Proteomics and Mass Spectrometry (BMG 744)

Mitochondrial Proteomics
March 15, 2005

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Mitochondria function

“Classical”
- ATP
- Heme & porphyrin synthesis
- Urea cycle
- β-oxidation of fatty acids
- \( \text{H}_2\text{O}_2 \rightarrow \text{Oxidative Damage} \)

“Novel”
- Generation of \( \text{H}_2\text{O}_2 \rightarrow \text{redox cell signaling} \)
- NO-cytochrome c oxidase signaling pathway
- Necrosis & Apoptosis
Mitochondria Dysfunction Leads to Disease

- Diabetes
- Ischemia/Reperfusion Injury
- Major neurodegenerative diseases – Parkinson’s, Alzheimer’s, ALS, Multiple Sclerosis, Huntington’s
- Cardiomyopathy
- Sepsis
- Cancer
- Alcohol-induced liver disease & other tissues
- Aging
**Mammalian mitochondrion**

**Outer membrane** - quite permeable; contains pores, which allow diffusion of molecules <1000 molecular weight

**Inner membrane** - invaginations called cristae; site of oxidative phosphorylation system
- metabolite transport across membrane - specific carriers

**Matrix** - contains enzymes of tricarboxylic (Kreb’s) cycle, enzymes for fatty acid oxidation, some enzymes for amino acid oxidation; these enzymes - involved in energy metabolism; mtDNA and mtRibosomes
Sequence and organization of the human mitochondrial genome.

Anderson S, Bankier AT, Barrell BG, de Bruijn MH, Coulson AR, Drouin J, Eperon IC, Nierlich DP, Roe BA, Sanger F, Schreier PH, Smith AJ, Staden R, Young IG.

12S, 16S rRNAs
22 tRNAs
COX I, II, and III
ATPase 6 and 8
Cytochrome b
7 complex I subunits
Respiratory Complexes are Coded by Nuclear and Mitochondrial DNA

- 10-12 copies per mitochondrion
- In the matrix (bound to the IMM)
- Maternally inherited
- Virtually “intron-less”, 93% coding

13 polypeptides:
- 7 subunits of Complex I
- 3 subunits of Complex IV
- 2 subunits F\textsubscript{0} ATP synthase
- Cytochrome b

Courtesy of V Darley-Usmar
mtDNA Mutations → Disease

Point mutations of tRNAs or OXPHOS genes
Maternally inherited
Decreased OXPHOS activity, pyruvate & fatty acids accumulate,
leading to lactate acidosis and accumulation of TGs
Rate of ATP synthesis is decreased – muscle weakness and exercise intolerance

- **Leber’s Hereditary Optic Neuropathy (LHON)**
  Single base change in genes encoding 3 complex I subunits (ND1, 4, 6) resulting in decreased complex I activity
- **Mitochondrial Encephalomyopathy, Lactic Acidosis, Stroke (MELAS)** – mutation tRNA for leucine
- **Myoclonic Epilepsy and Ragged Red Fibers (MERRF)**
  mutation in tRNA for lysine
- **Kearn’s-Sayre Syndrome (KSS)**
- **Neuropathy Ataxia Retinitis Pigmentosa (NARP)**
- **Hypertrophic Cardiomyopathy**
- **Leigh’s Syndrome**

http://www.neuro.wustl.edu/neuromuscular/mitosyn.html
Organization of Oxidative Phosphorylation Complexes

Complex I (NADH dehydrogenase)
- 43 subunits • 880 kDa
- FMN • 9 Fe-S

Complex II (Succinate dehydrogenase)
- 4 subunits • 130 kDa
- FAD • 3 Fe-S • heme

Complex III (Ubiquinone-cytochrome c oxidoreductase)
- 11 subunits • 245 kDa dimer
- 3 heme • Fe-S

Complex IV (Cytochrome c oxidase)
- 13 subunits • 200 kDa
- 2 Cu • 2 heme

Complex V (ATP synthase)
- 16 subunits • 660 kDa

4 H+ 4 H+ 2 H+
Q

FADH2

NADH

Ubiquinone

4 H+ Cytochrome c
12k Da • heme

2 H+

O2

H2O

ADP ATP

3 H+

Courtesy of PS Brookes
Glycolysis, TCA cycle, and β-oxidation of fatty acids make NADH & FADH$_2$ (reducing equivalents)

