Unit 2: simulation iGEM brainstroming

Content (for a double hour, 90 min)

1. Greeting and revision on last weeks topic
2. iGEM research – purpose and methods
3. group disposition and work order
4. group working phase
5. presentation of topics, discussion
6. feedback and evaluation

Attachment: material and commentary

1. Greeting and revision on last weeks topic

What exactly is synthetic biology – ask pupils, take notes on the board

Which projects last week: topics, problems and resolutions

2. iGEM research – purpose and methods

short introduction on iGEM (with reference to unit one): “student group (mainly bachelor), almost one year, presentation on the very end of this year, competition conditions, different criteria for success: solving of real world problem ect)

Criteria for successful topic: divisible in subgroups, actual and relevant, feasible in a short amount of time

3. group disposition and work order

„Elaborate a solution approach for the given problem regarding the target and scientific fundamentals of the subproject, methods and evidence of success. Consider how the work can be presented (which sketches facilitate the understanding). Various card types are provided for this purpose. They are the starting material. In addition, there are card types with basic knowledge about the relevant biological processes. The texts are in English, important words are listed below, any questions possible at any time.”

4. Group work phase

Do help the pupils in this phase by drawing their attention to different aspects they might benefit from (perhaps help with translations)

5. Presentation of topics, discussion

Whole groups together or a representative present the idea and the corresponding basic principles (they should draw on the board). Queries from the others, discussion on feasibility and relevance, possibly comparison with our “artico”-subprojects or other iGEM teams

6. feedback and evaluation

The pupils may fill out the evaluation questionnaire (in the attachment).

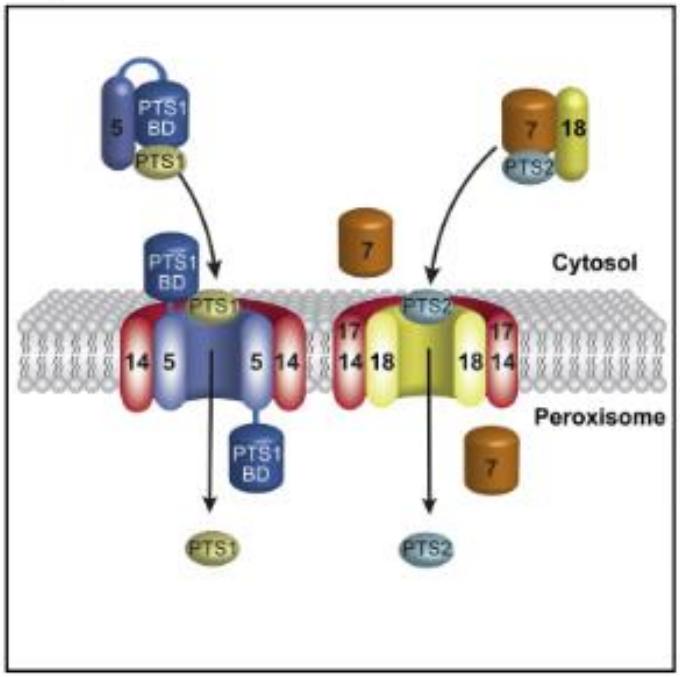
Index card: general

Report

Cell Reports

Distinct Pores for Peroxisomal Import of PTS1 and PTS2 Proteins

Graphical Abstract



Authors

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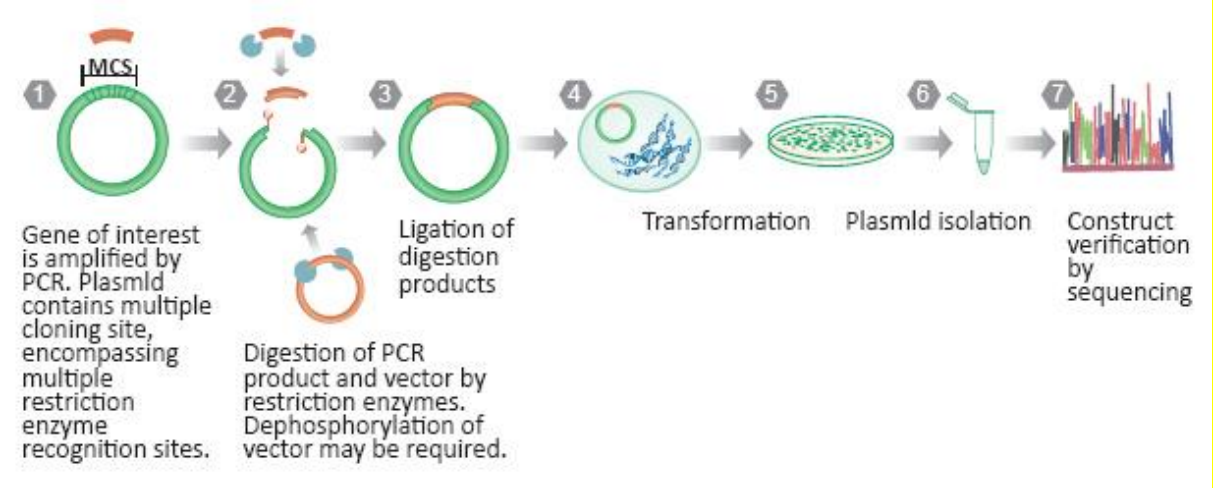
In Brief

Two peroxisomal targeting signals, PTS1 and PTS2, direct folded proteins to the peroxisomal matrix. Montilla-Martinez et al. (2015) identify a PTS2-specific pore, which contains the PTS2 co-receptor Pex18 and the Pex14/Pex17-docking complex as major constituents. The data demonstrate that import of PTS1 and PTS2 proteins is performed by distinct pores.

