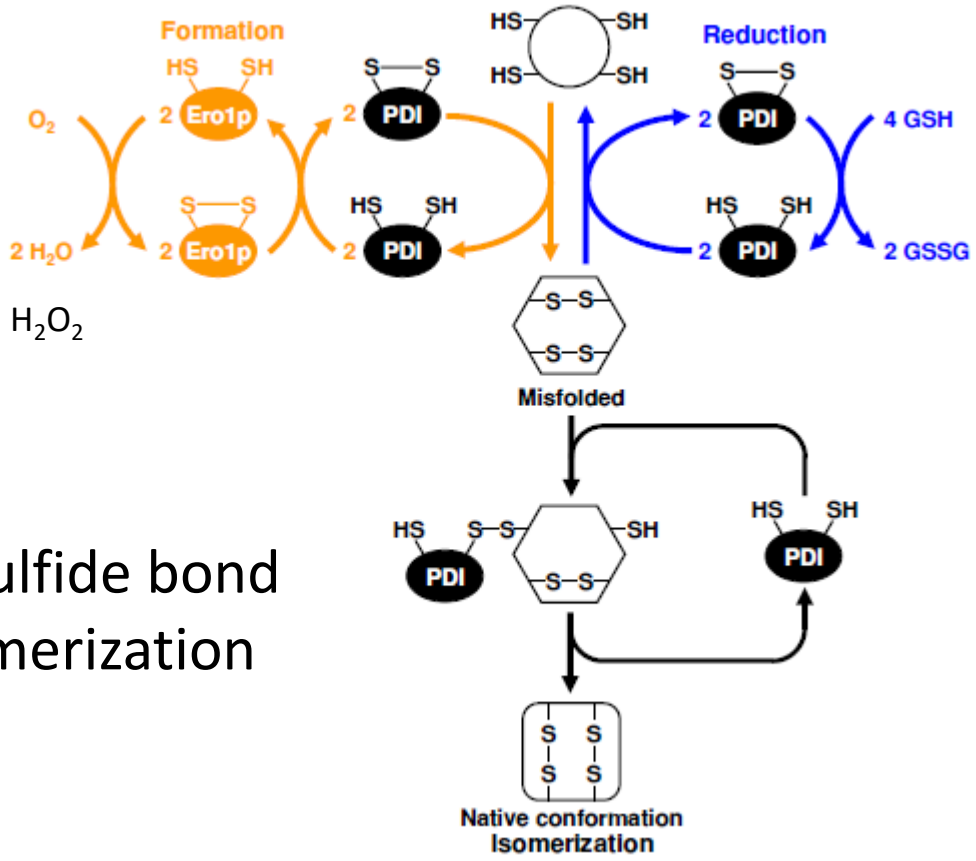
PDI

- PDI has four thioredoxin domains
- Domains a and a' are catalytically active containing the CXXC motif
  - Formation of intra- and intermolecular S-S bonds
- All PDI/Ero/Erv protein family members contain thioredoxin domains

Disulfide bond formation and isomerization reactions catalyzed by PDI and Ero1

Disulfide bond formation

Disulfide bond donation



Disulfide bond reduction

Disulfide bond isomerization

Disulfide bond donation and generation

- Disulfide bond donation: PDI
  - PDI recognizes hydrophobic patches of unfolded proteins – relaxed protein substrate specificity
  - High redox potential
- Disulfide bond generation: Ero1p
  - uses O_2 as electron acceptor, leading to formation of H_2O
  - Narrow substrate specificity -> PDI