1. Electrons from dehase’s are transported to NADH and FADH$_2$
2. These electron are then passed into the electron transport chain, where through the reoxidation of NADH and FADH$_2$, they participate in several sequential oxidation-reduction rxns. before reducing O$_2$ to H$_2$O.
3. In this process H$^+$ are pumped across the inner mito. membrane
4. The free energy stored in the resulting electrochemical gradient that drives the synthesis of ATP from ADP + P$_i$ via Oxidative Phosphorylation.
Mitochondrial $\text{Ca}^{2+}$ handling

1. $\text{Ca}^{2+}$ uptake transporter
   Sensitive to cytosolic [Ca$^{2+}$]
   Essentially non-saturable

2. $\text{Na}^+/\text{Ca}^{2+}$ exchanger
   Always works at $V_{\text{max}}$

3. $\text{Na}^+/\text{H}^+$ exchanger
   Always works at $V_{\text{max}}$

• $\Delta$ cytosolic [Ca$^{2+}$] mirrored by $\Delta$ matrix [Ca$^{2+}$]

• Control of ATP synthesis – Ca$^{2+}$ activates Krebs’ cycle
  Increase NADH supply to respiratory chain
  Increase ATP generation

Courtesy of PS Brookes
Mitochondria and Calcium Toxicity

Fig. 1. Two-hit hypothesis for mitochondrial Ca$^{2+}$ in physiology and pathology. Under physiological conditions, Ca$^{2+}$ is beneficial for mitochondrial function. However, in the presence of an overriding pathological stimulus, Ca$^{2+}$ is detrimental. Similarly, Ca$^{2+}$ can potentiate a subthreshold pathological stimulus, resulting in pathogenic consequences. See text for full explanation. [Ca$^{2+}]_m$, mitochondrial matrix Ca$^{2+}$ concentration; ROS, reactive oxygen species.
Mitochondrial sites of reactive oxygen species production

- < 5% electrons "leak" from etc to form $O_2$.
- ROS damage proteins, lipids, and mtDNA
- Complex I - NADH dehydrogenase
- Complex III - Ubiquinone cytochrome c reductase
- Presence of stable semiquinone intermediate in enzyme complex
Mitochondrial $\text{O}_2\cdot^-\text{ Generation – Complex I and III}$

$\text{NADH} \xrightarrow{\text{e}^-} \text{Quinone} \xrightarrow{\text{e}^-} \text{Semiquinone – Stable free radical species} \xrightarrow{\text{e}^-} \text{Quinol}$

$\text{O}_2 \xrightarrow{\text{e}^-} \text{O}_2\cdot^-$

$\text{H}^+ \xrightarrow{\text{e}^-} \text{H}_2\text{O}$

Courtesy of PS Brookes
**B) Mitochondrial Events During Cell Death**

**NECROSIS**

(Cell ATP Reserves Depleted)

Severe Cell Injury
(Anoxia, Cyanide)

Electron Transport Chain Inhibited (Terminal Steps)

Onset of MPT

Membrane Potential Decreases

$F_0F_1$ Switches from an Active ATP Synthase to a very Active ATPase

Cell ATP Rapidly Depleted

Other Deleterious Events

Plasma Membrane Rupture

**APOPTOSIS**

(Cell ATP Reserves Partially Retained and Required)

Death Stimulus

Signaling Pathways

Onset of MPT

Membrane Potential Decreases

$F_0F_1$ Switches from an ATP Synthase to a "Low Activity" or Inhibited ATPase

Cytochrome $c$ and AIF Release

Electron Transport Chain Inhibited (Terminal Steps)

Caspase Cascade Activates ATP

Other Deleterious Events

Engulfment By Other Cells
Mitochondria Function & Cell Viability & Pathology: Linked to alterations in Mitochondria Proteome
How to study the mitochondrial proteome?