Highlights

- Import of folded proteins into peroxisomes requires distinct PTS-specific pores
- PTS2 pore is formed by the cytosolic co-receptor Pex18 and docking complex Pex14/Pex17
- The PTS2 receptor is not part of the unloaded pore
- Complex gating of the PTS2 channel is voltage and cargo dependent

Index card: cloning strategy



https://www.genscript.com/molecular-cloning-strategy.html

Definitions:

Gene of interest: gene coding for the wanted function

PCR: polymerase chain reaction, multiplies the DNA fragment

Restriction enzyme: cuts DNA at a unique recognition site, generates specific overhangs

Ligation: combining DNA fragments depending on their specific overhangs

Transformation: transfer of a plasmid (DNA) into (bacteria) cells

Index card 1: secretion

The work on the secretion of compounds produced in modified compartments.

For industrial production of biological compounds one important part of the process is the downstream processing. For most biotechnological produced compounds, downstream processing is the most expensive part of the production (Keller et al. 2001). One step to decrease the costs is to secrete the products into the supernatant (Berlec, Strukelj 2013). After secretion, it is possible to remove most compounds of the cells with one simple centrifugation step. Due to this the secretion is not only a great tool for a compartment toolbox but also has an economic value.

To focus on the whole project our sub project is an important part for making the compartment more applicable. Here we go a step further and think about the extraction of products after the production.

At the end of this sub project it should be possible to secrete every compound which is produced in the modified compartment to the supernatant. The secretion of products from the compartment is not trivial. By using peroxisomes as a chassis there is no natural secretion described.

We overcome this problem by using the concept of Sagt and colleagues named peroxicretion (Sagt et al. 2009). They used a vesicle snare (v-Snare) fused to a peroxisome membrane-protein to secrete the content of peroxisomes. V-Snares interact with the target Snare (T-Snare) at the cell membrane, which leads to a fusion of the vesicle with the membrane (Chen, Scheller 2001). They applied this system in *Aspergillus niger*. We will adapt this system to secret the content of our modified compartments.

For the application of this system in *Saccharomyces cerevisiae* we use the v-Snare Snc1 to decorate our compartments. The v-Snare is used truncated without the transmembrane Domain (Gerst 1997). To target the compartments with the snare we use a peroxisomal transmembrane protein. In our case we use the protein Pex15 and fuse Snc1 to the N-terminus. The version we use is the truncated version Pex15 (315-383) (Figure 1).

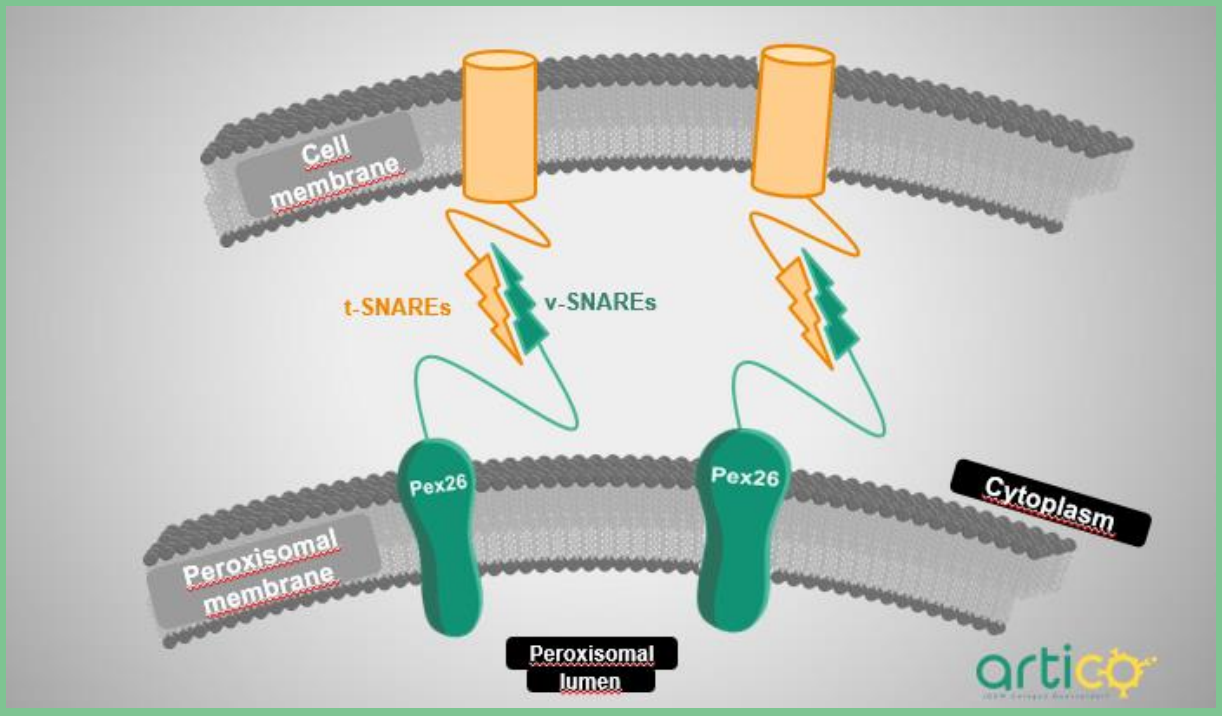


Figure 1 Concept of secreting peroxisomal contents to the supernatant. (A) For the secretion, the membrane anchor Pex15 in a truncated version is used (red). This anchor is used to anchor the v-Snare Snc1 (green) to peroxisomes or our modified compartments. (B) For the secretion Snc1 interacts with the T-Snares (green) in the cell membrane. Induced from this interaction the vesicle and cell membrane fuse and the content of the compartment is secreted to the supernatant.

