# Introduction

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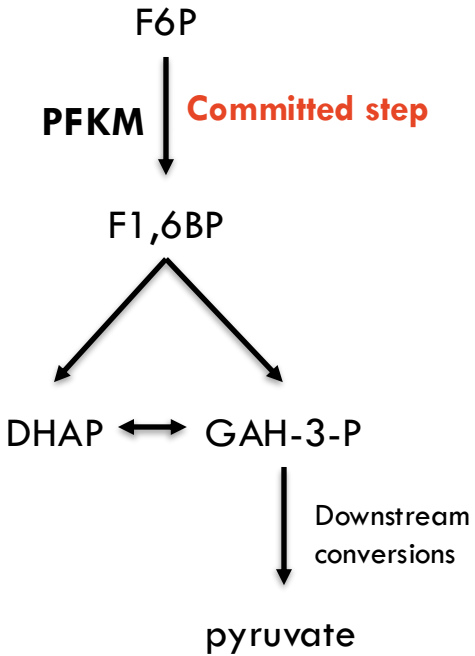
Nicole Lavinia and Cristina Yazbeck

# Glycogen Storage Disease 7 (GSD)

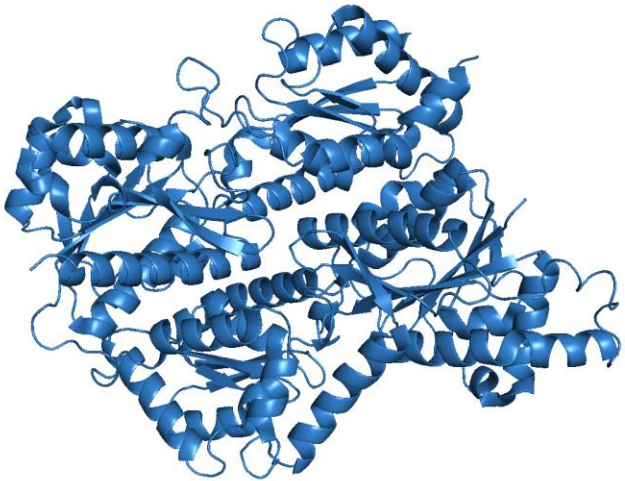
## (Tarui Disease)

| | |
|---|---|
| Inheritance | Autosomal recessive disorder |
| Symptoms | Exercise intolerance, cramps, myoglobinuria, mild hemolysis<br>Musumeci *et al.* (2012) |
| Cause | **Mutations** in PFKM gene<br>e.g. Missense Mutations in binding sites of PFKM<br>• Altered allosteric regulation of PFK leads to reduced enzyme activity<br>Bioinformatics work – variants in P39, *57*,180, 209, 309 are all within the human *N*-terminal catalytic PFK domain 1 (P2-390). |

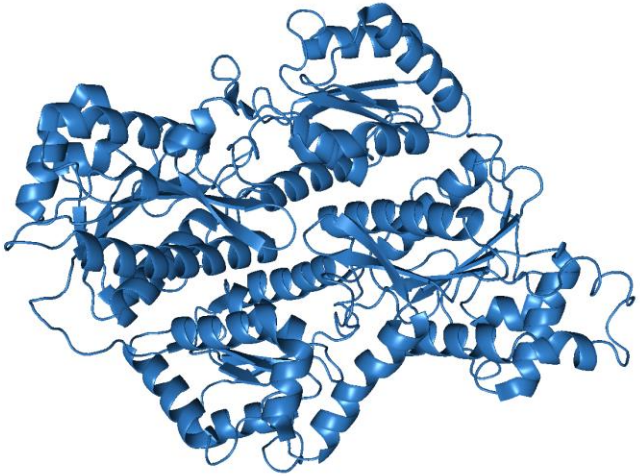



(Brüser, Kirchberger and Schöneberg, 2012)

# ΔPFK2 *S. cerevisiae* as a Model for Human GSD



~43% identity



*S. cerevisiae* PFK2
PDB Accession: 3O8O

Produces a potent allosteric modulator of PFK1 activity (F2,6-BP)

Human PFK (Muscle Isoform)
PDB Accession: 4OMT

Catalyses conversion of F6P to F1,6-BP

*Can we effectively model human Glycogen Storage Disease 7 using an S. cerevisiae ΔPFK2 model?*

## Inquiry Question

## Hypotheses

1. If PFK2 deletion creates a deficit in the glycolytic pathway, then we should observe increased flux towards the pentose phosphate pathway (PPP) to ensure stable ATP/NAPDH production.

2. If this expected metabolic reprogramming alters or prevents glucose-based signalling (e.g. via TORC1), then we should observe an increase in closely-associated cellular processes including fatty acid oxidation, lipid biosynthesis, and autophagy.

3. If we can correlate these cellular changes to an absence of PFK2, then we can assume a similar phenotype in GSD7; however, we expect that some physiological complexity will not be recapitulated by a yeast model.

# Project Aims

1. Infer any **metabolic flux** reprogramming that occurs in *S. cerevisiae* when glycolysis is impaired

2. Apply **network theory** to identify new gene/protein targets which report a high centrality relative to PFK2, and therefore, when PFK2 is removed
*(Collaboration with MATH3888)*

3. Validate whether the PFK2 deletion is a **comprehensive model system** for human GSD

**Pho85:** Negative regulator of glycogen synthase and starvation-induced autophagy

**Tkl1:** Transketolase of the non-oxidative branch of the pentose-phosphate pathway

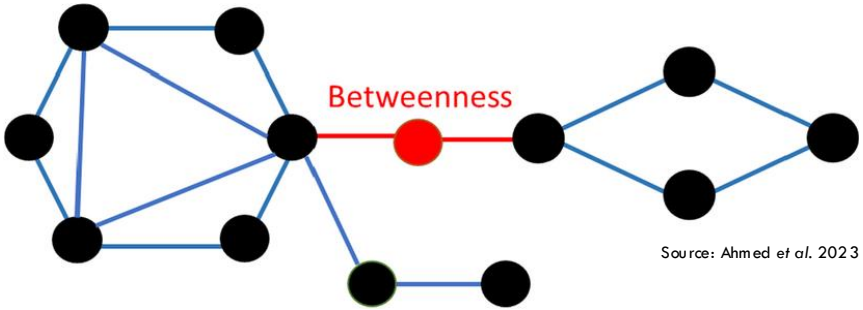
**Revise current experimental approaches**

# Further Network Theory

1. Betweenness Centrality
2. Percolation Centrality

Cristina Yazbeck

# Betweenness Centrality




Source: Ahmed *et al.* 2023

$$g(v) = \sum_{s \neq v \neq t} \frac{\sigma_{st}(v)}{\sigma_{st}}$$

Useful for identification of:

- Bottlenecks
- Biologically important participants

# Key Parameter: Inclusion of Essential Proteins (EPs)*

Where EPs are maintained in the network:

| Gene | Distance from PFK2 | Importance | Essential |
|---|---|---|---|
| ACT1 | 2 | 0.0515 | Y |
| YPT1 | 4 | 0.0329 | Y |
| CDC28 | 3 | 0.0250 | Y |
| RPS31 | 3 | 0.0232 | Y |
| UBI4 | 2 | 0.0201 | N |
| HHT1 | 2 | 0.0192 | Y |
| RPS3 | 2 | 0.0154 | Y |
| PUF3 | 3 | 0.0150 | N |

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| Gene | Distance from PFK2 | Importance |
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| UBI4 | 3 | 0.0456 |
| HHT1 | 2 | 0.0428 |
| TOR1 | 3 | 0.0262 |
| BI4 | 4 | 0.0213 |
| TRP5 | 3 | 0.0207 |
| URA3 | 2 | 0.0201 |
| GUA1 | 3 | 0.0200 |
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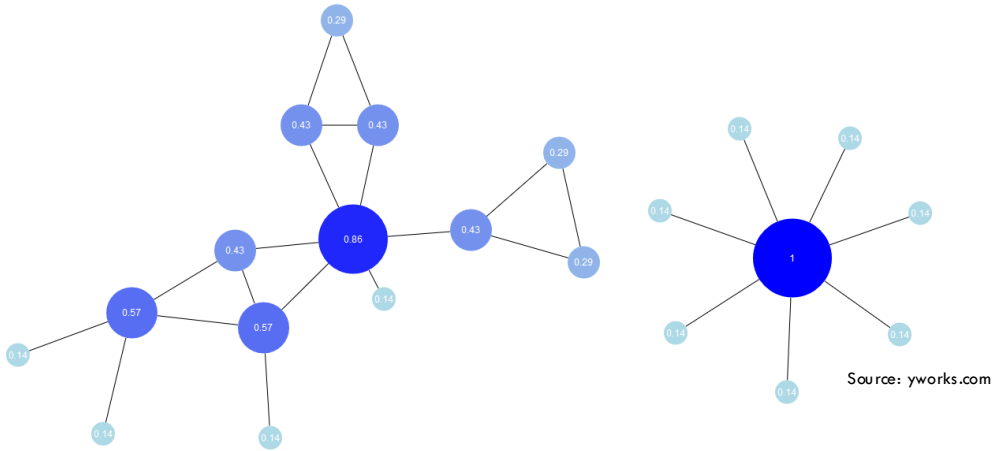
Non-essential; Confers oxidative stress resistance (Consistent with preliminary viability assay results)

PFK2 may be linked to 'salvage pathways' at the genome level

*EPs refer to 'Inviable Proteins' on the Saccharomyces Genome Database

# Percolation Centrality

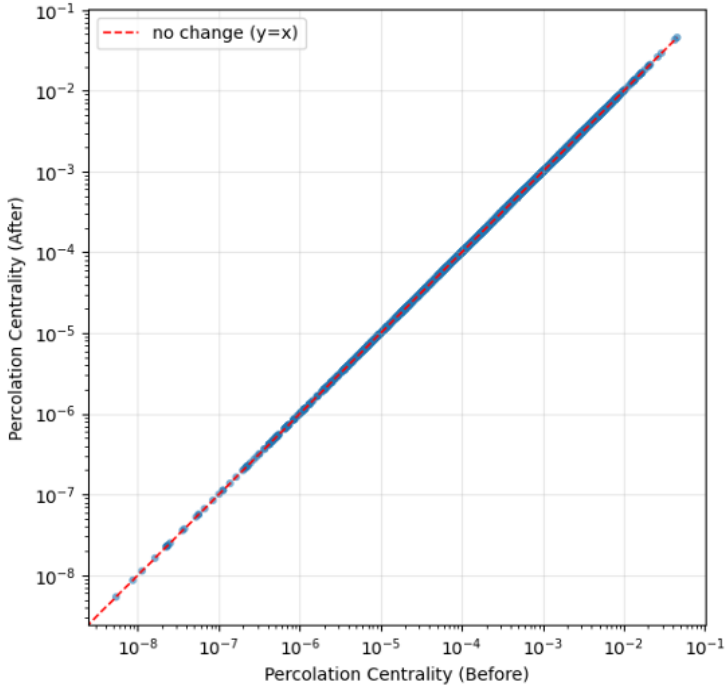



$$PC(v) = \frac{1}{N-2} \sum_{s \neq v \neq t} \frac{\sigma_{st(v)}}{\sigma_{st}} \frac{x_s}{[\sum_i x_i] - x_v}$$