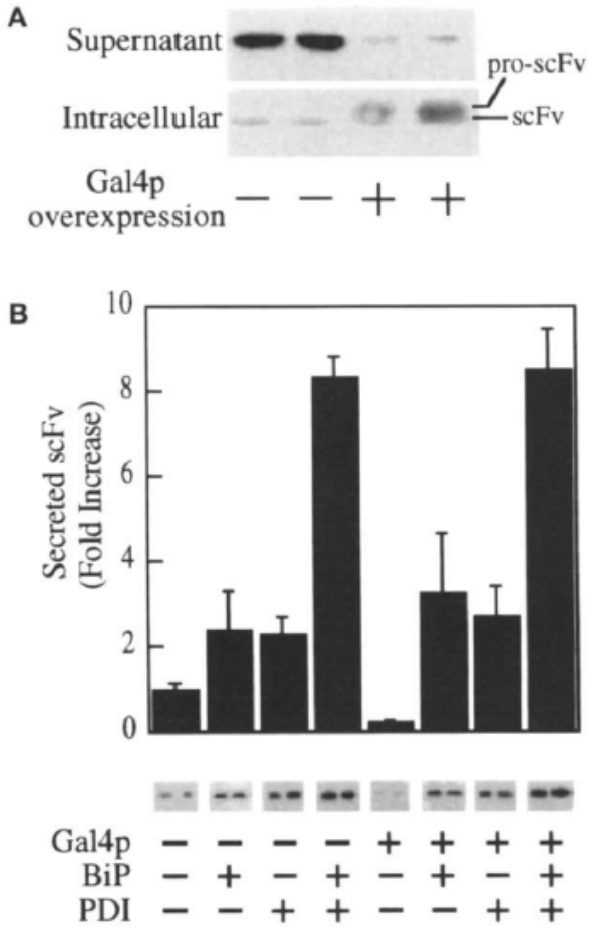
Increasing the secretory capacity of *Saccharomyces cerevisiae* for production of single-chain antibody fragments

Eric V. Shusta, Ronald T. Raines[1], Andreas Plückthun[2], and K. Dane Wittrup*

*Department of Chemical Engineering, University of Illinois, Urbana, IL 61801. [1]Departments of Biochemistry and Chemistry, University of Wisconsin, Madison, WI. [2]Biochemisches Institut, Universität Zürich, CH-8057 Zürich, Switzerland. *Corresponding author (e-mail: wittrup@uiuc.edu).*

Received 23 February 1998; accepted 29 June 1998

Effect on the yeast secretory pathway of transcription factor overexpression and BiP and/or PDI overexpression



Questions:

- The figure summarizes the data from two strategies for increasing productivity
- Overexpression of the transcriptional regulator Gal4p
- Overexpression of BiP and/or PDI

- What is the effect of Gal4p overexpression alone (visible in panel A) and in combination with Bip/PDI?

- What are the effects of BiP and PDI

Unfolded protein response (UPR)

Recap: The unfolded protein response (UPR)