We check our secretion with two different steps. First, we do a Western Blot. For this Western Blot, we use sfGFP which is targeted to the peroxisome by a Pts1 sequence. We will use the supernatant of a culture to check if sfGFP is secreted succesfully.

Second, we will use Beta-Glucuronidase (Gus) as a reporter protein. In 2012 Stock and colleagues described the Gus reporter assay for unconventional secretion. With the Gus reporter assay it is possible to determine if a Protein is secreted conventional and *N*-glycosylated or secreted unconventional, bypassing the endomembrane system and is not *N*-glycosylated. Gus is a bacterial protein with a *N*-glycosylation-site, which is just active if the protein is not *N*-glycosylated. The activity from Gus could be measured with different reagents in plate or liquid assays. Liquid assays can be applied qualitatively as well as quantitatively to measure differences in the activity. If Gus is secreted by the conventional pathway the *N*-glycosylation leads to an inactivation of the enzyme. Gus will be imported to the peroxisome with the Pts1 sequence and measured quantitatively in the supernatant (Fig 2) (Stock et al. 2012).

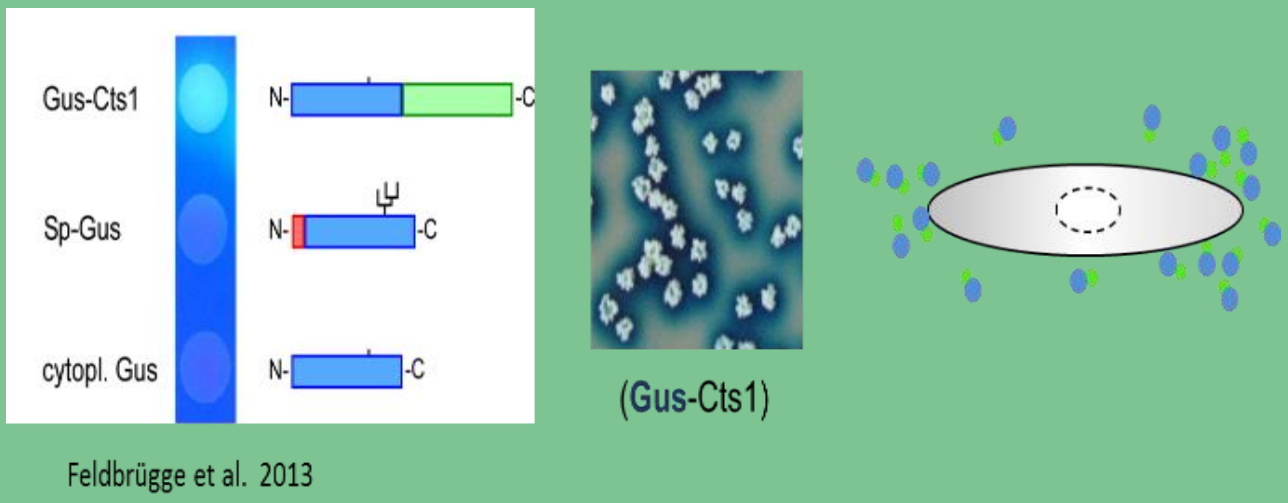


Figure 2: the Gus Assay. Gus secreted with an unconventional secreted protein like Cts1 from *Ustilago maydis* active in the supernatant. Gus secreted with a conventional Signal Peptide (SP) is not active in the supernatant. If Gus is in the cytoplasm there is also no activity (Lysis control) (Feldbrügge et al. 2013).

Literature

Berlec, Aleš; Strukelj, Borut (2013): Current state and recent advances in biopharmaceutical production in *Escherichia coli*, yeasts and mammalian cells. In *Journal of industrial microbiology & biotechnology* 40 (3-4), pp. 257–274. DOI: 10.1007/s10295-013-1235-0.

Chen, Y. A.; Scheller, R. H. (2001): SNARE-mediated membrane fusion. In *Nature reviews. Molecular cell biology* 2 (2), pp. 98–106. DOI: 10.1038/35052017.

Feldbrügge, Michael; Kellner, Ronny; Schipper, Kerstin (2013): The biotechnological use and potential of plant pathogenic smut fungi. In *Applied microbiology and biotechnology* 97 (8), pp. 3253–3265. DOI: 10.1007/s00253-013-4777-1.

Gerst, Jeffrey E. (1997): Conserved α-Helical Segments on Yeast Homologs of the Synaptobrevin/VAMP Family of v-SNAREs Mediate Exocytic Function. In *J. Biol. Chem.* 272 (26), pp. 16591–16598. DOI: 10.1074/jbc.272.26.16591.

Keller, K.; Friedmann, T.; Boxman, A. (2001): The bioseparation needs for tomorrow. In *Trends in biotechnology* 19 (11), pp. 438–441.

Sagt, Cees M. J.; Haaft, Peter J. ten; Minneboo, Ingeborg M.; Hartog, Miranda P.; Damveld, Robbert A.; van der Laan, Jan Metske et al. (2009): Peroxicretion: a novel secretion pathway in the eukaryotic cell. In *BMC biotechnology* 9, p. 48. DOI: 10.1186/1472-6750-9-48.

Stock, Janpeter; Sarkari, Parveen; Kreibich, Saskia; Brefort, Thomas; Feldbrügge, Michael; Schipper, Kerstin (2012): Applying unconventional secretion of the endochitinase Cts1 to export heterologous proteins in *Ustilago maydis*. In *Journal of biotechnology* 161 (2), pp. 80–91. DOI: 10.1016/j.jbiotec.2012.03.004.