All paths are important, but some paths are more important than others…

# Percolation Centrality Shift

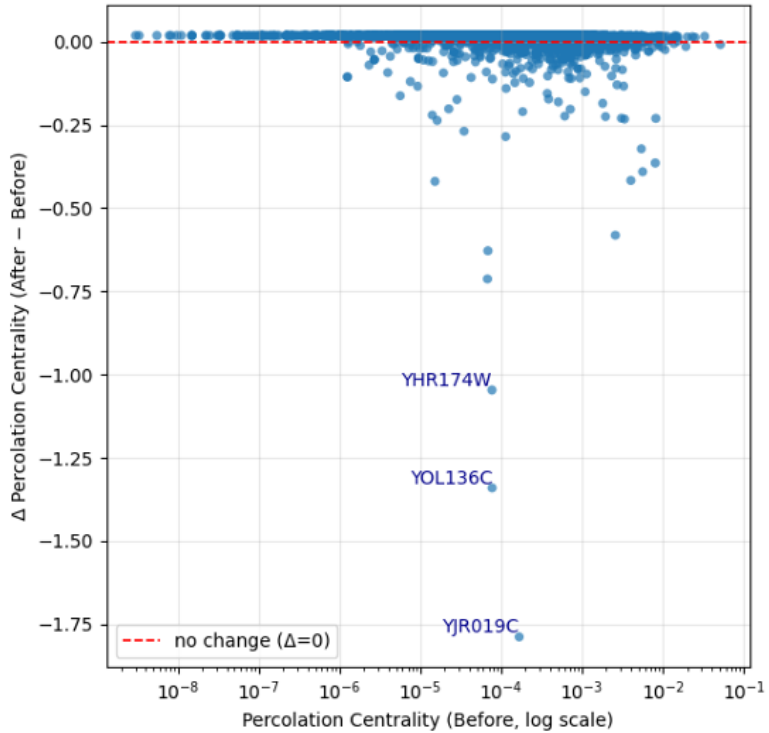
- Knocking out PFK2 from the network does not have a significant mathematical effect on the system



# Percolation Centrality Shift

- Knocking out PFK2 from the network does not have a significant mathematical effect on the system
- **Centrality** of other nodes after PFK2 is inactivated is **biochemically useful**

| | |
|---|---|
| YHR174W | *Enolase II* |
| YOL136C | *PFK27* (PFK2 alias) |
| YJR019C | *Peroxisomal acyl-CoA thioesterase* |



# ΔPFK2 *S. cerevisiae* Network

| EPs Maintained | | | | EPs Removed | | | |
|---|---|---|---|---|---|---|---|
| Gene | Distance from PFK2 | % Change | Centrality | Gene | Distance from PFK2 | % Change | Centrality |
| YPT1 | 4 | +0.0157 | 0.0329 | UBI4 | 3 | +0.0154 | 0.0456 |
| RPS31 | 3 | +0.0145 | 0.0232 | TOR1 | 3 | +0.0180 | 0.0262 |
| CDC28 | 4 | +0.0125 | 0.0250 | PUF3 | 3 | +0.0161 | 0.0292 |
| TDH3 | 1 | -0.4172 | 0.0040 | TRP5 | 3 | +0.0205 | 0.0207 |
| TDH2 | 1 | -0.3220 | 0.0054 | GCN4 | 3 | +0.0224 | 0.0164 |
| PGK1 | 1 | -0.2309 | 0.0082 | GLK1 | 1 | -0.2568 | 0.0041 |
| PYK2 | 1 | -0.3908 | 0.0056 | TDH1 | 1 | -0.4656 | 0.0045 |
| CDC19 | 1 | -0.3647 | 0.0080 | PYK2 | 1 | -0.4049 | 0.0165 |

*Mostly conserved from Betweenness Centrality analysis*

# ΔPFK2 *S. cerevisiae* Network

**EPs Maintained**

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**EPs Removed**

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| **TDH1** | **1** | **-0.4656** | **0.0045** |
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*Aligns with our initial hypotheses*

*Consistent with computational Metabolic Flux Balance simulations*

# Biochemical and Experimental Validation of New Targets: Lipid Biosynthesis

Nara Wu

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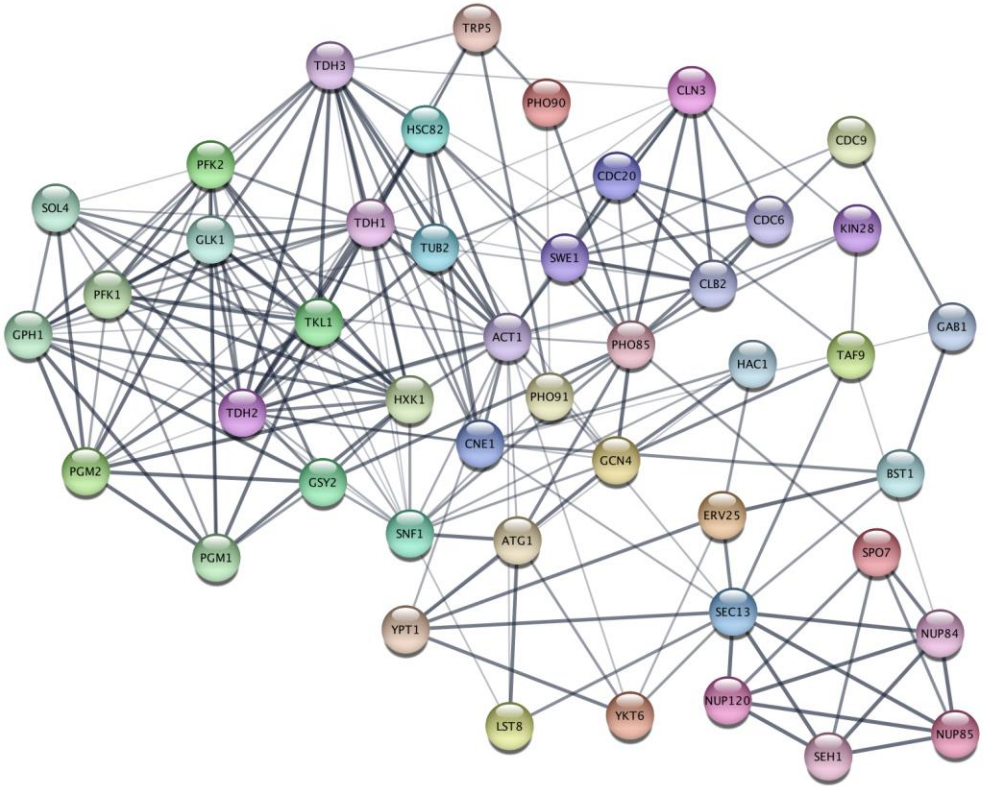
# Rationale for Target Confirmation

1. Do we expect the proteins to interact **directly** (i.e. physically) or **indirectly** based on literature evidence/pathway analysis?
2. Are the proteins localised in the same **subcellular** compartment?
3. Are there specific **conditions** under which the proteins are expressed, or are they expressed **constitutively**?
4. Can we correlate changes in the **transcription/expression** of these proteins to a **deletion** of PFK2?

# Pairwise Shortest Paths in *S. cerevisiae*

**Objective:** Identification of downstream pathways which are perturbed by PFK2 deletion



# Pairwise Shortest Paths in *S. cerevisiae*

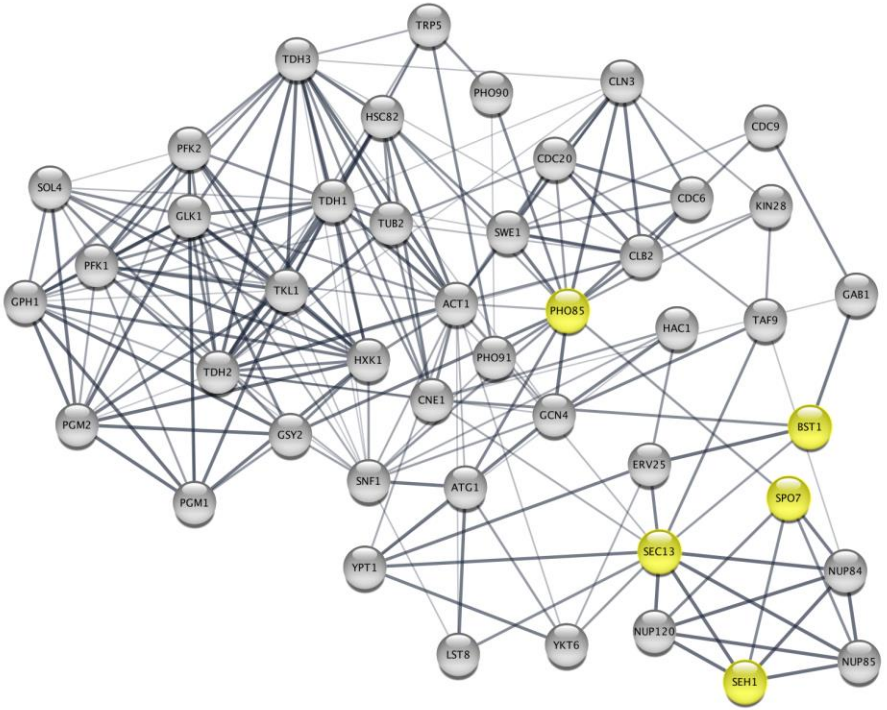
*BST1* — GPI inositol-deacylase of ER

*SEC13* — COPII-coated vesicle budding (ER-Golgi);
TORC1 activation (via inhibition of SEACIT)

*SEH1* — NPC subunit associated with ER
Positive regulation of TORC1

*SPO7* — Regulation of nuclear growth;
Activation of Pah1 which contributes to phospholipid/lipid droplet biosynthesis

*PHO85* — Cell cycle progression and response to nutrients;
Phosphorylation of Pah1



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| *SPO7* | Regulation of nuclear growth;<br>Activation of Pah1 which contributes to phospholipid/lipid droplet biosynthesis |
| *PHO85* | Cell cycle progression and response to nutrients;<br>Phosphorylation of Pah1 |

By qPCR of YMR205C, we have observed that **PHO85** is:

- **Downregulated** under low glucose conditions
- **Upregulated** under high glucose conditions

Secondary Hypothesis:

*YMR205C exhibits altered lipid biosynthesis, and this is dependent on cellular glucose levels*

# Lipid Biosynthesis in GSD

- Pah1 regulates lipid synthesis by dephosphorylating phosphatides to diacyl- and triacylglycerol (DAG/TAG)
- Pho85 phosphorylates (inactivates) Pah1, sequestering it within the cytosol
- Spo7 dephosphorylates (activates) Pah1 in conjunction with Nem1 on the ER/nuclear membrane

Association between PFK2 deletion and altered lipid biosynthesis

Low Glucose ↓ Pho85 → ↓ Pah1 phosphorylation ↑ activation → ↑ DAG/TAG synthesis

High Glucose ↑ Pho85 → ↑ Pah1 phosphorylation ↓ activation → ↓ DAG/TAG synthesis

PFK2 dysfunction indirectly influences lipid synthesis via Pho85-Nem1–Spo7–Pah1 signalling pathway

Khondker *et al.* Adv. Bio. Reg. **2022,** 84.
Su *et al.* J. Biol. Chem. **2014,** 289 (50).

