Investigating important residues for Dnf1 phospholipid selection and flip by incorporation of UV-activated unnatural amino acids in *S. cerevisiae*

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Honors Thesis
Outside the Cell

Protein embedded in the membrane

Cell Membrane

Inside the Cell (Cytoplasm)

1. Blood clotting
2. Recognition and removal of apoptotic cells
3. Signal transduction
4. Cytokinesis
P4-ATPases are responsible for generating and maintaining this asymmetry.
**S. cerevisiae** P4-ATPases

- Membrane asymmetry
- Vesicle-mediated protein transport

Characteristics of the yeast P4-ATPases

- Dnf1 $\rightarrow$ Lem3
- Drs2 $\rightarrow$ Cdc50
Characteristics of the yeast P4-ATPases

- 10 transmembrane segments for catalytic α-subunit
- Actuator, Phosphorylation, and Nucleotide Binding domain
Homology Modeling of Dnf1 from Na+/K+ ATPase

Type I: Heavy Metal
Type II: Cation
Type III: Proton
Type IV: Phospholipid
Type V: Eukaryotic
Unknown


Baldridge and Graham (2012) PNAS

Na+/K+ ATPase
Dnf1 (P4-ATPase)
P4-ATPase Family in Yeast

Dnf1 vs. Drs2

31% identity
48% similarity

PC and PE

PS

Lumen / extracellular space

Cytoplasm

Drf1

Drs2
Residues defining substrate specificity of Dnf1

Baldridge and Graham (2013) PNAS
Two gate Mechanism for Phospholipid Flip

Baldridge and Graham (2013) PNAS
Positions on TM1 and 3
Positions on TM1 and 3

- N550Bpa
- L549Bpa
- Y618
- F551Bpa
- N220Bpa
- S552Bpa
- L549Bpa
- N220Bpa
- S552Bpa
- L549Bpa
- N220Bpa
- F551Bpa
- N550Bpa
Use of p-Benzoyl-L-phenylalanine to evaluate orientation of TM3

- Crosslinks when exposed to UV light
Daggett and Sakmar (2011)
Unnatural Amino Acid Incorporation

• Inserted into the cell’s proteins by replacing three nucleotides to form the TAG codon (amber codon)
• Transformation with a plasmid that has the ability to synthesize the tRNA with the amber codon
• Stop codon suppression yields incorporation

Hui-wang Ai (2012)
Detection of TM orientation with UAA cross-linking
Detection of crosslink
pRS313 GPD
Flag3 Dnf1 TEV
11256 bp

* = relative position of amber codon insertions
Other Plasmids Used

**pRS416 GPD**
**myc LEM3**
~5000 kb

**pEcTyrRS/tRNA_{CUA}**
~8000 bp
Growth Curve
Without Bpa in 2x media

<table>
<thead>
<tr>
<th>313 Plasmid</th>
<th>Growth Rate (# doublings/min)</th>
<th>Doubling Time (min)</th>
</tr>
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<tbody>
<tr>
<td>Empty Vector</td>
<td>0.0021</td>
<td>330.07</td>
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<tr>
<td>DNF1</td>
<td>0.0017</td>
<td>407.73</td>
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<tr>
<td>DNF1 TEV2</td>
<td>0.0022</td>
<td>315.07</td>
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<tr>
<td>N220a</td>
<td>0.0013</td>
<td>533.19</td>
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<tr>
<td>N550a</td>
<td>0.0014</td>
<td>495.11</td>
</tr>
<tr>
<td>F551a</td>
<td>0.0012</td>
<td>577.62</td>
</tr>
<tr>
<td>S552a</td>
<td>0.0016</td>
<td>433.22</td>
</tr>
</tbody>
</table>
Effect of tRNA Synthase Plasmid

*Experiment used S552a Dnf1 allele
Effect of Bpa on Growth

- 414 GAL
- 414 GAL +Bpa
- pEcTyrs/tRNAcua
- pEcTyrs/tRNAcua +Bpa
Protein Expression Profile of Amber Codon Alleles
Steps for Evaluating Bpa crosslinking

1. Grow cells in 1 mM Bpa
2. UV irradiate
3. TEV treatment
4. SDS-PAGE and Western blot for FLAG
TEV Protease Experiment

- Empty +UV+TEV
- dnfl TEV2 +UV+Bpa
- F551a TEV2 +TEV
- F551a TEV2 +Bpa
- F551a TEV2 +UV +Bpa
- F551a TEV2 +UV+Bpa+TEV

Diagram:
- 71 kDa
- 180 kDa
- FLAG

Graph:
- Gel experiment results with different conditions.
Future Directions

• Incorporate the orthogonal tRNA synthase into the yeast genome
• Purify Dnf1 with the FLAG tag to have purer protein samples
Thanks!

• Dr. Graham
• Ryan Baldridge
• The Graham Lab
Background Slides
## Use of UAAs

### Model Organisms/Systems
- *Pichia Pastoris*
- *Escherichia coli*
- *Saccharomyces cerevisiae*
- *Xenopus laevis*
- *Drosophila melanogaster*
- *Caenorhabditis elegans*
- HeLa Cells

### Uses
- G-protein- ligand interactions
- Protein-DNA interactions
  - Chromatin remodeling system
- Protein-Protein interactions
  - RNA Pol III and TF
  - GPCR and G-protein
  - Grb2 and binding to SH2 domains
Amber Codons were introduced by primer manipulation in a pGEM plasmid

pGEM
Dnf1 (1-2569)
Dnf1/Lem3 Crosslink?
Expression of Truncated Dnf1 Alleles
pRS313 HA
Dnf1 TEV
11,136 bp

EcoRI

TEV

1.7 kB

MfeI

SpeI

SphI

NotI
Drs2: VEKIINROIIRLFTVLIVLLISSLSSIGNVIASMADAKHLSYLYLEGTKAGLFF--KDFLTFWILFSNLVIPISLFVTVELIKYYQAFMIG

Dnf1: ISRELNSVVINFVLLFLICFVSIGANGVYYDKGSRFSYEFGTIAGSAATNGFVSVVAVILYQSLVPSLYISVEIKTAQAFAYQ

L549amb: ISREXNSVVINFVLLFLICFVSIGANGVYYDKGSRFSYEFGTIAGSAATNGFVSVVAVILYQSLVPSLYISVEIKTAQAFAYQ

N550amb: ISRELXSVVINFVLLFLICFVSIGANGVYYDKGSRFSYEFGTIAGSAATNGFVSVVAVILYQSLVPSLYISVEIKTAQAFAYQ

F551amb: ISRELNXSVVINFVLLFLICFVSIGANGVYYDKGSRFSYEFGTIAGSAATNGFVSVVAVILYQSLVPSLYISVEIKTAQAFAYQ

S552amb: ISRELFXVVINFVLLFLICFVSIGANGVYYDKGSRFSYEFGTIAGSAATNGFVSVVAVILYQSLVPSLYISVEIKTAQAFAYQ

N220amb: QYPNKIRTTPYTPTFLPKNILFQFHNFAVYFLVLIILGAFQIIFGVTNPGLSAVPLVVIIITAISKDAIEDSRTVALDLEVNNTKTH

TM1: Potential phospholipid translocation pathway

TEV2 site

TM3: LL3-4
Gel Extractions using MfeI and SphI digest

**pGEM digest**
- Uncut
- MfeI
- SphI
- both

**pRS313 HA dnf1 TEV2 digest**
- Uncut
- MfeI
- SphI
- both

1.7 kB

~10 kB
To ensure correct incorporation, a digest of EcoRI was run...
Protein Expression Profile of Amber Codon Alleles