Index card 2: Violacein pathway

Metabolic engineering of microorganisms promises to enable the environmental friendly production of fuels, bulk chemicals and therapeutics. A chief consideration for optimizing production in microbial hosts is a limiting crosstalk between high-flux engineered metabolic pathways and the native cellular processes of the production host. Eukaryotes address the problem of metabolic crosstalk by partitioning proteins and metabolites in membrane-bound organelles to sequester toxic compounds, direct the activity of enzymes towards specific substrates, and establish distinct chemical environments (for example, altered pH or redox state).

Although the optimal parameters for peroxisomal protein import remain an open question, the biological mechanism has been extensively studied. Peroxisomal matrix proteins generally contain one of two peroxisomal targeting signals (PTS1 or PTS2) that are recognized in the cytosol by corresponding receptor proteins, Pex5p and the Pex7p/Pex18p/Pex21p complex, respectively. Upon recognition, these proteins are recruited to the Pex13p/Pex14p/Pex17p import pore complex and translocated into the peroxisomal lumen while remaining in the folded state. The majority of native cargo proteins enter via some variant of the PTS1 tag, which canonically consists of Ser-Lys-Leu (SKL) at the carboxy-terminus. Numerous studies have demonstrated that fusion of this three-amino-acid tag is sufficient to redirect cytosolic proteins into the peroxisome [...].

Development of a sensitive peroxisomal protein import assay: The first step in expressing a heterologous pathway in the peroxisome is ensuring that each enzyme is effectively compartmentalized. Although protein import is commonly monitored using fluorescence

microscopy, we sought a method that would directly test the primary metric of interest for compartmentalizing new enzymatic pathways—cytosolic activity of an enzyme before peroxisomal import. For this purpose, we turned to a three-enzyme pathway from the bacterium Chromobacterium violaceum that converts tryptophan to the green pigment prodeoxyviolacein (PDV).

"Towards repurposing the yeast peroxisome for compartmentalizing heterologous metabolic pathways" WC Deloache, ZN Russ, JE Dueber; 11 Nov 2015; nature communications

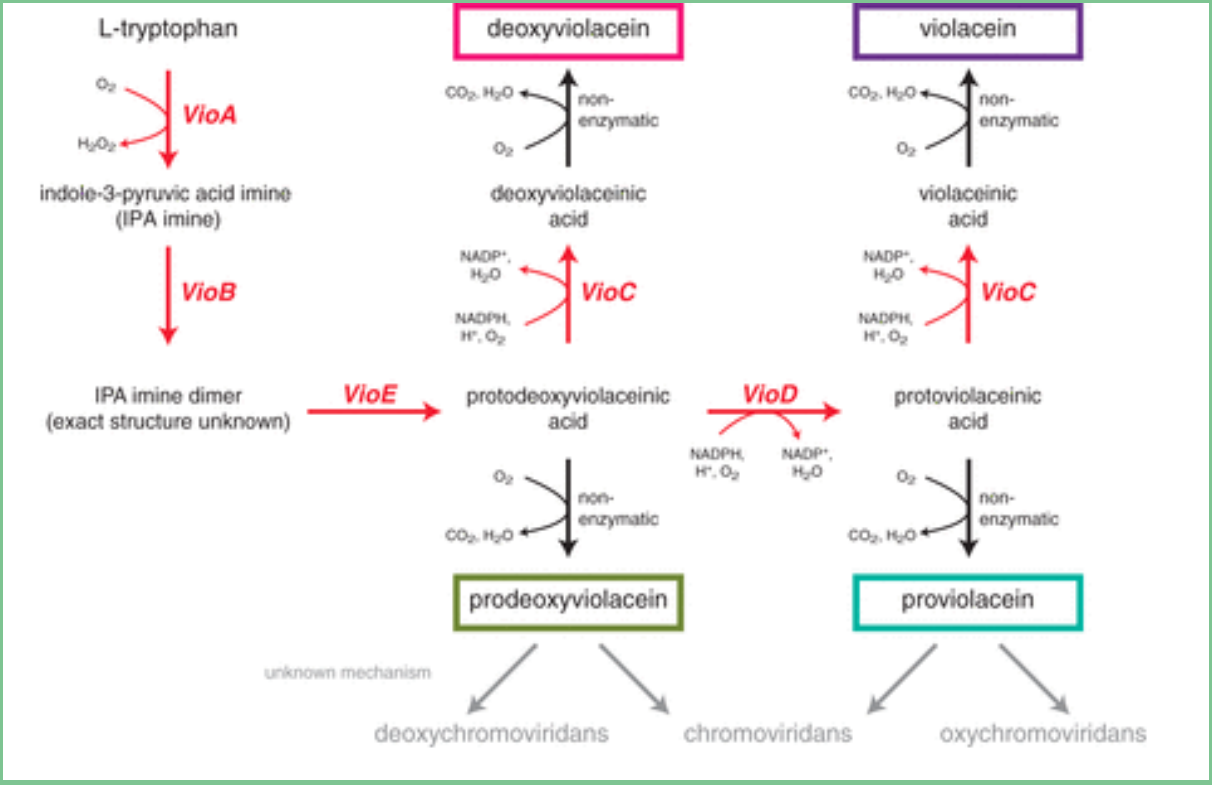


Figure 1: Expression-level optimization of a multi-enzyme pathway in the absence of a high-throughput assay