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Pathway Conservation in Humans

- NEP1-R1 forms stabilizing complex with CTDNEP1 to activate Lipin-1 in a similar manner to Nem1-Spo7-Pah1 pathway in *S. cerevisae*
- Defects in CTDNEP1-NEP-R1-Lipin-1 pathway linked to disruption in lipid synthesis; Parallels *spo7Δ* and *pah1Δ* phenotypes

| Yeast Protein | Human Homologue |
|---|---|
| Pah1 | Lipin-1 |
| Spo7 | NEP1-R1 |
| Nem1 | CTDNEP1 |

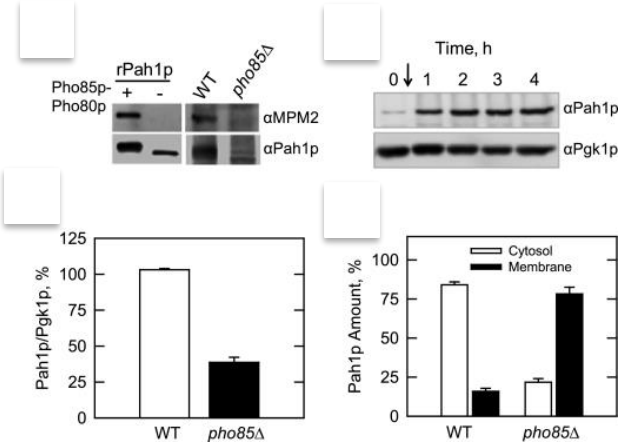


Han *et al.* J. Biol. Chem. **2012**, 287 (5).

# Lipid Biosynthesis in GSD

Existing literature

- *Pho85* mutation affects phosphorylation, abundance, and localization of Pah1
- 62% increase in Pah1 associated with membranes (activated) in Δ*pho85*



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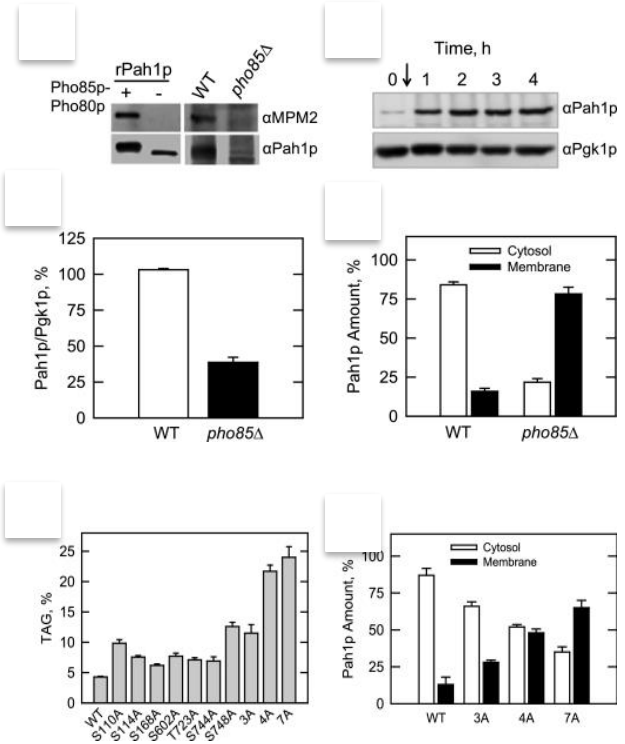
- *Pho85* mutation affects phosphorylation, abundance, and localization of Pah1
- 62% increase in Pah1 associated with membranes (activated) in Δ*pho85*
- Pho85-phosphorylation site mutations in Pah1 revealed increased TAG content
- Confirms phosphorylation-deficient forms of Pah1 bypass the requirement of Nem1-Spo7 dephosphorylation for activation

In low glucose conditions with ↓ Pho85, is Pah1 activated without Nem1-Spo7 dephosphorylation?



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# Lipid Biosynthesis in GSD

Experiments

**Aim:** Confirm whether Δ*pfk2* cells have altered lipid synthesis via Pho85-Nem1-Spo7-Pah1 activation axis

- Lipid Droplet Quantification via BODIPY Staining/Microscopy
- Determination of Pah1 Localisation (Indirect Immunofluorescence and Fixed Cell Analysis)
- Determination of Pah1 Phosphorylation (Phos-tagged SDS-PAGE)



Han *et al*. J. Biol. Chem. **2012**, 287 (5).

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Expected Results

| Condition | Pah1 Phosphorylation | Lipid Droplets | Pah1 Localization |
|---|---|---|---|
| wild-type<br>low glucose | Basal Pho85 phosphorylation and Spo7 dephosphorylation | Control | Control |
| Δ*pfk2* low glucose | ↓ Pho85 phosphorylation + Spo7 dephosphorylation | Highest lipid accumulation | Most Pah1 localized on membrane |
| Δ*pfk2* + Δ*spo7*<br>low glucose | ↓ Pho85 phosphorylation but no Spo7 dephosphorylation | High lipid accumulation<br>(same as Δ*pfk2*?) | Pah1 mainly localized on membrane |



Han *et al. J. Biol. Chem.* **2012,** 287 (5).

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| Δ*pfk2* + Δ*spo7* low glucose | ↓ Pho85 phosphorylation but no Spo7 dephosphorylation | High lipid accumulation | Pah1 mainly localized on membrane |

Similar outcomes between Δ*pfk2* and Δ*pfk2* + Δ*spo7* cells validate the hypothesis that Δ*pfk2* have ↑ lipid biosynthesis due to ↓ Pho85, allowing Pah1 activation with Spo7 under low glucose conditions



Han *et al.* J. Biol. Chem. **2012**, 287 (5).

# Biochemical and Experimental Validation of New Targets: Autophagy

Nicole Lavinia

THE UNIVERSITY OF SYDNEY

# Pairwise Shortest Paths in *S. cerevisiae*

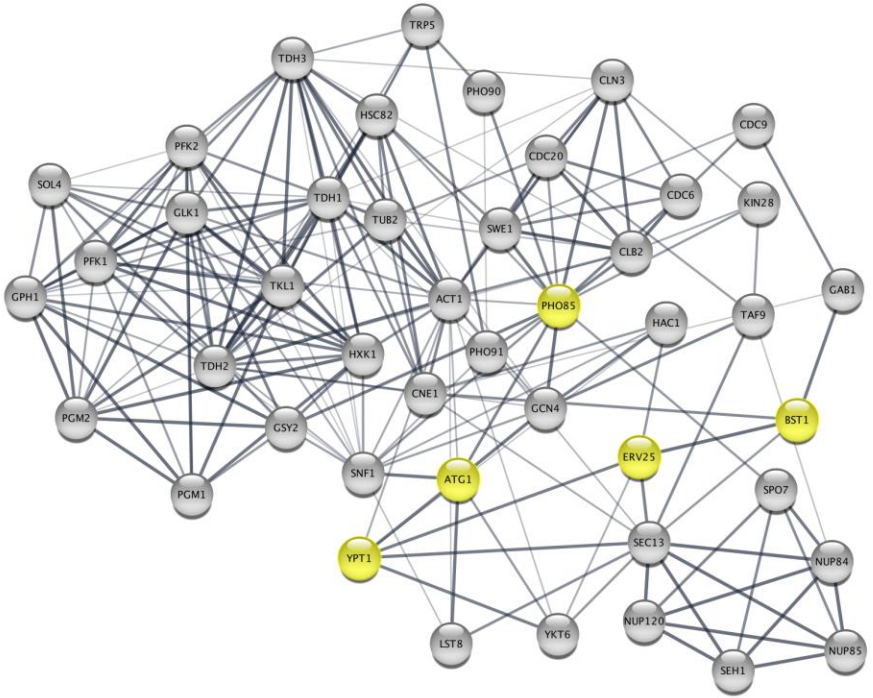
*BST1* — GPI inositol-deacylase of ER

*ERV25* — Cargo receptor complex (Emp24p); Selection & Package of cargo into COPII (ER-Golgi)

*YPT1* — Regulates vesicle docking and COPII vesicle fusion (cis-Golgi); Positive regulator of autophagy

*ATG1* — Autophagy initiation and phagophore nucleation

*PHO85* — Sensitive to nutrient signalling; Negative regulator of autophagy under nutrient-rich conditions



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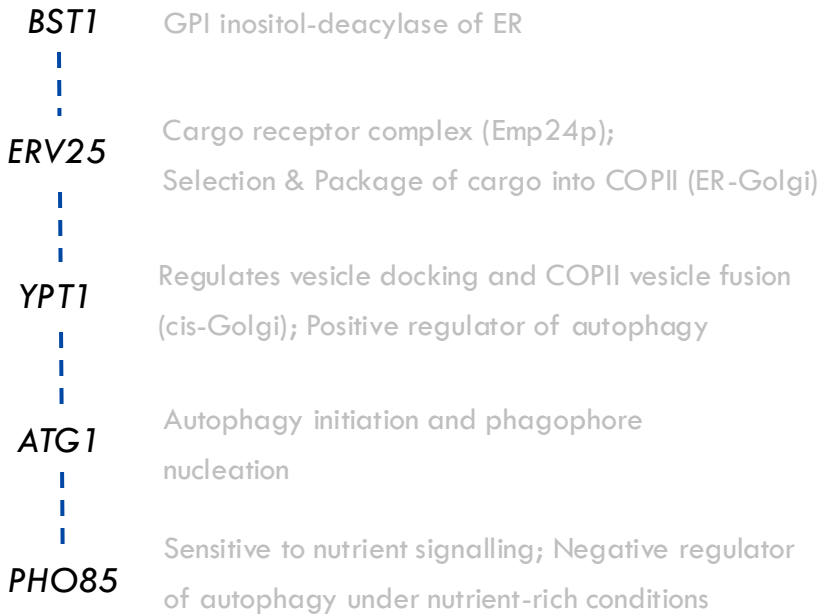



Link between Autophagy and Glycogen metabolism:

Starvation → ↓ PHO85 → ↑ Autophagy and ↑ Glycogen synthesis

<u>Secondary Hypothesis:</u>

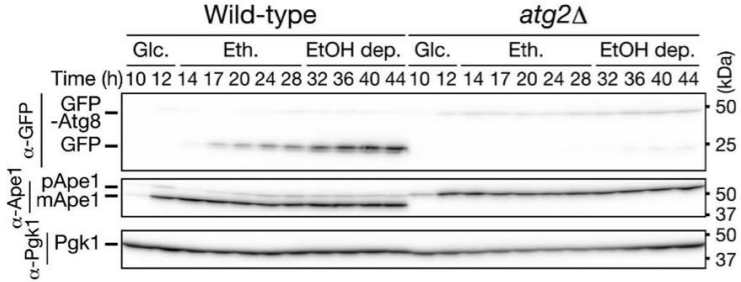
*YMR205C exhibits increased autophagy, in response to impaired glucose utilisation and low glucose levels*

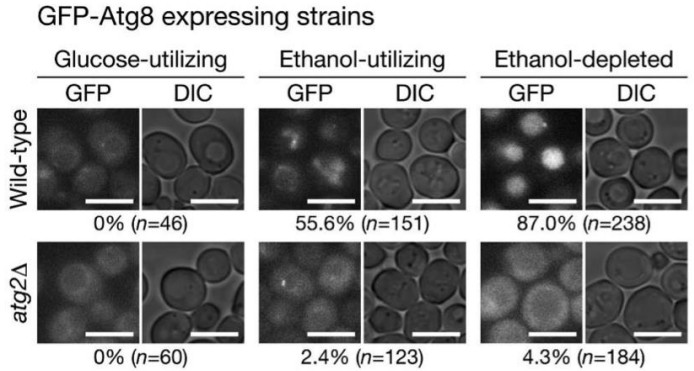
Wang *et al.*, 2001

# Autophagy in GSD

## Previous Findings

- Autophagy induced after glucose depletion in *S. cerevisiae*
- Activation observed during ethanol-utilizing and ethanol depleted phases (glucose source removed)
- GFP-Atg8 cleavage and vacuolar localization confirmed autophagy activation
- Atg2-dependent process; confirms canonical machinery
- Carbon source limitation acts as key trigger for autophagy






Iwama & Ohsumi, 2019

# Autophagy in GSD

1st stage: Immunoblot and Fluorescence Microscopy

**Measurements:**

Autophagy initiation marker: p-Atg1

Fluorescence marker: GFP-Atg8

**Aim:** Confirm that even under normal glucose, YMR205C induces increased autophagy

- Determination of Atg1 Phosphorylation
- Fluorescent Microscopy of GFP-Atg8 vacuolar localisation

| Conditions | Immunoblot (p-Atg1) | Expected Band Intensity | Fluorescent Localisation (GFP-Atg8) |
|---|---|---|---|
| WT – Normal glucose | Low/basal phosphorylation | Faint band | Diffuse cytoplasmic distribution |
| WT – Low glucose (0.05%) | Moderate increase | Moderate band | Partial vacuolar localisation |
| KO – Normal glucose | Mild increase (↓ PHO85) | Slightly stronger band | Mild vacuolar puncta |
| KO – Low glucose | Strong phosphorylation | Intense distinct band | Prominent vacuolar accumulation |

# Autophagy in GSD

1st stage: Immunoblot and Fluorescence Microscopy

2nd stage: ATG1 Knockdown

Mechanistic Confirmation: Validate autophagy observed is canonical

**Measurements:**

Autophagy initiation marker: p-Atg1

Fluorescence marker: GFP-Atg8

| Conditions | Immunoblot (p-Atg1) | Expected Band Intensity | Fluorescent Localization (GFP-Atg8) |
|---|---|---|---|
| WT – Normal glucose | Basal phosphorylation | Faint band | Diffuse cytoplasmic distribution |
| WT – Low glucose | Moderate increase | Moderate band | Basal vacuolar puncta |
| KO – Normal glucose | Mild increase (↓ PHO85) | Slightly stronger band | Mild Vacuolar puncta |
| KO 0 Low glucose | Strong phosphorylation | Intense distinct band | Prominent vacuolar accumulation |

**Experimental Flow**

1. Generate ATG1 knockdown
2. KD confirmation
3. Repeat Stage 1 readouts

**Expected Results with ATG1 KD**

- No band (absent or low p-Atg1)
- No vacuolar puncta indicating canonical machinery

# Biochemical and Experimental Validation of New Targets: Beta-oxidation

Nicole Lavinia

THE UNIVERSITY OF SYDNEY

# Pairwise Shortest Paths in *S. cerevisiae*

*PHO85*

Sensitive to nutrient signalling; Acts antagonistically to SNF1 under nutrient-rich conditions

*SNF1*

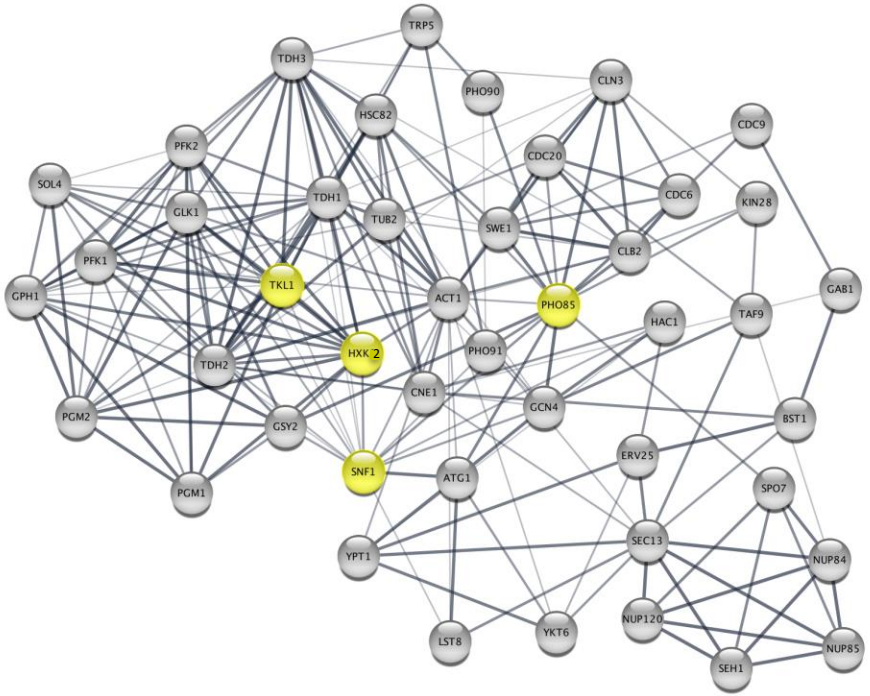
Activated under low glucose/stress; Stimulates expression of glucose-repressed genes via Adr1p (downstream activation of peroxisomal FA-oxidation)

*HXK2*

Kinase sensitive to glucose; Glucose-dependent transcriptional repressor that is Inhibited by SNF1

*TKL1*

PPP (non-oxidative) enzyme, NADPH Production; redirects carbon flux to the PPP–an alternative path supporting survival under metabolic stress



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Kinase sensitive to glucose; Glucose-dependent transcriptional repressor that is Inhibited by SNF1

*TKL1*
PPP (non-oxidative) enzyme, NADPH Production; redirects carbon flux to the PPP—an alternative path supporting survival under metabolic stress

Since PHO85 and SNF1 work antagonistically, we could assume that,

↓ PHO85 → ↑ SNF1 → ↑ Adr1p

Secondary Hypothesis:

*YMR205C exhibits increased fatty acid oxidation via SNF1 activation under impaired glycolytic flux and low glucose levels*

Simon *et al.*, 1992

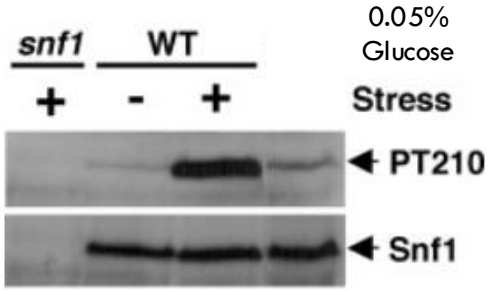
# Beta-oxidation in GSD

## Previous Findings

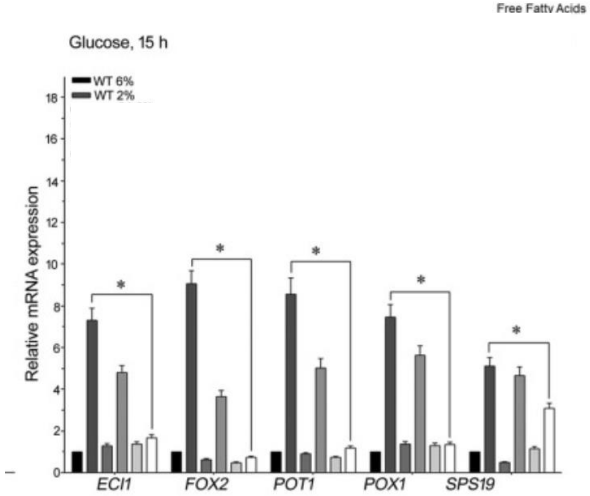
- Under low glucose (0.05%), SNF1 is phosphorylated at Thr210, indicating activation during metabolic stress (Orlova et al., 2008).
- SNF1 positively regulates Adr1p, stimulating expression of FOX1&2 and POT1 — key peroxisomal enzymes for β-oxidation (Gurvitz et al., 2001).