**Separation Techniques**

2D IEF/SDS-PAGE – hydrophilic (matrix) proteins

**Affinity fractionation (Lopez and Kristal)**
- calcium binding proteins, glycosylated proteins, hydrophobic proteins

**Sucrose density gradient centrifugation (Capaldi)**
- separate intact protein complexes via sucrose gradient fractionation

**Gel filtration (Mootha)**
- size separation using gel filtration chromatography into 15-20 fraction, digested, and analyzed via LC-MS/MS

**Immunocapture (Capaldi)**
- monoclonal antibodies against complexes (complex I, ATP synthase, PDH)

**BN-PAGE (Schagger & von Jagow)**
- separate OXPHOS complexes intact under non-denaturing conditions (1-D) followed by denaturing conditions to separate individual polypeptides (2-D)
Sucrose density gradient centrifugation (Capaldi)

- Mitochondrial extracts (1% LM) loaded onto 10-35% step fraction sucrose gradient, centrifuged overnight, fractions collected from bottom of tube
- Large protein complexes (complex I) found in higher density sucrose fractions, whereas free proteins found in lighter sucrose fractions
- Protein fractions analyzed via 1-D or 2-D electrophoresis
- MALDI and LC/MS/MS identified 615 distinct proteins, 19% previously undefined.

Size dependent fractionation of mitochondrial complexes by sucrose gradient

Bovine heart
9 fractions

Hanson et al Electrophoresis 22:950, 2001
Mitochondria Proteome
**Immunocapture (Capaldi) - rapid, small amts. tissue**

Mitochondria treated with 1% LM

Extract incubated (10 mg) with monoclonal ND6 antibody-crosslinked to protein G-agarose beads, beads washed, immunocaptured complex I eluted, and run on 1-D and 2-D gels

MALDI and LC MS/MS to identify proteins

29 protein bands

Using MALDI & LC MS/MS - Identified 42 of 45 proteins

**Immunopurified human heart complex I**

By 2-D IEF/SDS-PAGE - 10 μg, 1-D linear pH 3-10 strip, 2-D 15% gel (Sypro ruby), MALDI

Murray et al JBC 278:13619, 2003
Immunocapture - ATP synthase

Human Heart – 16 subunits

Immunocaptured ATP synthase is active

Aggeler et al. JBC 277:33906, 2002
Application of mitochondrial proteomics:

Chronic alcohol-induced mitochondrial dysfunction & liver disease
Chronic Alcohol

ROS/RNS stress

Mitochondria Dysfunction

Inflammatory Response

Pathology -
Steatosis
Hepatitis
Fibrosis
Effects of Chronic Ethanol Consumption on Mitochondrial Energy Metabolism

Structural abnormalities

Normal mitochondria

Alcoholic mitochondria
Decrease in state 3 respiration by 25-40%

↓ NADH-linked substrates & fatty acids

↓ succinate-driven

↓ cytochrome oxidase

Decrease in the rate of ATP synthesis

Defects in complexes I, III, IV, and ATP synthase
13 mtDNA encoded polypeptides:
- 7 subunits of Complex I
- 3 subunits of Complex IV
- 2 subunits F₀ ATP synthase
- Cytochrome b

In ethanol mitochondria, there is a decrease in the concentration of all 13 mitochondrial-encoded polypeptides
Decreased translation capacity of mitochondrial ribosomes due to chronic alcohol-associated modifications in the structure and function of mitochondrial ribosomes (Arch Biochem Biophys 398:41, 2002)

Damage to mtDNA by chronic alcohol (JBC 279: 22092, 2004)
Chronic alcohol-induced lesions

**Decreased**
- Activity & heme of IV cytochrome b
- Fe-S centers of I
- ATPase and ATP-P$_i$ (F$_0$)

**No change**
- Cytochrome c and c$_1$
- Ubiquinone
- Succinate dHase
- Catalytic F$_1$ portion
- ANT & carriers
Strategy to reveal chronic alcohol-related alterations to mitochondria proteome