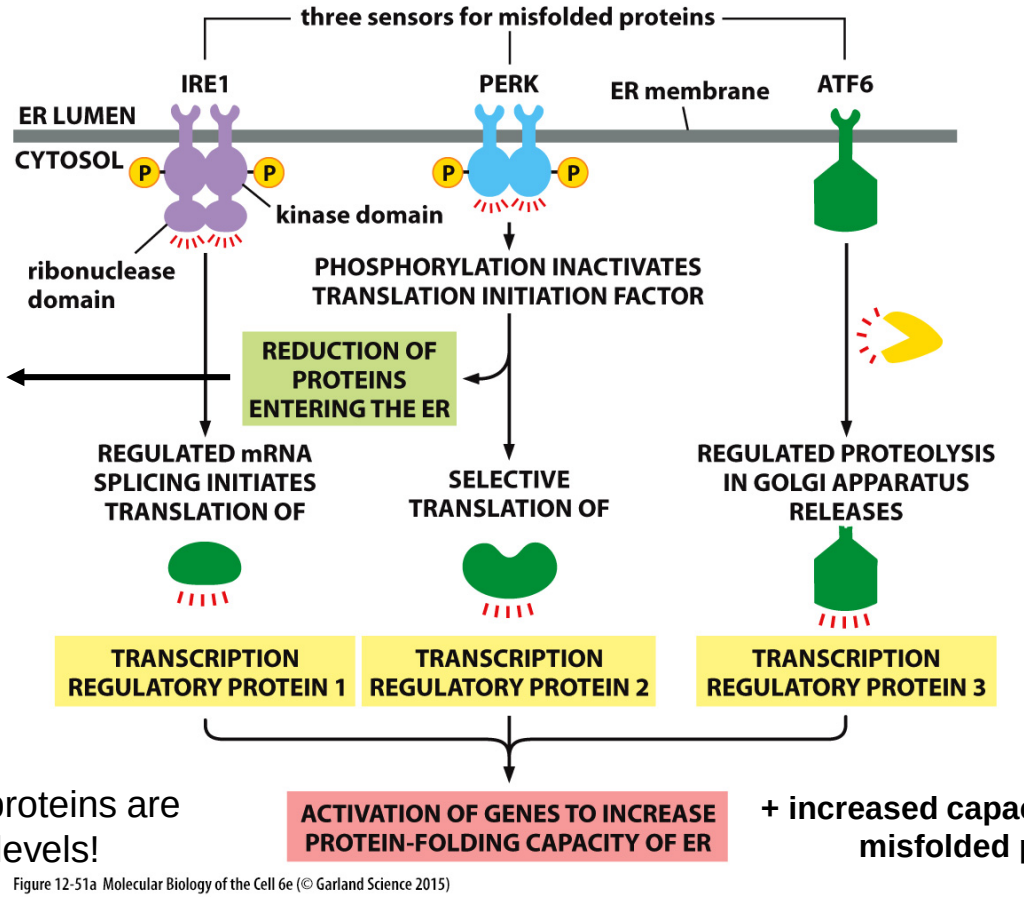


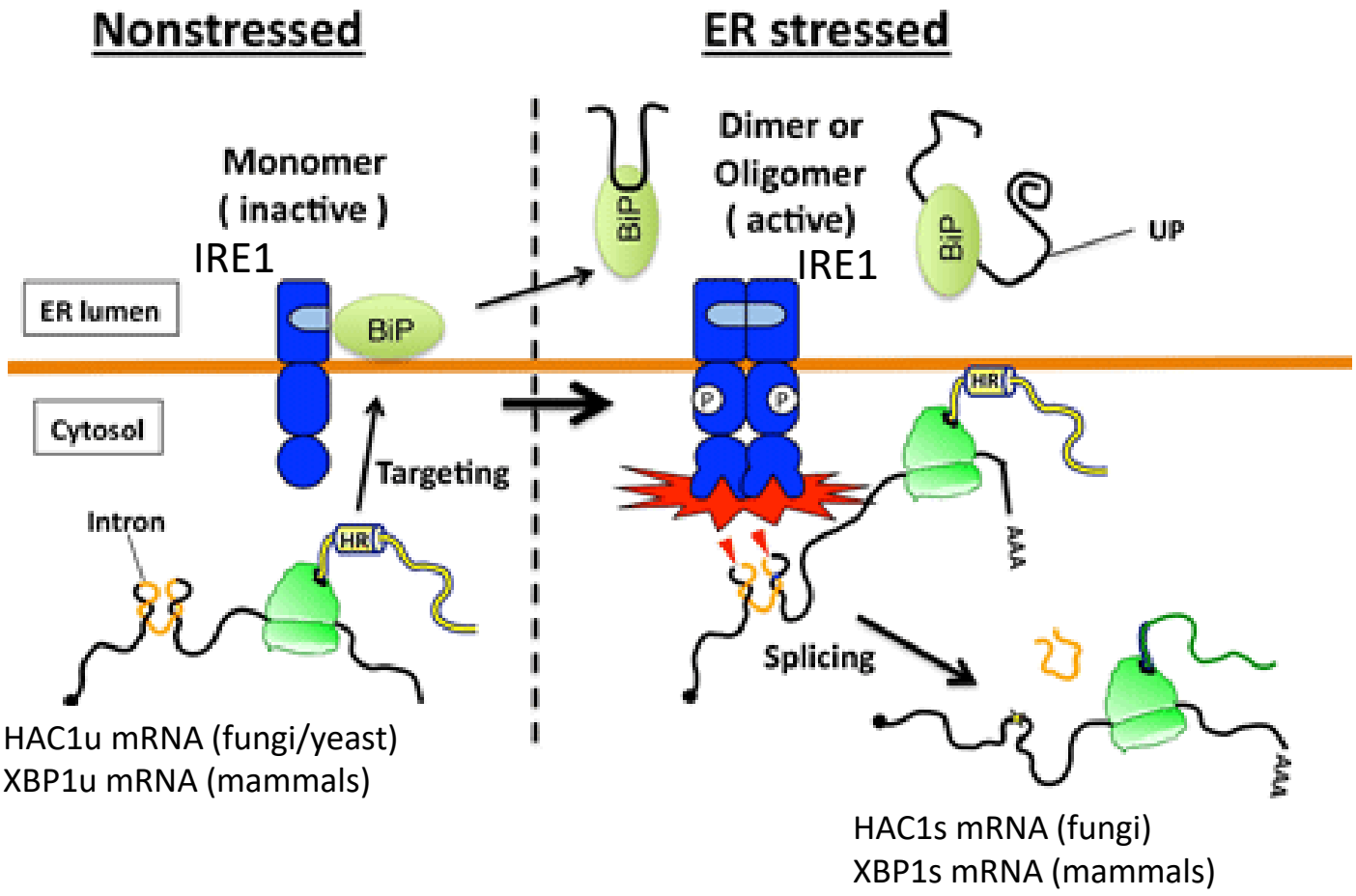
Figure 12-51a Molecular Biology of the Cell 6e (© Garland Science 2015)

- Relative importance of the 3 pathways for UPR activation differs in different cell types
- In lower eukaryotes only IRE1 pillar exists!