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4997675/#CR14

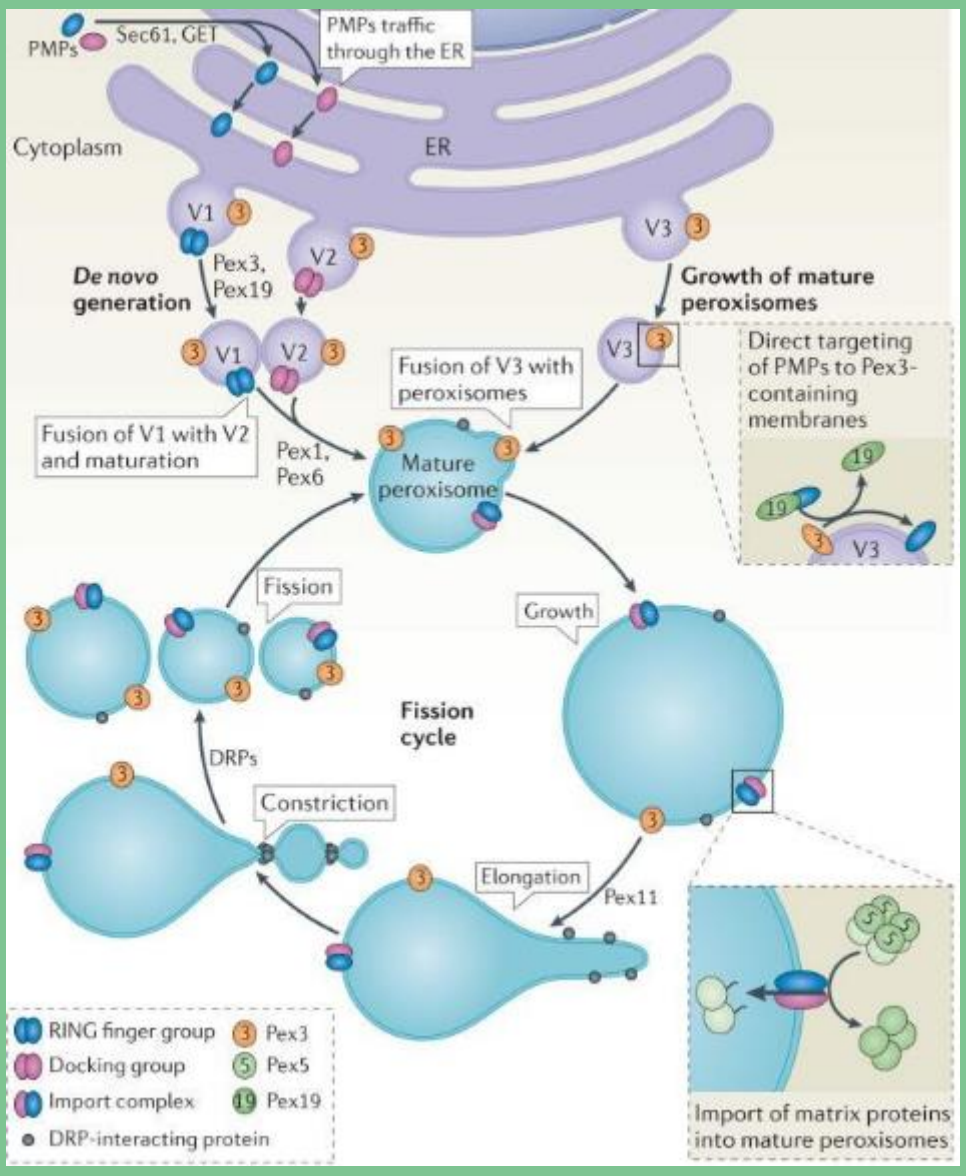
Index card 3: Size and Number

The PEX11 peroxisomal membrane proteins are the only factors known to promote peroxisome division in multiple species. It has been proposed that PEX11 proteins have a direct role in peroxisomal fatty acid oxidation, and that they only affect peroxisome abundance indirectly. Here we show that PEX11 proteins are unique in their ability to promote peroxisome division, and that PEX11 overexpression promotes peroxisome division in the absence of peroxisomal metabolic activity. We also observed that T mouse cells lacking PEX11 display reduced peroxisome abundance, even in the absence of peroxisomal metabolic substrates, and that PEX11/ mice are partially deficient in two distinct peroxisomal metabolic pathways, ether lipid synthesis and very long chain fatty acid oxidation. Based on these and other observations, we propose that PEX11 proteins act directly in peroxisome division, and that their loss has indirect effects on peroxisome metabolism.

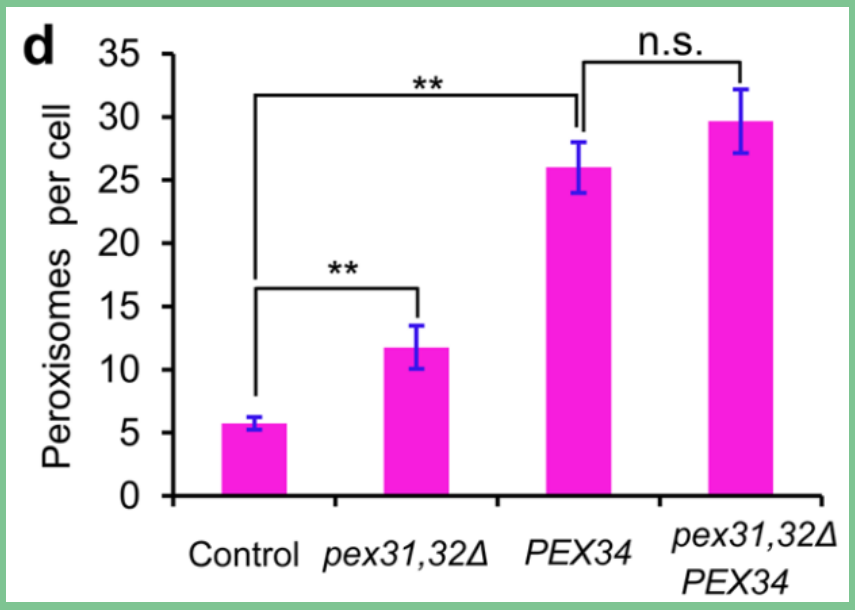
"PEX11 promotes peroxisome division independently of peroxisome metabolism", *Xiaoling Li and Stephen J. Gould, Department of Biological Chemistry, The Johns Hopkins University School of Medicine, Baltimore, MD 21205*

The Saccharomyces cerevisiae peroxisomal membrane protein Pex11p has previously been implicated in peroxisome proliferation based on morphological observations of PEX11 mutant cells. Pex11p-deficient cells fail to increase peroxisome number in response to growth on fatty acids and instead accumulate a few giant peroxisomes.