**Aims:**

- Confirm activation of SNF1 pathway under low glucose and impaired glycolytic flux conditions (ΔPFK2)
- Determine whether the PFK2 deletion promotes Beta-oxidation downstream of SNF1 activation




(Orlova, Barrett and Kuchin, 2008)




(Rajvanshi, Arya and Rajasekharan, 2017)

# Beta-oxidation in GSD

## Western blotting of SNF1

**Protein Target:**

SNF1 activation marker: Phosphorylated SNF1 (p-T210)

Control: Total SNF1 or ACT1 (loading control)

## Gene Expression Analysis via qPCR

**Genes of Interest:** POT1, FOX1, FOX2

**Reference gene:** ACT1

| Conditions | Western blot (p-T210) | Expected Band Intensity | Gene Expression of POT1, FOX1, FOX2 |
|---|---|---|---|
| WT – Normal glucose | Low/basal phosphorylation | Faint band | Low baseline expression (glucose-repressed) |
| WT – Low glucose | Strong phosphorylation | Intense distinct band | Increased expression of genes |
| KO – Normal glucose | Mild SNF1 activation (due to altered flux) | Slightly stronger band than WT-normal | Mild upregulation of genes |
| KO – Low glucose | Strong SNF1 phosphorylation (synergistic) | Very intense band | High induction of gene expression indicating increased FA-oxidation |

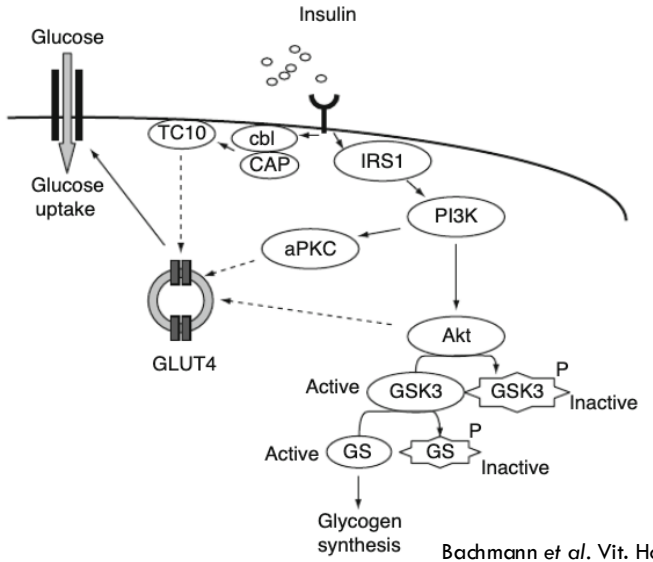
# Towards a Suitable Model System for Glycogen Storage Disease 7

Cristina Yazbeck

THE UNIVERSITY OF SYDNEY

# Limitations of ΔPFK2 *S. cerevisiae*

- *S. cerevisiae* relies primarily on intracellular Snf1/TORC1 nutrient-sensing for glycogen metabolism
- Absence of **human insulin signaling cascade** in *S. cerevisiae*
  Lacks **insulin-mediated kinase-phosphatase interactions** (RTK and P13K/PTEN/Akt pathway) leading to GSK3 inhibition → GS activation
- Unable to reproduce hormonal regulation and downstream multi-layered phosphorylation cascades controlling glycogen metabolism and synthesis in human tissues




Bachmann *et al.* Vit. Horm. **2009,** 80.

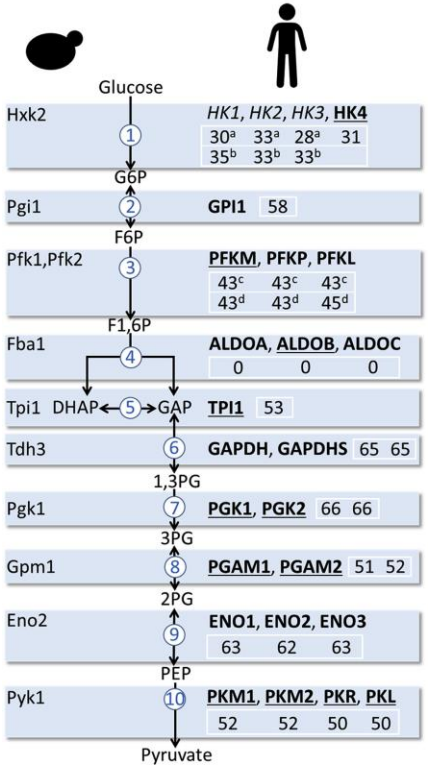


Coronas-Serna *et al. Inter. Micro.* **2020,** *23* (1).

# Full Humanisation of Glycolysis in *S. cerevisiae*

- 43-66% **identity** at the protein level
- Minimised set of yeast glycolytic genes has been fully relocated to a **single chromosomal locus**
- Complementation of **moonlighting** functions of HXK, ALDO, and ENO, e.g. assembly of vacuolar ATPases, Crabtree effect
- $\mathbf{V_{max}}$ of glycolytic enzymes was same order of magnitude in humanised yeast and muscle cells
- **Turnover rates** of proteins were not substantially different

**Challenge:** No human homologue for *S. cerevisiae* HXK1/2, which have been identified as central by network analysis.






Boonekamp *et al. Cell.* **2022,** *39* (13)

# Future Directions: Validation

Yuanjun (Sam) Sun

# Further Validation of ΔPFK2 *S. cerevisiae*

- Excess purine degradation occurred in the exercising muscles of patients with GSD7[1]
- Purine degradation produces fumarate fueling citric acid cycle[2]
  - AMP → IMP → Adenylosuccinate → AMP + **Fumarate**
- Purine degradation under low ATP:AMP leads to uric acid formation
  - AMP → IMP → Inosine → Hypoxanthine → Xanthine → Uric Acid
- Yeast also possess purine catabolic pathway but lacks xanthine oxidoreductase[3]
  - AMP → IMP → Inosine → Hypoxanthine (excreted)



1. Mineo *et al. Journal of Clinical Investigation.* **1985**, *76* (2), 556–560.
2. Garrett *et al.* Biochemistry, 2nd ed.; Chapter 27, Fort Worth : Saunders College Pub, **1999**.
3. Daignan *et al.* Cells **2019**, 8 (1), 67.

# Further Validation of ΔPFK2 *S. cerevisiae*

- Exactly how purine catabolism behaves in yeast is unknown
- Examine the purine catabolic pathway in KO yeast under extreme caloric restriction (model exercise in GSD7)
    1. Simulate high AMP:ATP ratio (inducible by 2-deoxyglucose (100 mM) followed by incubation (30 min)[1]
    2. Sample coupled to LC-MS/MS
        - Enrichment by hydrophilic interaction chromatography (HILIC)[2]
        - Orbitrap or QTOF mass spectroscopy
    3. Compare metabolite concentration (inosine and hypoxanthine) between WT and KO yeast
    4. If inosine and hypoxanthine concentration increased in KO yeast → upregulated purine catabolism → increased confidence for ΔPFK2 yeast as GSD7 model
    5. Compare the magnitude of metabolite changes with healthy and GSD7 patient


1. O'Donnell *et al.* Molecular and Cellular Biology **2015**, 35 (6), 939–955.

# Future Directions: Proteomics

Yuanjun (Sam) Sun

# Identification of Novel Altered Pathways

- Many enzymes are functionally regulated by kinases/phosphatases
- Identify altered pathway in Δ*PFK2* yeast that were previously unknown
- Similar workflow in past literature to identify novel protein biomarkers in GSD5[1]
- Identification of these novel pathway might enhance our understanding on GSD7
- **Comparative phosphoproteomics:**
    - Protein labelling (SILAC or TMT)
    - Enrichment through $TiO_2$ chromatography
    - LC MS/MS for quantitation and identification
    - Western blot to validate protein candidate


1. García-Consuegra *et al.* International Journal of Molecular Sciences **2022,** *23* (9), 4650–4650.

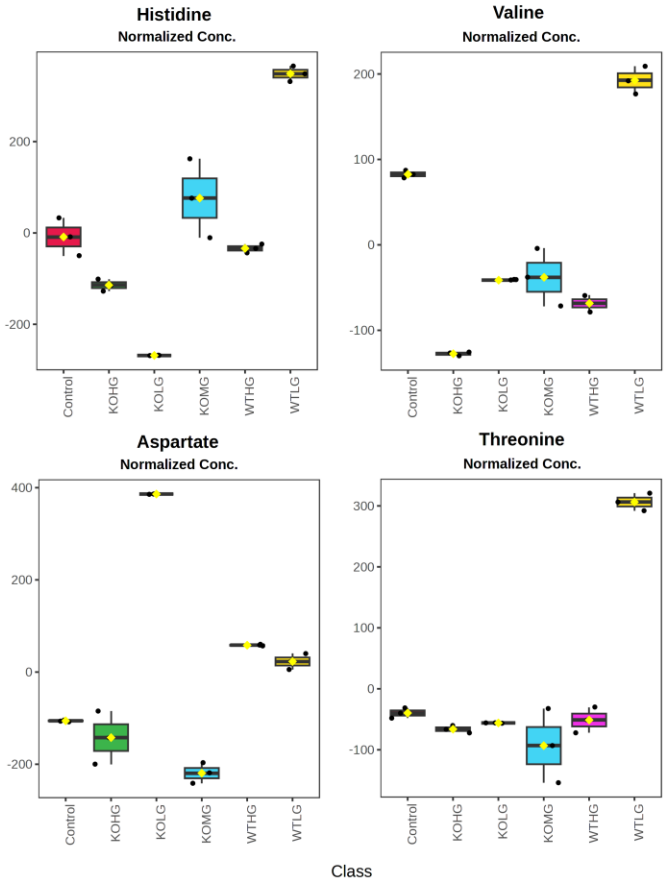
# Future Directions: Metabolomics

Yuanjun (Sam) Sun

THE UNIVERSITY OF SYDNEY

# Metabolomics Results

- Past research simulated caloric restriction by rapamycin to investigate how amino acid profile shifts[1]
- Increase in valine and histidine abundance observed in low glucose WT
- Elevated threonine level doesn't align with past literature
- Elevated aspartate also observed in KO






1. Mülleder *et al.* Cell **2016**, 167 (2), 553-565.e12.

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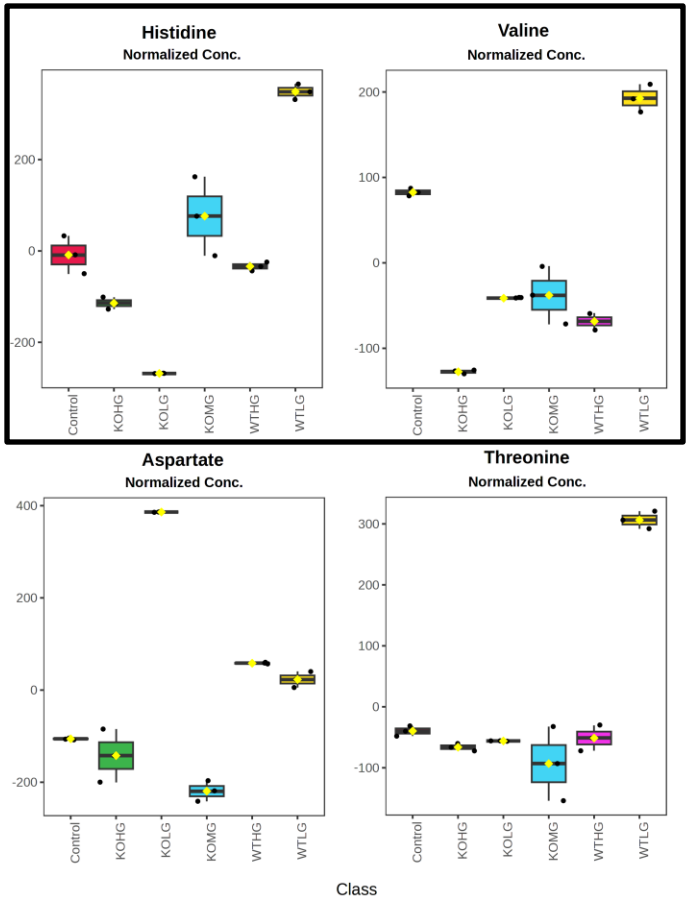