UPR activation by activation of sensor IRE1

Endoribonuclease activity of IRE

Cytoplasmic splicing!



Functions upregulated by UPR in yeast

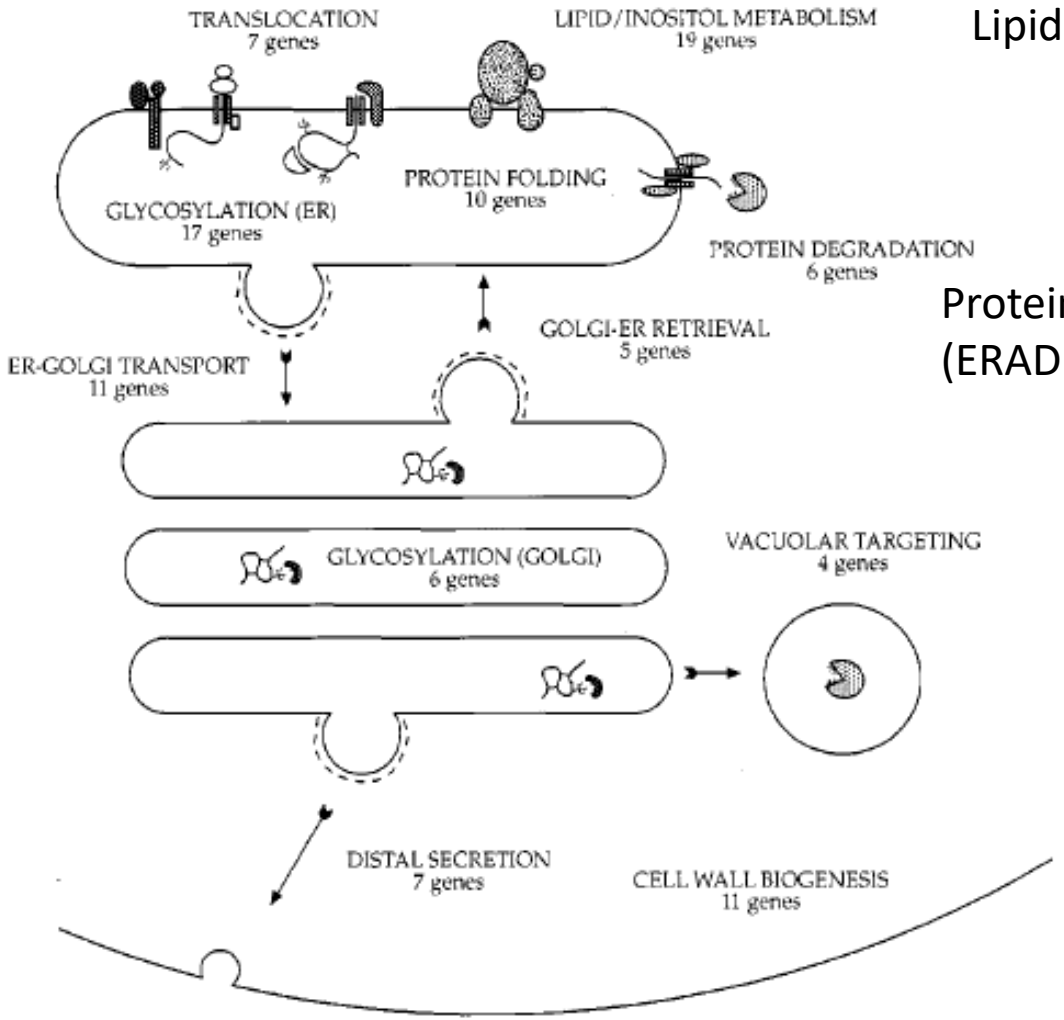
Translocation

Glycosylation, LLO biosynthesis

Protein transport & trafficking

Glycosylation

Cell wall synthesis

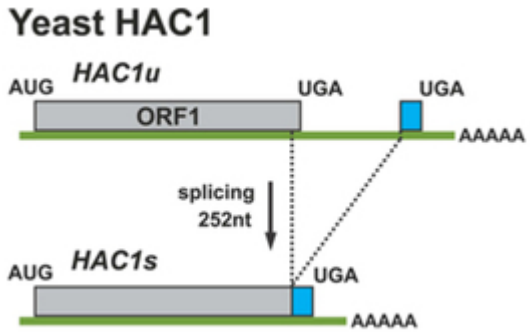


Lipid biosynthesis

Protein degradation (ERAD)