A. 2-D IEF/SDS-PAGE

B. 1-D BN-PAGE

C. 2-D BN-PAGE
Protein Changes with Chronic Ethanol Consumption

Venkatraman et al. JBC 279:22092, 2004
Proteins with different abundances in liver mitochondria following chronic ethanol consumption – identification of proteins from 2-D IEF/SDS-PAGE gels.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Mass calc. (kDa)</th>
<th>MOWSE score</th>
<th>Mean fold change(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>acyl-Coenzyme A dehydrogenase, very long chain</td>
<td>70.7</td>
<td>72</td>
<td>1.31</td>
</tr>
<tr>
<td>acyl-Coenzyme A dehydrogenase, medium chain</td>
<td>46.5</td>
<td>94</td>
<td>0.78</td>
</tr>
<tr>
<td>acyl-CoA dehydrogenase, short-chain specific</td>
<td>44.9</td>
<td>67</td>
<td>0.81</td>
</tr>
<tr>
<td>β-ketoacyl CoA thiolase</td>
<td>41.8</td>
<td>84</td>
<td>0.12</td>
</tr>
<tr>
<td>Δ(^3), Δ(^2)-enoyl-CoA isomerase</td>
<td>32.2</td>
<td>100</td>
<td>1.58</td>
</tr>
<tr>
<td>2,4-dienoyl-CoA reductase (NADPH)</td>
<td>36.1</td>
<td>78</td>
<td>1.70</td>
</tr>
<tr>
<td>oxoglutarate dehydrogenase (lipoamide); α-ketoglutarate dehydrogenase</td>
<td>116.0</td>
<td>64</td>
<td>2.87</td>
</tr>
<tr>
<td>Glutamate dehydrogenase</td>
<td>61.4</td>
<td>101</td>
<td>0.56</td>
</tr>
</tbody>
</table>

Respiratory Complexes I,III,IV,V

β-oxidation

TCA cycle-related

Ox-phos

Chaperones
EtOH metabolism
AA catabolism

<table>
<thead>
<tr>
<th>Protein</th>
<th>Mass calc. (kDa)</th>
<th>MOWSE score</th>
<th>Mean fold change(^a)</th>
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</thead>
<tbody>
<tr>
<td>ATP synthase beta subunit</td>
<td>51.1</td>
<td>144</td>
<td>0.65</td>
</tr>
<tr>
<td>Chain A, Rat liver F(_{1})-ATPase (alpha aubunit)</td>
<td>55.2</td>
<td>156</td>
<td>0.71</td>
</tr>
<tr>
<td>Ubiquinol-cytochrome c reductase iron-sulfur subunit</td>
<td>27.7</td>
<td>108</td>
<td>0.48</td>
</tr>
<tr>
<td>ubiquinol-cytochrome c reductase, core protein II precursor</td>
<td>48.3</td>
<td>67</td>
<td>0.21</td>
</tr>
<tr>
<td>60kDa heat shock protein (Hsp60)</td>
<td>60.9</td>
<td>115</td>
<td>0.84</td>
</tr>
<tr>
<td>dnaK-type molecular chaperone grp75 (Hsp70/GRP75)</td>
<td>73.7</td>
<td>121</td>
<td>1.80</td>
</tr>
<tr>
<td>aldehyde dehydrogenase</td>
<td>48.2</td>
<td>75</td>
<td>0.44</td>
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<tr>
<td>3-hydroxyisobutyrate dehydrogenase</td>
<td>35.3</td>
<td>67</td>
<td>1.41</td>
</tr>
<tr>
<td>3-mercaptopropionate sulfurtransferase</td>
<td>32.9</td>
<td>78</td>
<td>1.82</td>
</tr>
</tbody>
</table>

\(^a\) Mean fold change was determined by averaging the fold change observed from 5 pairs of control and ethanol-fed animals.

\(^b\) p values were determined using a two-tailed paired Student’s t-test on the normalized protein spot densities obtained using PDQuest.

Venkatraman et al. JBC 279:22092, 2004
Human mtDNA

13 proteins
35S label
(cycloheximide)

rRNA
Large subunit (16S)
Small subunit (12S)

tRNA
22

7 subunits
Complex I

Cytochrome b subunit
Complex II

Subunits I, II & III
Complex III

Subunits 6 & 8
Complex IV

Complex V
Visualization of $^{35}$S-labeled mitochondrial encoded subunits

A. 1-D SDS-PAGE  

B. 2-D IEF/SDS-PAGE

Legend:
- Lane 1
- Lane 2
- IP Complex IV

- ND5 - COI - ND4 - Cyt b - ND2 - ND1 - COII - COIII - A 6 -
- ND6 - ND3 - A 8 - ND4 -

- CO I
- ND I
- Cyt b
- CO II
- A 8

Bailey et al. FRBM 38:175, 2005
Solution... Functional 2D Proteomics: BN-PAGE

0.25% Coomassie Blue
1.25% Laurylmaltoside

Intact protein complexes

Native gradient Gel. 20V, 15h, 4°C

Cx I (880)
Cx V (600)
Cx III (460)
Cx IV (200)
Cx II (140)
1-D Blue Native gels

Venkatraman et al. JBC 279:22092, 2004
Specific Protein Subunits Decreased by Chronic Alcohol