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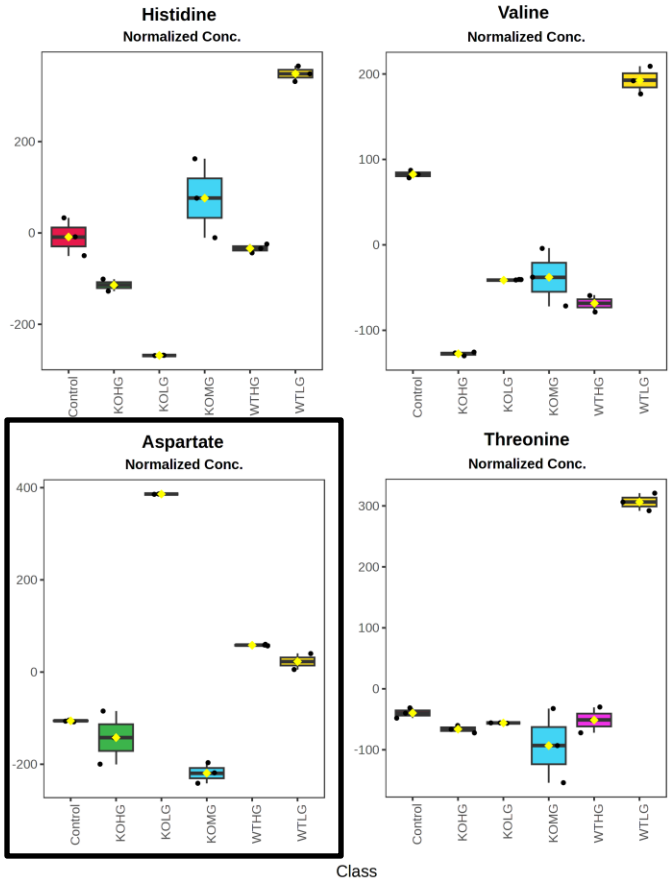




1. Mülleder *et al.* Cell **2016,** 167 (2), 553-565.e12.

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- Increase in valine and histidine abundance observed in low glucose WT
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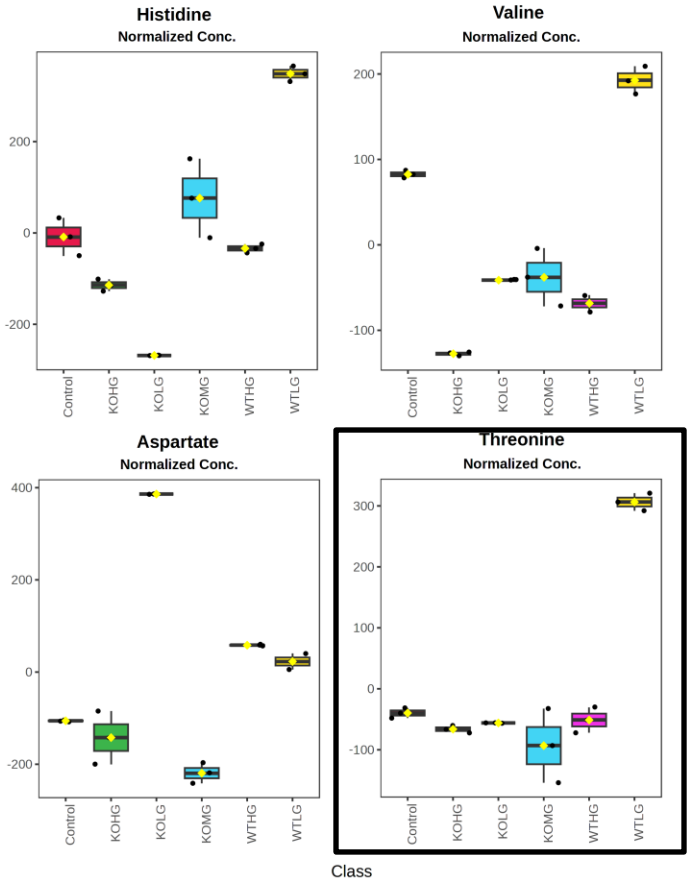
- **Elevated threonine level in WT doesn't align with past literature**

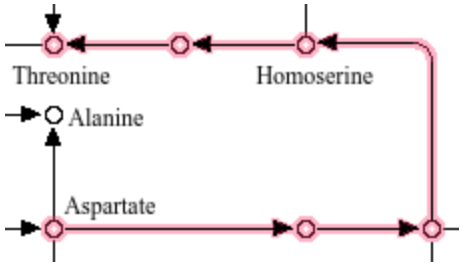


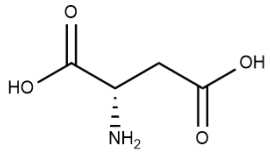


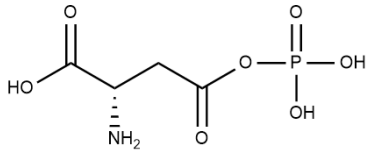

1. Mülleder *et al.* Cell **2016**, 167 (2), 553-565.e12.

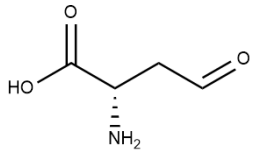
# Metabolomics Future Directions

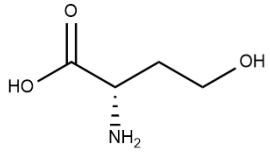
- High [Aspartate] but low [Threonine] in KO
- High [Threonine] in WT
- Metabolomics investigating threonine biosynthesis pathway to identify rate limiting step

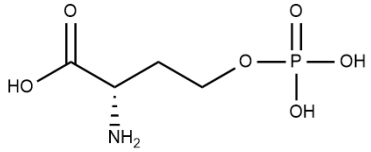


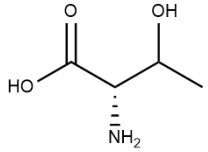


Aspartate


4-Phospho-L-aspartate


L-Aspartate 4-semialdehyde


Homoserine

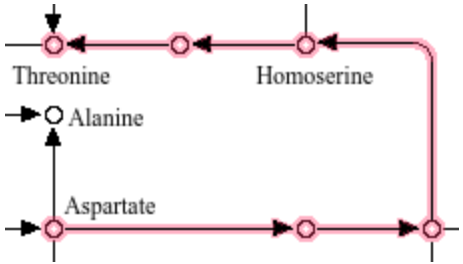

O-Phospho-L-homoserine

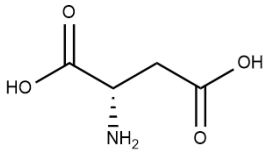

Threonine

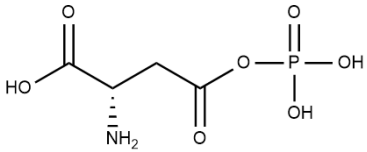

1. Mülleder *et al.* Cell **2016**, 167 (2), 553-565.e12.

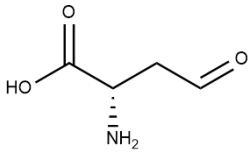
# Metabolomics Future Directions

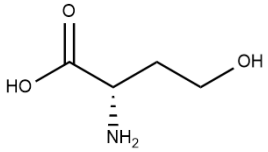
- All intermediates are polar
- Adopting very similar metabolomics procedure as previous
  - LC-MS/MS (HILIC and orbitrap MS)
- Metabolite concentration compared between KO and WT low glucose

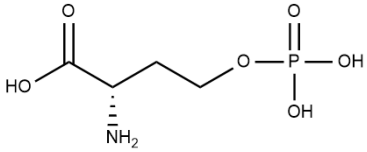


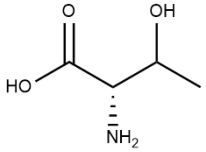


Aspartate


4-Phospho-L-aspartate


L-Aspartate 4-semialdehyde


Homoserine

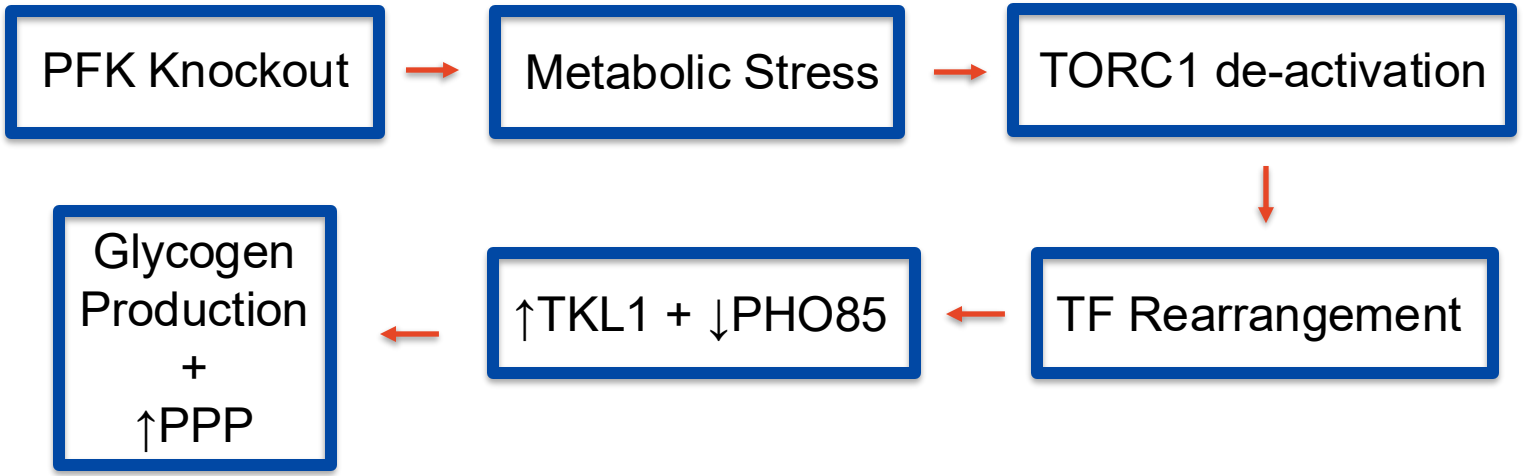

O-Phospho-L-homoserine


Threonine


1. Mülleder *et al.* Cell **2016**, 167 (2), 553-565.e12.

# Future Directions: Epigenetics

# Original Hypothesis: Erroneous qPCR




- Simplified hypothesis = TORC induced transcription factors
- Unexpected qPCR results
- Epigenetic machinery also regulates transcription