"Pex11p Plays a Primary Role in Medium-Chain Fatty Acid Oxidation, a Process that Affects Peroxisome Number and Size in Saccharomyces cerevisiae" *Carlo W.T. van Roermund,* Henk F. Tabak,‡ Marlene van den Berg,‡ Ronald J.A. Wanders,*§and Ewald H. Hettema‡ *Department of Clinical Chemistry, ‡ Department of Biochemistry and § Department of Pediatrics, Emma Children's Hospital, University of Amsterdam, Academic Medical Center, 1105 AZ Amsterdam, The Netherlands*



"Peroxisomes can form through two pathways" Nat Rev Mol Cell Biol. 2013 Dec; 14(12): 803–817.



"Harnessing Yeast Peroxisomes for Biosynthesis of Fatty-Acid-Derived Biofuels and Chemicals with Relieved Side-Pathway Competition" Zhou Y. J., Buijs N. A., (2016)

Harnessing Yeast Peroxisomes for Biosynthesis of Fatty-Acid-Derived Biofuels and Chemicals with Relieved Side-Pathway Competition

As S. cerevisiae contains only a very small number of peroxisomes under glucose-rich conditions (Figure S2), it is feasible to increase the peroxisome population for enhanced biosynthesis. Here we show that deletion of PEX31 and PEX32 resulted in more and larger peroxisomes and further increased the biosynthesis of fatty acyl-CoA-derived fatty alcohols (Figure 4b and d). However, there was much less of a benefit for alkane production, whereas the byproduct fatty alcohols increased by 50% (Figure 4c). Furthermore, the peroxisomal membrane protein Pex3-GFP was not properly targeted to the peroxisomal membrane (Figure 4d). All these results suggested that the pex31,32Δ strain may have an altered peroxisomal membrane

structure, which could have resulted in an increased leakage of fatty aldehydes to the cytosol for ALR/ADHs-catalyzed fatty alcohol biosynthesis. As the peroxisome membrane is permeable for metabolites smaller than 400 Da, 13 the fatty aldehydes could partly diffuse across the peroxisome membrane. This is in agreement with the fact that a small amount of fatty alcohol accumulation was observed for the peroxisomal pathways and with the observation that deletion of HFD1 is still crucial for peroxisomal alkane biosynthesis (Figure 3c). In contrast, PEX34 overexpression improved alkane production without elevating fatty alcohol accumulation, which might indicate that this constitutively expressed peroxin promoted peroxisome proliferation without increasing membrane permeability. During the preparation of our manuscript, a similar study highlighted the peroxisomal membrane permeability by constructing the peroxisomal prodeoxyviolacein biosynthetic pathway. 52 Our observations here further bring to the attention that engineering peroxisome proliferation may affect the peroxisome (membrane) structure and function, which could affect primary metabolism beyond fatty acid degradation, because peroxisomes play an essential role in those processes, too. 53 Our study therefore also provides new understanding of the biogenesis and metabolism of peroxisomes in yeast.

Index card 4: Import via Pex5

Compartmentalization of enzymes into organelles is a promising strategy for limiting metabolic crosstalk and improving pathway efficiency, but improved tools and design rules are needed to make this strategy available to more engineered pathways.

The Difference in Recognition of Terminal Tripeptides as Peroxisomal Targeting Signal 1 between Yeast and Human Is Due to Different Affinities of Their Receptor Pex5p to the Cognate Signal and to Residues Adjacent to it.

"Towards repurposing the yeast peroxisome for compartmentalizing heterologous metabolic pathways." DeLoache, William C., Zachary N. Russ, and John E. Dueber. *Nature communications* 7 (2016).

Compartmentalization of metabolic processes in eukaryotic cells requires that participating enzymes be transported to their correct subcellular location. Proteins destined to the peroxisomal matrix are imported from the cytosol (1) in a process involving specific targeting signals within the primary structure. So far, two different signals that are sufficient for transporting proteins to the peroxisomal matrix have been defined: a C-terminal peroxisomal targeting signal 1 (PTS1)1 that is present in the majority of peroxisomal matrix proteins and first identified in luciferase, and a PTS2 that is usually located within the N-terminal 30 amino acids of some peroxisomal proteins.

"The difference in recognition of terminal tripeptides as peroxisomal targeting signal 1 between yeast and human is due to different affinities of their receptor Pex5p to the cognate signal and to residues adjacent to it." Lametschwandtner, Guenther, et al. *Journal of Biological Chemistry* 273.50 (1998): 33635-33643.

The import of folded matrix proteins is mediated by cycling receptors that shuttle between the cytosol and peroxisomal lumen. Receptor release back to the cytosol represents the ATP-

dependent step of peroxisomal matrix protein import, which consists of two energy-consuming reactions: receptor ubiquitination and dislocation.

"The peroxisomal protein import machinery." Platta, Harald W., and Ralf Erdmann. *FEBS letters* 581.15 (2007): 2811-2819.

Peroxisomal matrix proteins have to be imported into their target organelle post-translationally. The major translocation pathway depends on a C-terminal targeting signal, termed PTS1. Our previous analysis of sequence variability in the PTS1 motif revealed that, in addition to the known C-terminal tripeptide, at least nine residues directly upstream are important for signal recognition in the PTS1–Pex5 receptor complex.