Travers et al., 2000, Cell, 101: 249

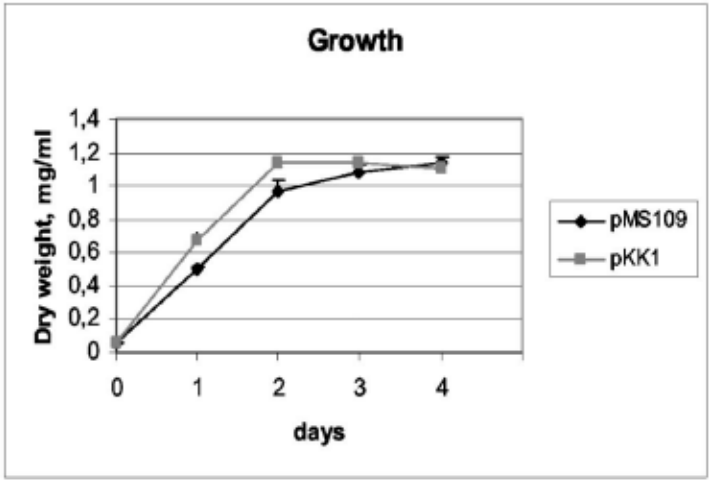
Effects of *HAC1* overexpression on growth and secretion of *B. amyloliquefaciens* –amylase in yeast



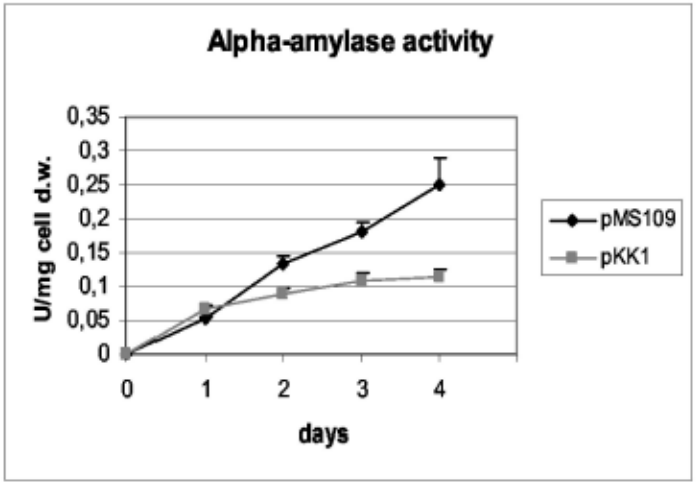
HAC1-overexpressing strain: pMS109

Control strain: pKK1

A.



B.

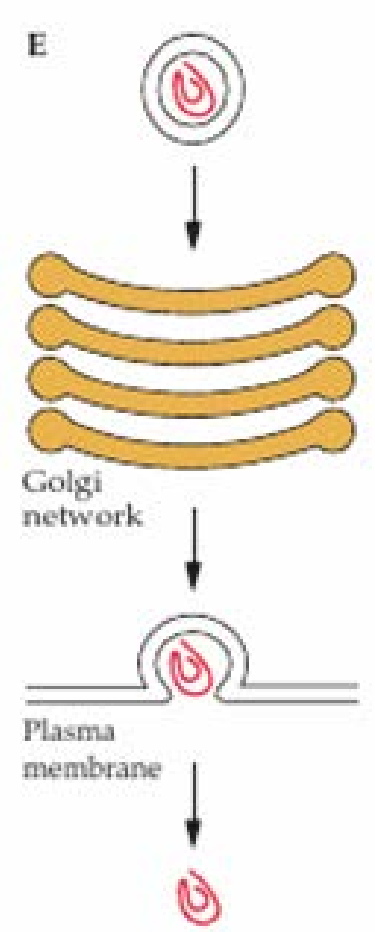


Valkonen et al., 2003, Environ. Appl. Microbiol, 69:2065

Up to 300 genes are under control of UPR -> more targeted approaches feasible?