Venkatraman et al. JBC 279:22092, 2004
2-DBN-PAGE – Loss of OXPHOS subunits by chronic alcohol

Venkatraman et al. JBC 279:22092, 2004
Molecular Chaperones
Hsp60
Hsp70/GRP75

Mitochondria
Ribosome

Ethanol metabolism
Aldehyde dehase

Fatty acid metabolism
Acyl-CoA dehase, SC
Acyl-CoA dehase, MC
Acyl-CoA dehase, VLC
2,4-Dienoyl CoA reductase
β-Ketoacyl-CoA thiolase
Δ³,Δ²-Enoyl-CoA isomerase

TCA cycle &
amino acid metabolism
α-Ketoglutarate dehase (lipoamide)
Glutamate dehase
3-Hydroxyisobutyrate dehase
Methylmalonate-semialdehyde dehase

Oxidative phosphorylation

<table>
<thead>
<tr>
<th>NADH dehase</th>
<th>Ubiquinol-cyto c reductase</th>
<th>Cytochrome c oxidase</th>
<th>ATP synthase</th>
</tr>
</thead>
<tbody>
<tr>
<td>ND1</td>
<td>Core protein 1</td>
<td>Subunit I</td>
<td>α &amp; β</td>
</tr>
<tr>
<td>ND2</td>
<td>Core protein 2</td>
<td>Subunit II</td>
<td>γ</td>
</tr>
<tr>
<td>ND3</td>
<td>Heme protein</td>
<td>Subunit III</td>
<td>B-chain</td>
</tr>
<tr>
<td>ND4</td>
<td>Fe-S subunit</td>
<td>Subunit IV</td>
<td>OSCP</td>
</tr>
<tr>
<td>ND4L</td>
<td>14 kDa protein</td>
<td>Subunit V</td>
<td>D-chain</td>
</tr>
<tr>
<td>ND5</td>
<td>Cyto b</td>
<td>Subunit VI</td>
<td>F-subunit</td>
</tr>
<tr>
<td>ND6</td>
<td></td>
<td>Subunit VII</td>
<td>ATPase 6 &amp; 8</td>
</tr>
</tbody>
</table>

Bailey et al. FRBM 38:175, 2005
Role of oxidative stress in Alcoholic Liver Disease

Chronic ethanol feeding increases hepatocyte ROS

Mitochondrial ROS production

Unpaired electron transferred to molecular oxygen to form the superoxide anion free radical

\[
\text{O}_2 + e^- \rightarrow \text{O}_2^-. 
\]
Hepatic Redox State:

ADH and ALDH reactions use NAD+ and produce NADH. Increase in the NADH/NAD+ ratio in the cytosol and mitochondria leads to disruption of liver metabolism, resulting in toxicity.
Reoxidation of NADH necessary for maintenance of normal liver function

- There is a need to reoxidize NADH back to NAD+
- NADH is reoxidized by the mitochondrial electron transport chain
Ethanol stimulates ROS production at Complex I and III

NADH → FMNH₂ → O₂

Complex I

O₂ → O₂⁻⁻ → MnSOD → H₂O₂ → GPx-1 → 2H₂O

H₂O₂ → ONOO⁻ → NO⁻ → ONOOO⁻ → Nitrotyrosine formation

Protein oxidation
Lipid peroxidation
DNA oxidation

Mitochondria Dysfunction
Role of post-translational modifications in alcoholic liver disease

Nitration

P-NO$_2$

(Nitration)

ONOO$^-$

Nitrosation

P-SNO

(S-nitrosothiols)