"Prediction of peroxisomal targeting signal 1 containing proteins from amino acid sequence." Neuberger, Georg, et al. *Journal of molecular biology* 328.3 (2003): 581-592.

Originally, the peroxisomal targeting signal 1 (PTS1) was defined as a tripeptide at the C-terminus of proteins prone to be imported into the peroxisomal matrix. The corresponding receptor PEX5 initiates the translocation of proteins by identifying potential substrates via their C-termini and trapping PTS1s through remodeling of its TPR domain. Thorough studies on the interaction between PEX5 and PTS1 as well as sequenceanalytic tools revealed the influence of amino acid residues further upstream of the ultimate tripeptide. Altogether, PTS1s should be defined as dodecamer sequences at the C-terminal ends of proteins. These sequences accommodate physical contacts with both the surface and the binding
cavity of PEX5 and ensure accessibility of the extreme C-terminus.

"Peroxisome targeting signal 1: is it really a simple tripeptide?" Brocard, Cécile, and Andreas Hartig. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research* 1763.12 (2006): 1565-1573.

Index card 5: Nootkatone

Extraction of (+)-nootkatone from natural sources, e.g. citrus fruits, typically suffers from inadequate yields owing to slow biomass accumulation, low overall (+)-nootkatone concentrations and annual harvest fluctuations. Thus, chemical methods for (+)-nootkatone synthesis have been applied to satisfy the high industrial demand. As chemical synthesis often involved toxic heavy metals, highly flammable compounds or strong oxidants, more attention is being paid to environment-friendly and safe methods for (+)-nootkatone synthesis. Several approaches for de novo (+)-nootkatone synthesis or the biotransformation of the abundantly available (+)-valencene to the rare (+)-nootkatone have been reported. Whole-cell systems employing bacteria, fungi and plants or applications of cell extracts and/or purified proteins as biocatalysts have been described. Many of these biotransformation reactions are catalyzed by enzymes of the cytochrome P450 monooxygenase (CYP) superfamily, although the screening and identification of efficient and regio-selective P450 enzymes for commercial applications is still challenging."

"Production of the sesquiterpenoid (+)-nootkatone by metabolic engineering of Pichia pastoris." Wriessnegger, Tamara, et al. *Metabolic engineering* 24 (2014): 18-29.

(+)-Nootkatone is an important oxidised sesquiterpene for the flavour and fragrance industry. It has a characteristic grapefruit-like flavour and a low odour threshold. Natural (+)-nootkatone can be extracted from grapefruit. Since grapefruit material is limited on the world market, synthetic (+)-nootkatone produced from (+)-valencene is predominantly used in commercial applications, also for the flavour market. Chemical oxidation of valencene requires the use of tert-butyl chromate, which is a carcinogenic substance. Alternatively non-carcinogenic tert-butyl peracetate or tert-butyl hydroperoxide can be used which are

highly flammable and corrosive compounds. (+)-Nootkatone may also be produced from (+)-valencene via biotechnological approaches in recombinant organisms using the enzymes involved in its biosynthesis. The biosynthetic route to (+)-nootkatone in grapefruit has not been established experimentally, but has been suggested to start from valencene on which a regioselective allylic hydroxylation results in formation of 2-hydroxyvalencene (or nootkatol), followed by oxidation to (+)-nootkatone.

"A chicory cytochrome P450 mono-oxygenase CYP71AV8 for the oxidation of (+)-valencene." Cankar, Katarina, et al. *FEBS letters* 585.1 (2011): 178-182.

Bioconversion assays led to production of β-nootkatol and nootkatone, but with low yields that decreased upon increase of the substrate concentration. The reasons for this low bioconversion efficiency were further investigated and several factors potentially hampering industry-compatible valencene bioconversion were identified. One is the toxicity of the products for yeast at concentrations exceeding 100 mg L−1. The second is the accumulation of β-nootkatol in yeast endomembranes. The third is the inhibition of the CYP71D51v2 hydroxylation reaction by the products.

"Challenges and pitfalls of P450-dependent (+)-valencene bioconversion by Saccharomyces cerevisiae." Gavira, Carole, et al. *Metabolic engineering* 18 (2013): 25-35.

Terpenes are structurally diverse compounds that are of interest because of their biological activities and industrial value. These compounds consist of chirally rich hydrocarbon backbones derived from terpene synthases, which are subsequently decorated with hydroxyl substituents catalyzed by terpene hydroxylases. Availability of these compounds is, however, limited by intractable synthetic means and because they are produced in low amounts and as complex

mixtures by natural sources. We engineered yeast for sesquiterpene accumulation by introducing

genetic modifications that enable the yeast to accumulate high levels of the key intermediate farnesyl diphosphate (FPP). Co-expression of terpene synthase genes diverted the enlarged FPP pool to greater than 80 mg/L of sesquiterpene. Efficient coupling of terpene production with hydroxylation was also demonstrated by coordinate expression of terpene hydroxylase activity, yielding 50 mg/L each of hydrocarbon and hydroxylated products. The yeast now provides a convenient format for investigating catalytic coupling between terpene synthases and hydroxylases, as well as a platform for the industrial production of high value, single-entity and stereochemically unique terpenes.

"Metabolic engineering of sesquiterpene metabolism in yeast." Takahashi, Shunji, et al *Biotechnology and bioengineering* 97.1 (2007): 170-181.