Schematic representation of the secretion pathway in eukaryotes

- The ER-released vesicle carrying the secretory protein fuses with the Golgi network at the cis face and passes through the Golgi stack
- Protein can be further modified
- a plasma membrane-specific vesicle is formed at the trans face of the Golgi network
- The secretory transport vesicle fuses with the plasma membrane and releases the secretory protein to the extracellular environment.



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Bernard R. Glick, Jack J. Pasternak, and Cheryl L. Patten

Recap: Vesicle mediated transport through the secretory pathway

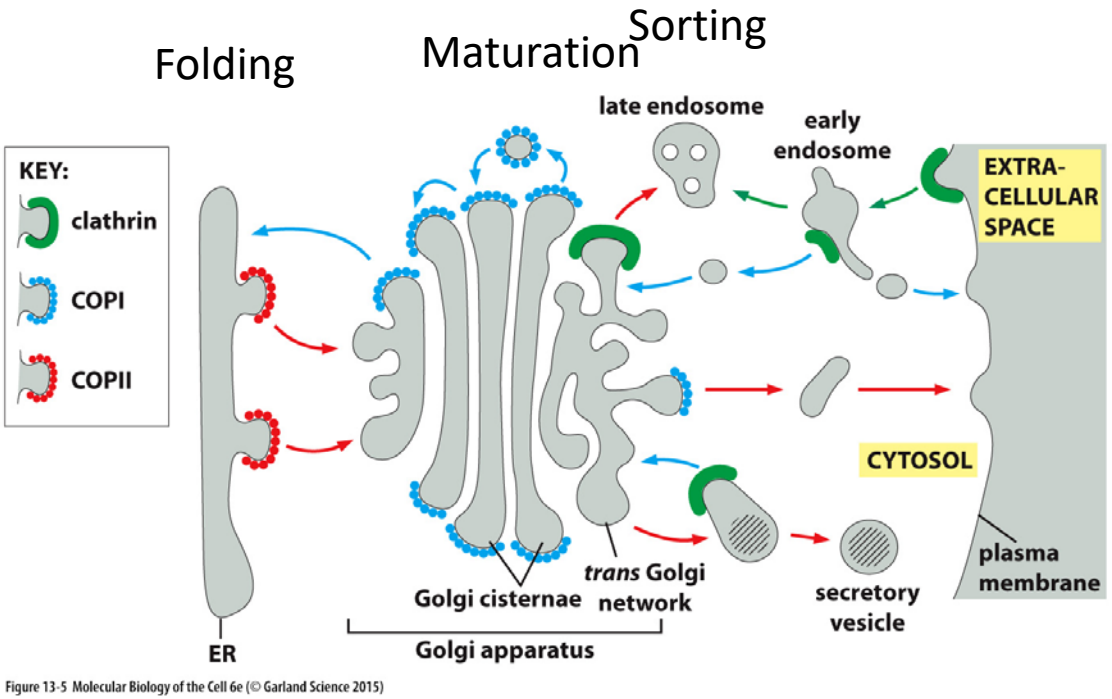


Figure 13-5 Molecular Biology of the Cell 6e (© Garland Science 2015)

- After synthesis in the ER, proteins are sorted into vesicles by the coat protein complex-II (COPII) machinery and delivered to the Golgi complex by vesicle transport (Tethers and SNARE machineries required)
- Intra-Golgi transport and retrograde transport from the Golgi to the ER are regulated by the COPI machinery.
- At the trans-Golgi network (TGN), proteins are sorted into vesicles by intrinsic sorting motifs, and are transported along cytoskeletal elements to the plasma membrane.
- Protein delivery to the plasma membrane is mediated by vesicle transport (Tethers and SNARE machineries required)

Engineering of vesicle trafficking improves heterologous protein secretion in *Saccharomyces cerevisiae*

Jin Hou [a], Keith Tyo [a,b], Zihe Liu [a], Dina Petranovic [a], Jens Nielsen [a,*]

[a] Department of Chemical and Biological Engineering, Chalmers University of Technology, SE-41296 Göteborg, Sweden
[b] Department of Chemical and Biological Engineering, Northwestern University, 2145 Sheridan Road, Evanston, IL 60208-3120, USA