Oxidation/modification

Oxidized thiols (P-SOH, P-SO$_2$H, P-SO$_3$H)

Protein Carbonyls (P-CHO)

Lipid peroxidation adducts – 4HNE

Mixed disulfide (P-SSG)

Mitochondria inner membrane
Post-Translational Modifications

- \( \text{O}_2 \cdot^- \)
- \( \cdot \text{NO} \)
- \( \text{ONO}_2 \text{H} \)
- \( \text{N}_2\text{O}_3 \)
- \( \text{NO}_2^- \)

- Nitration: -\( \text{NO}_2 \)
- Nitrosation: -\( \text{NO} \)
- Nitrosylated thiols: \( \text{S-nitrosothiols} \)
- Oxidation: Oxidized thiols, Carbonyls
- Nitratated proteins: Nitratated proteins
Mitochondria & Protein Thiol Status

Changes in mitochondrial protein thiol status

- MPT
- Cell death
- Oxidative stress
- NO responsiveness
- TNFα signaling
- Regulation of respiratory chain function

**Hypothesis**
Post-translational modification to mitochondrial protein thiols disrupts mitochondrial function and contributes to cell injury in response to chronic alcohol.
Labeling Mitochondrial Protein Thiols with IBTP

pH 8
100 - 500x Accumulation

pH 7.2

5 - 10x Accumulation

Δψ_mito

Δψ_plasma

IBTP
IBTP Labeling to Mitochondrial Protein Thiols in Cells

Experimental Design – IBTP labeling of mitochondrial protein thiols

Mito isolated from liver of control and ethanol-fed rats

Mito (1 mg/mL) in respiration buffer (succinate/ADP)

Incubation with IBTP (5 µM) for 10 min

Reaction stopped with iodoacetate

Mito centrifuged, pellets stored -80°C

Gel electrophoresis and immunoblotting with anti-TPP
Immunoblot detection of IBTP-labeled proteins

Decrease in labeling indicates oxidized/modified thiols
Labeling of Mitochondria Protein Thiols with IBTP
Decreased IBTP Immunoreactivity in GRP78 and ALDH
Mitochondrial proteins identified as containing IBTP-reactive thiols

<table>
<thead>
<tr>
<th>Spot no.</th>
<th>Mass (kDA)</th>
<th>MOWSE score</th>
<th>No. peptides matched/unmatched</th>
<th>Fold change ethanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>129.6</td>
<td>195</td>
<td>28/49</td>
<td>1.43(0.2)</td>
</tr>
<tr>
<td>2 GRP78</td>
<td>72.1</td>
<td>194</td>
<td>28/74</td>
<td>0.57(0.1)</td>
</tr>
<tr>
<td>3</td>
<td>60.9</td>
<td>90</td>
<td>8/13</td>
<td>0.91(0.1)</td>
</tr>
<tr>
<td>4</td>
<td>56</td>
<td>98</td>
<td>8/15</td>
<td>0.53(0.2)</td>
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<tr>
<td>5</td>
<td>56.9</td>
<td>123</td>
<td>10/9</td>
<td></td>
</tr>
<tr>
<td>6 ALDH</td>
<td>53</td>
<td>135</td>
<td>12/15</td>
<td>0.56(0.1)</td>
</tr>
<tr>
<td>7</td>
<td>41.8</td>
<td>79</td>
<td>7/13</td>
<td>1.67(0.7)</td>
</tr>
</tbody>
</table>
Chronic alcohol decrease low $K_m$ ALDH activity

Irreversible oxidation to active site Cysteine
Protein Thiols As A Molecular Switch

Regulate mitochondria function

Ethanol

I

II

III

cytc

IV

V

Mitochondria inner membrane

Ethanol

O$_2^-$

NO

Nitration

P-NO$_2$

(Nitration)

ONOO$^-$

Nitrosation

P-SNO

(S-nitrosothiols)

Oxidation/modification

Oxidized thiols (P-SOH, P-SO$_2$H, P-SO$_3$H)

Protein Carbonyls (P-CHO)

Lipid peroxidation adducts – 4HNE

Mixed disulfide (P-SSG)

Histidine

adduct

Cysteine or Lysine

adduct
Mitochondria Proteomic Approaches

1. Abundances
2. PTMs