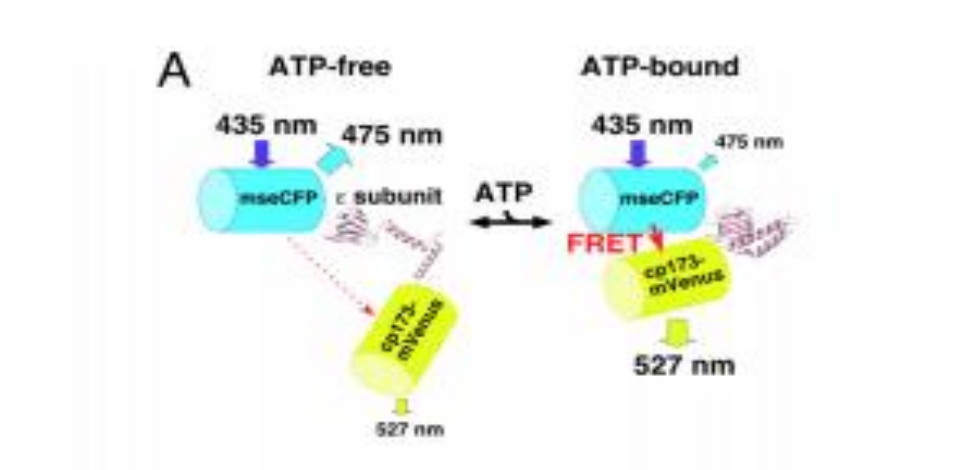
Index card 6: Biosensors

Genetically encoded Förster resonance enrgy transfer(FRET) nanosensors provide a unique tool enabeling dynamic quantitation of metabolite analysis with subcellular resolution.The nanosensors have been developed for a variety of sugars and amino acids. […]

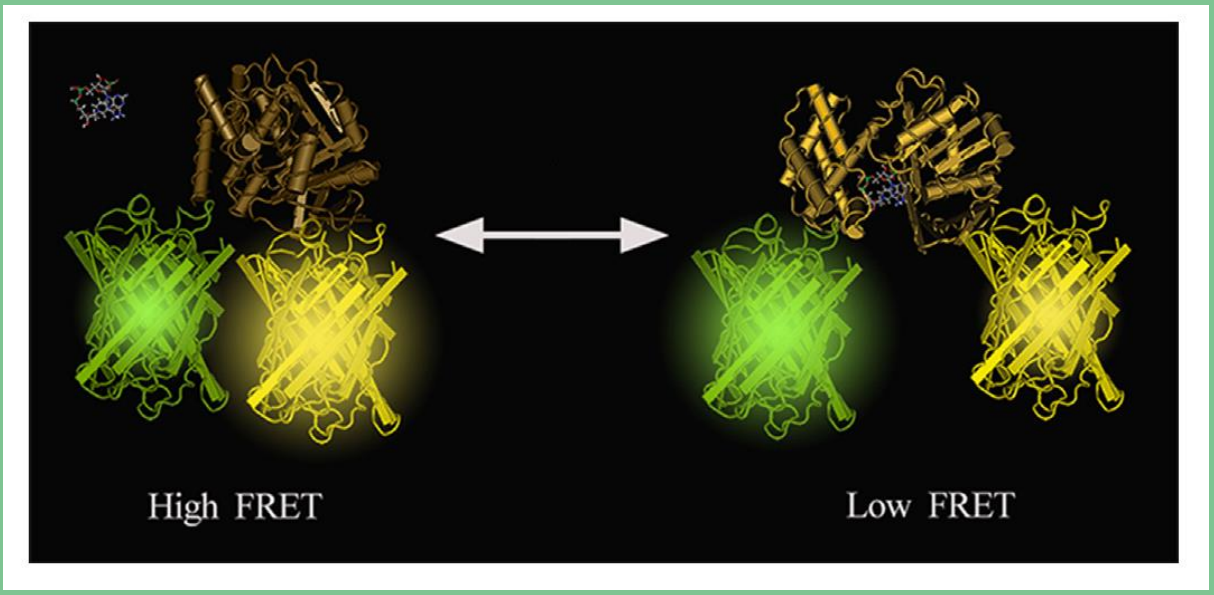
Visualisation of ATP levels inside single living cells with fluorescence resonance energy transfer-based genetically encoded indicators.

For example, Ateam might become a strong tool to study energy metabolism and mitochondrial function. In addition, estimation of intracellular ATP levels by Ateam might be a good way to evaluate cell viability or toxicity of chemicals, because dead cells lose intracellular ATP.

"Dynamic analysis of cytosolic glucose and ATP levels in yeast using optical sensors." Bermejo, Clara, et al. *Biochemical Journal* 432.2 (2010): 399-406.



"Visualization of ATP levels inside single living cells with fluorescence resonance energy transfer-based genetically encoded indicators." Imamura, Hiromi, et al. *Proceedings of the National Academy of Sciences* 106.37 (2009): 15651-15656.



"A genetically encoded biosensor for in vivo and in vitro detection of NADP, Biosensors and Bioelectronics", Feng-Lan Zhao, Chang Zhang, Chen Zhang, Yun Tang, Bang-Ce Ye, Volume 77, 2016, Pages 901-906, ISSN 0956-5663

Biosensors can be defined broadly as molecules (typically RNA's or proteins) or cells that report analysis or processes in live organisms or in their environment.

"Quantitative imaging with fluorescent biosensors." Okumoto, Sakiko, Alexander Jones, and Wolf B. Frommer. *Annual review of plant biology* 63 (2012): 663-706.

A significant advantage of biosensors is the ability to collect real time data to study the kinetics of metabolite variation. In this study we detected intracellular NADP+ in *E. coli* cells to verify the capabilities of a reducing equivalents pool for metabolic systems. The pathway known to yield NADP+ is the phosphorylation of NAD by NAD kinase.

"A genetically encoded biosensor for in vitro and in vivo detection of NADP+." Zhao, Feng-Lan, et al. *Biosensors and Bioelectronics* 77 (2016): 901-906.