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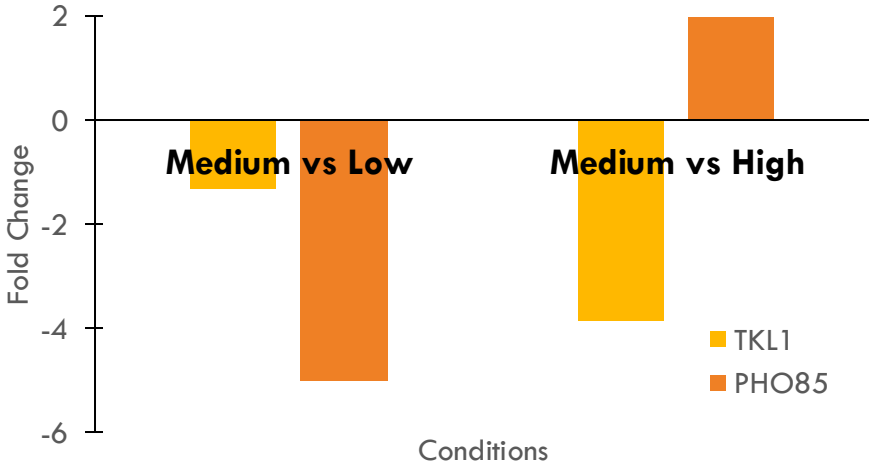
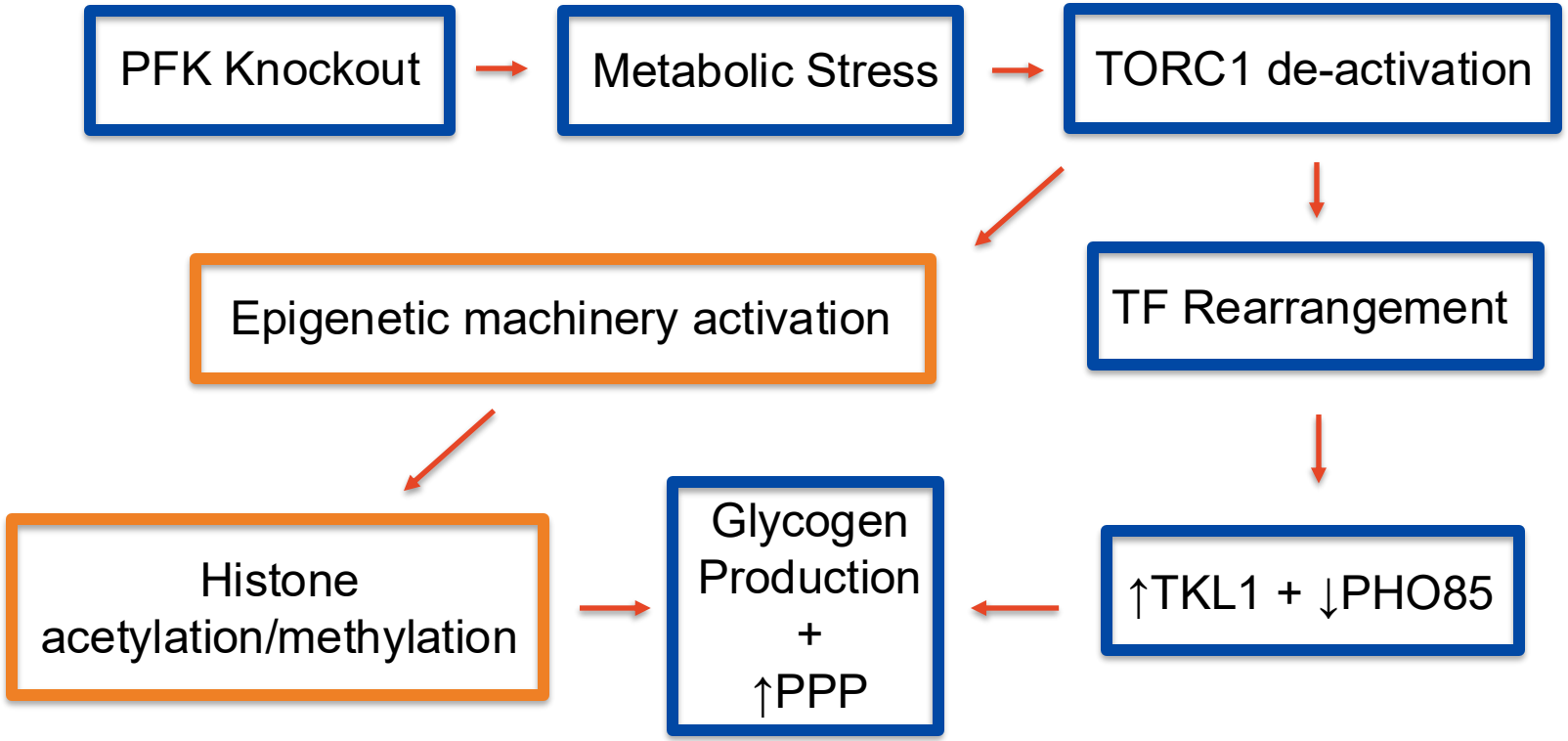



Figure 1: TKL1 and PHO85 gene expression level in KO yeast cultured in varying concentration of glucose, plotted using KO HG TKL 1:1

- Simplified hypothesis = TORC activation of transcription factors
- Unexpected qPCR results
- Epigenetic machinery also regulates transcription

# Alternative Hypothesis: Epigenetic Modulation



# Precedent: GSD1A and Starvation Induced Autophagy

GSD1A = G6Pase-α deficiency results in **IMPAIRED** autophagy

1. Afflicted cells activate CREBp, PPAR-γ and PPAR-α, lipogenic transcription factors and regulators
2. Expression and activation SIRT1 deacetylase downregulated
3. Deacetylation of autophagy-related proteins inhibited

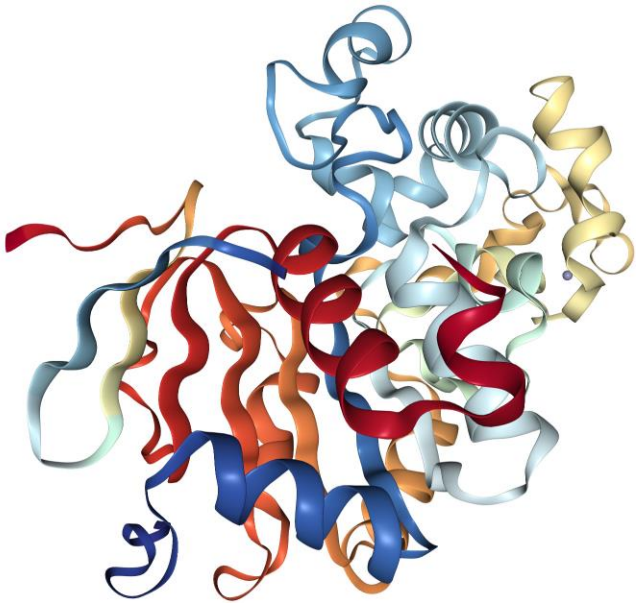


Figure 2: SIRT1 derived structure

# Precedent: GSD1A and Starvation Induced Autophagy

H4K16 acetylation regulates long term autophagy

- Autophagy is activated by TIP60
- Autophagy is repressed by SIRT1 activation
- GSD7 creates metabolic stress conditions similar to GSD1A

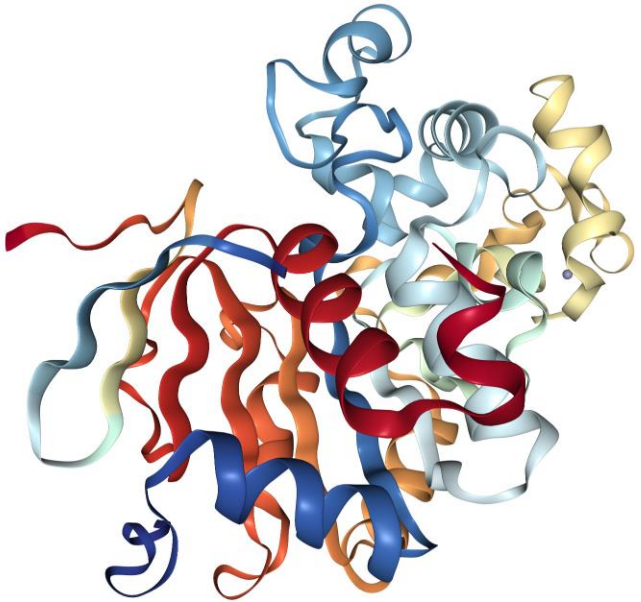


Figure 2: SIRT1 derived structure

# Precedent: Epigenetics of Glycogen Production

Glycogen production is also regulated by epigenetic machinery

- PRMT4 expressed abundantly in skeletal muscle cells
- Suppression of PRMT4 expression inhibited glycogen synthase 1 transcription
- PRMT4 positively regulated by AMPK nutrient sensing pathway

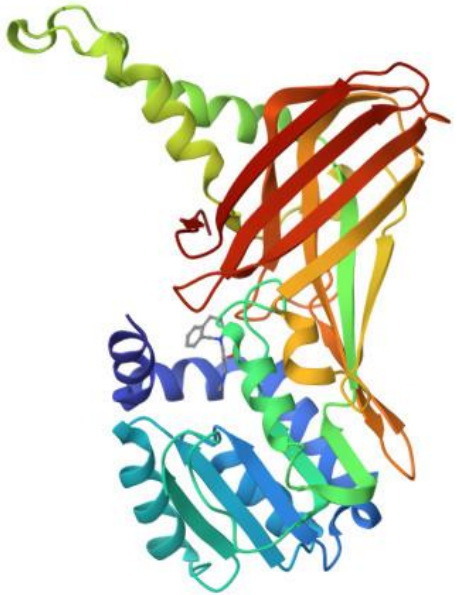


Figure 3: PRMT4 (CARM1) bound with a bicyclic compound

# Human – Yeast Epigenetic Homology

Most relevant epigenetic machinery between humans and yeast have high homology

- Autophagic response TFs differ slightly between organisms (FOXO vs FKH1/2)
- PRMT4 has low homology but is very important for epigenetic regulation, different protein may perform methyl transferase activity

# DiMelo-seq: Epigenetic Detection

GSD7 induced epigenetic activity can be investigated using a novel “DiMelo-seq” approach.