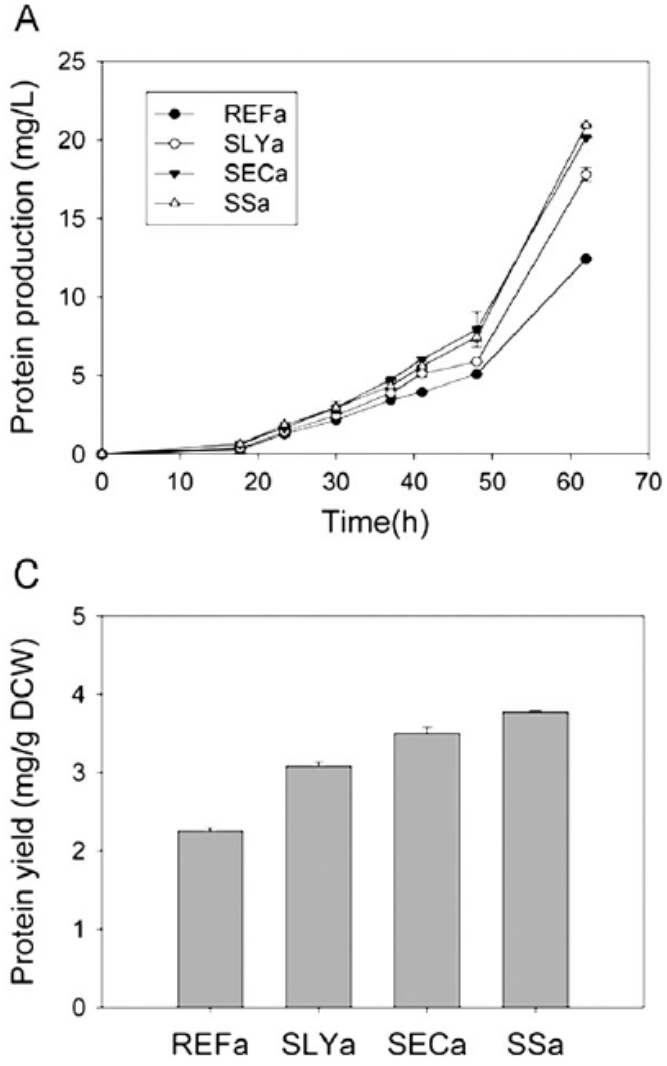
Metabolic Engineering 14 (2012) 120–127

SLY1: protein involved in ER/Golgi vesicle trafficking belonging to SM (Sec1/Munc-18) protein family. It binds the tSNARE Sed5p and stimulates its assembly into a trans-SNARE membrane-protein complex

SEC1: protein involved in docking and fusion of exocytic vesicles; binds to assembled SNARE complexes at the membrane and stimulates membrane fusion

Heterologous protein secretion of recombinant strains in batch cultivations.

a-amylase production



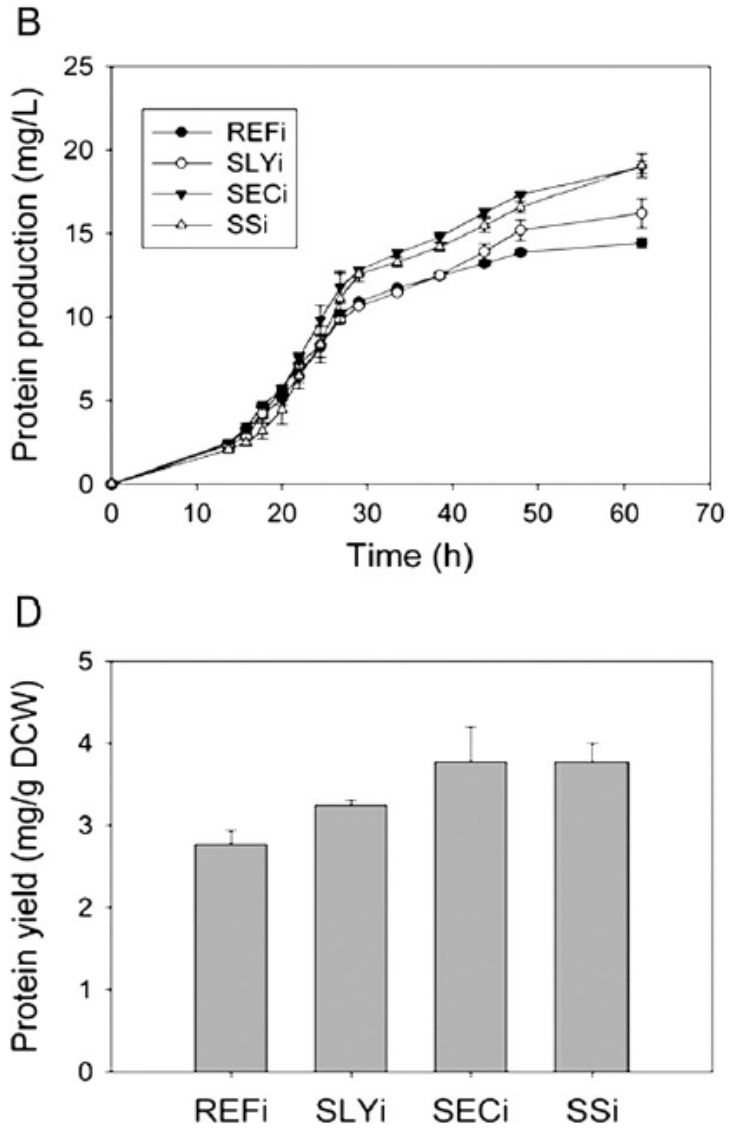
SLY1 overexpression: protein involved in ER/Golgi vesicle trafficking