1. An antibody targets a protein or histone modification of interest (H4K16)
2. A protein fused to the antibody methylates DNA surrounding the antibody target
3. LRS identifies the mark during sequencing

Figure 4: Oxford Nanopore flow cell

# Future Directions:
# Growth Condition Experiments

Matthew Wolfenden

# Growth Conditions Goals

- Our Knockout Condition $\Delta pfk2$ reduces the efficiency of glycolysis
- Aim to explore KO vs WT cell growth differences from a top-down perspective
- We chose varied glucose levels to assess the pathway's behaviour in excess glucose and starvation environments
- We also explored some alternate carbon sources to identify alternate paths and potential treatments for our human homolog disease modelled off GSD V treatment

# Which Conditions Did We Use?

- 2% Glucose
- 1% Glucose
- 4% Glucose
- 2% Pyruvate
- 2% Lactate

---------- PCR Confirmation, Yeast Viability

---------- qPCR, Metabolomics

------- Growth Plate, Glycogen Staining

# Briefly, GSD V

- GSD V is **NOT** modelled by our KO yeast strain
- GSD V is a block in glycogenolysis, whereas GSD VII is a block in glycolysis
- Both cause an increase in glycogen but for different reasons. In both cases, glycolysis cannot be relied upon for fast, reliable energy supply, especially in skeletal muscle
- Enter **LCKD**

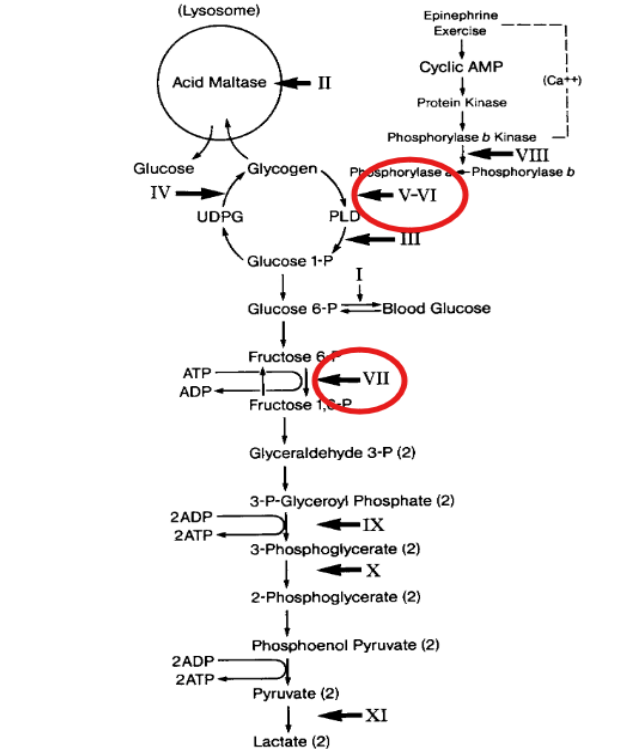



Fig. 1. Scheme of glycogen metabolism and glycolysis. Roman numerals refer to glycogen storage diseases resulting from deficiencies of the following enzymes: I, glucose-6-phosphotase; II, acid maltase; III, debrancher; IV, brancher; V, muscle phosphorylase; VI, liver phosphorylase; VII, phosphofructokinase (PFK); VIII, phosphorylase b kinase; IX, phosphoglycerate kinase (PGK); X, phosphoglycerate mutase (PGAM); XI, lactate dehydrogenase (LDH).

(DiMauro, S. et Al, 2005, American Journal of Medical Genetics)

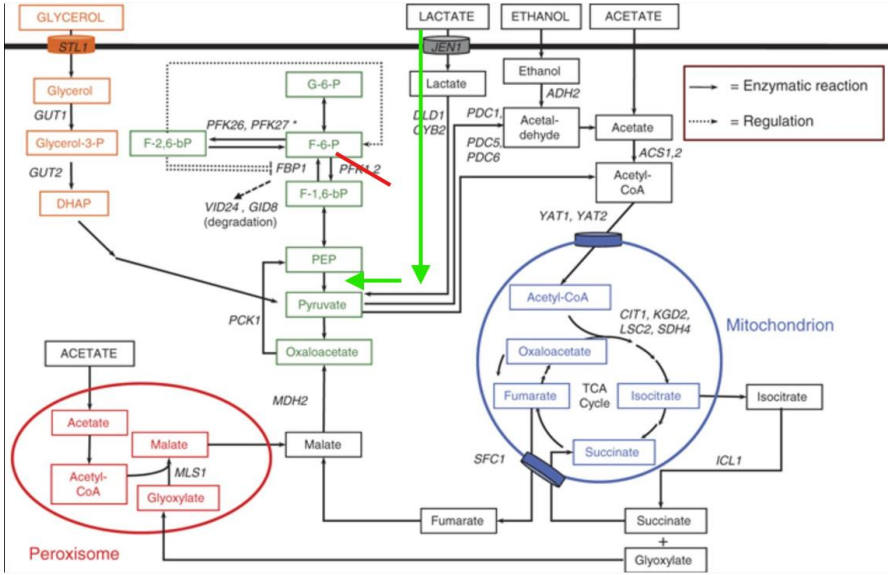
# LCKD: Low Carbohydrate Ketone Diet

- Ketone bodies originate primarily from the liver and are produced as an alternate energy source under carbohydrate restrictions due to fasting or prolonged exercise
- They have been explored as a potential treatment for metabolic disorders including for GSD V and GSD VII but not extensively
- We aimed to simulate this with our yeast KO strain
  - We utilised lactate and pyruvate here as they were a simpler option because they directly bypass glycolysis
  - Ketone bodies do not map perfectly onto S. *cerevisiae* which is a limit of our use of it as a model

(Lucia, A. et Al, 2021, Neuromuscular Disorders; Similiä, A. et Al, 2020, Front. Neurol.; Løkken, N. et Al, 2020, Journal of Inherited Metabolic Disease)

# Lactate and Pyruvate

- Both 3 C molecules brought into S. *cerevisiae* through Jen1 transporter
  - Lactate converted to pyruvate in cell
- Metabolites that slot in directly after glycolysis and are used in TCA Cycle
- Jen1 transporter analogous to MCT1, 4 transporters in human cells but not homologous and expressed on cell membrane in low glucose conditions. MCT transporters are also responsible for the intake of ketone bodies




Edited from Turcotte, B. et al 2009, FEMS Yeast Research

# Growth Plates

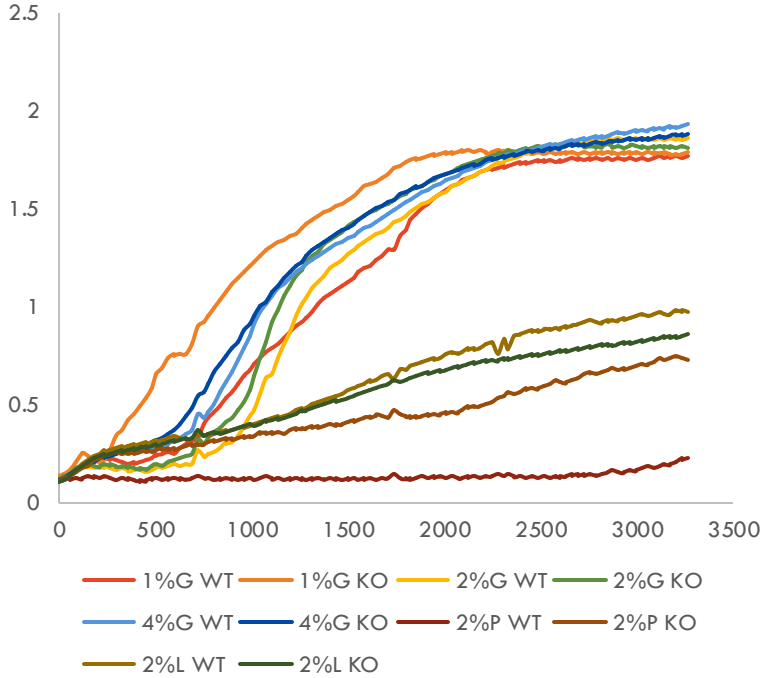
Matthew Wolfenden

# Hypotheses vs Our Results

- Growth could be solely dependent on [G] for KO
  - There was very little difference between the glucose level growth conditions to support this
- Low [G] could benefit KO growth by encouraging low glucose cell response
  - The low glucose growth condition did slightly benefit the KO strain as it grew the fastest, even compared to WT strains
- High [G] could have a negative effect through conflict between high glucose cell response and low ATP, or glycogen over synthesis
  - There was no evidence for this in our data
- Lactate and Pyruvate would be adequate alternative energy sources
  - While cell growth was observed, it was at about 50% the rate of the equivalent w/v% of glucose
  - Likely due to TCA cycle being slower than Glycolysis
  - Further investigation to see how this aligns with ketone dieting

# Limitations

- Glucose conditions all maxed out at A = 2
  - Likely limited by either absorbance measurement or by cell density, meaning [G] did not have as strong an influence
  - This was not consistent with the variations in OD observed in glucose cultures grown in flasks for our other experiments, which makes some of these results heavily contrast our expectations
- Lactate and pyruvate growth did not reach a stationary phase
- Very small volume used with better aeration in well plates
  - Not consistent with growth conditions of other experiments



# Next Steps

- Expand concentrations tested to lower concentration of glucose and higher for alternate carbon sources
  - When does low glucose become a limiting growth factor
  - When do higher 3C energy sources reach comparable growth to the 2% Glucose standard YPD condition
- Because we did not have time to assess the cell environment with our lactate and pyruvate growth conditions due to time, expanding our qPCR and metabolomics to include them could allow for better understanding of the internal cell environment
  - This will help connect these results to our other investigations which focus more on specific proteins – converge top-down and bottom-up experimental approaches

# Glycogen Staining

Matthew Wolfenden

# Hypotheses vs Results

- Higher Glycogen in 4%G and in KO strains
  - Hard to determine using stain. Very little differentiation
- No glycogen in Pyruvate and Lactate growth conditions
  - Appeared to be true but hard to validate due to microscopy quality

# Limitations and Improvements

- Our method was unreliable and qualitative
  - Stain method was old with an unclear methodology that we needed to test
  - We couldn't quantify the glycogen present in any way and it was hard to differentiate stain intensity due to microscopy resolution
- How we aim to improve
  - Use Periodic Acid Schiff (PAS) staining protocol instead
    - More complex and expensive than the Lugol's solution we used but have a more reliable "gold standard" method
    - Can be quantitatively measured at 555nm in a spectrometer

Kirkeby, S. et Al, 1992, Journal of Biochemical and Biophysical Methods

# Portfolio Conclusions

THE UNIVERSITY OF SYDNEY

# Conclusions I

- We have predicted phenotypic discrepancies between GSD7 and other glycogen storage diseases.
- Our preliminary results and network analyses are consistent with our hypothesis that the PFK2 deletion induces metabolic reprogramming; however, the experiment aspect requires more rigorous validation.
- So far, our results do not capture the extent of human signaling pathways which mediate glucose metabolism and glycogen formation. These limitations will be addressed with our ongoing experimental plans, including, but not limited to:
    - Modelling P13K/PTEN/Akt pathway to mimic human hormonal (insulin) signaling.
    - Complementing the PFK2 deletion with additional gene knockdown experiments to isolate key contributors to the GSD7 phenotype.
    - Conducting large-scale omics studies to better understand pathways of interest from preliminary glycogen staining, growth curves assays, and metabolomics.

# Conclusions II

- Further validation of yeast as model organism by looking at purine catabolism through metabolomics
- Comparative proteomics to identify novel pathobiology pathways
- Metabolomics focusing on threonine biosynthesis pathway
- Long Read Sequencing of KO and WT yeast conditions to investigate nutrient stress induced epigenetic acetylation/methylation
- Further analysis with alternate carbon source metabolism to expand limited understanding and bridge the gap to protein-protein interactions understanding in line with other research