SEC1: protein involved in docking and fusion of exocytic vesicles

SS: SLY1 and SEC1 overexpression
-> additive effects of SLY1 and SEC1

Heterologous protein secretion of the recombinant strains in batch cultivations

insulin precursor production

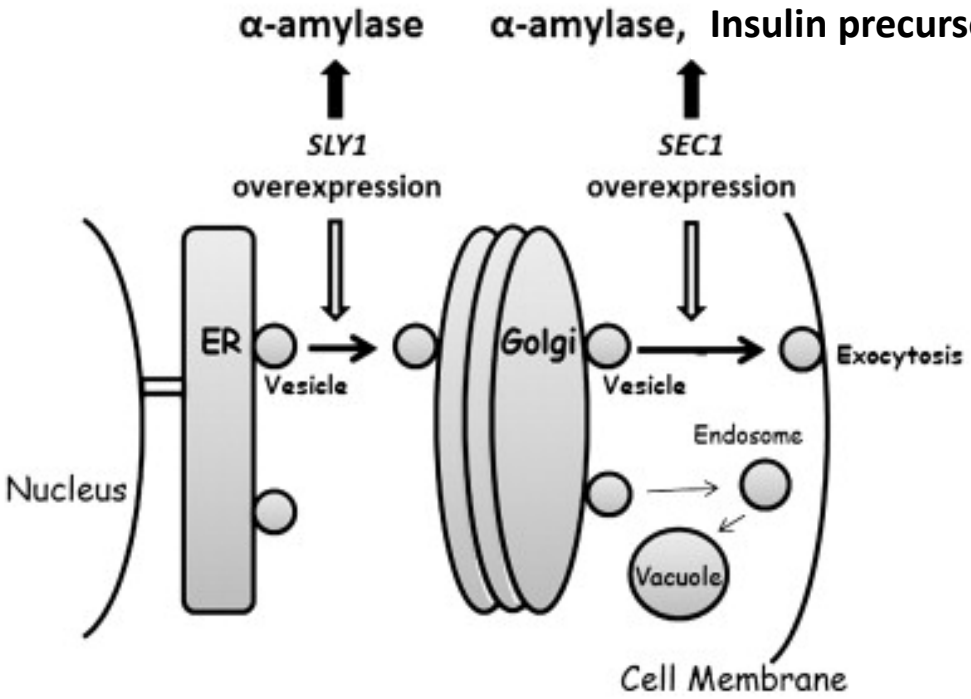


SLY1 overexpression: protein involved in ER/Golgi vesicle trafficking

SEC1: protein involved in docking and fusion of exocytic vesicles

SS: SLY1 and SEC1 overexpression

Engineering vesicle trafficking improves the production of a-amylase and insulin precursor



- study demonstrates the positive impact of SNARE-interacting SM proteins on heterologous protein secretion
- shows that engineering of the secretory pathway by targeting the vesicle trafficking is a promising engineering approach
- reports that protein trafficking engineering has different effects for protein with different properties

Summary of protein folding, quality control, degradation and secretion

- Nascent proteins are bound by the chaperone BiP and calnexin
- Protein disulfide isomerases (PDI) catalyze the formation & isomerization of disulfide bonds
- Quality control systems ensure that only correctly folded proteins are released from the ER -> ERAD
- Prolonged binding of BiP to misfolded proteins leads to activation unfolded-protein response (UPR)
- Proteins released from the ER are transported to the Golgi apparatus for further processing
- All process from folding to secretion are engineering targets to improve production (case by case evaluation needed)