O-linked β-N-acetylglucosamine

- Nucleocytoplasmic Glycosylation
  - O-GlcNAc

- Other Putative Nucleocytoplasmic Modifications
  - In Dicty (Slime Mold and other diatoms) there is Gal-Gal-Fuc-Gal-GlcNAc-HyPro on a E3 Ubiquitin ligase subunit (Skp1, Chris West)
  - O-GalNAc?? A reported nucleocytoplasmic hexosaminidase D (N-acetylgalactosaminidase)…likely ENGase involved in degrading misfolded glycoproteins
  - O-Glc-Tyr (glycogenin…building glycogen)
  - O-Man in S. Cerevisae (that lack O-GlcNAc)…search on for cycling enzymes
The majority of the Gal-capped GlcNAc structures are O-linked. Most are disaccharides!!! 1st Evidence for O-GlcNAc

Tracking the Label:

Intracellular Fraction: 72% of label

Majority of O-GlcNAc Modified Proteins are intracellular??!!??

Nuclear Pore Complex Glycoproteins Contain Cytoplasmically Disposed O-Linked N-Acetylglucosamine

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O-GlcNAc

O-linked β-N-Acetylglucosamine

![Chemical structure of O-GlcNAc]

Tyr-Ser-Pro-Thr-Ser-Pro-Thr

O-GlcNAc Transferase

1998

O-GlcNAcase

2001

O-GlcNAc Transferase (OGT)

- 110 kDa Polypeptide (multiple splice variants)
- Localized to the Nucleus/Cytoplasm/Mitochondria
- Functions as a Multimer
- 11.5 TPR Repeats In N-Terminus
- Several Binding Partners
- No Obvious Recognition Motif at the Primary Sequence Level
- Xq13-several neurological diseases map

- Unpublished (recent mutations found in patients with X-linked intellectual disability (XLID))
Regulation of OGT recruitment to substrates by binding partners

Random text...

Silver Staining and Putative Identification of O-GlcNAcase From a Cow Brain Purification

- Nanobore LC-ESI Mass Spectrometry
- Identification of Proteins (minimum of 5 Sequenced (MS/MS) Peptides)

# ID
1. TATA binding protein-interacting-protein (TIP-120)
2. Putative O-GlcNAcase (similar to hyaluronidase)
3. Heat shock protein 110
4. Cullin
5. DNA K-type molecular chaperone hsc70
6. Amphiphysin & Calcineurin A
7. Dihydropyrimidinase related protein-2
Cloned O-GlcNAcase is Specific for O-GlcNAc Peptides
- Not a hexosaminidase but an N-acetylglucosaminidase

- a, CTD-GalNAc, Mock
- b, CTD-GalNAc + O-GlcNAcase
- c, CTD-GlcNAc, Mock
- d, CTD-GlcNAc + O-GlcNAcase

Overexpression of O-GlcNAcase Lowers Global O-GlcNAc Levels
Localization of O-GlcNAcase

![Localization of O-GlcNAcase](image)

**O-GlcNAcase**

- A neutral, nucleocytoplasmic β-N-acetylglucosaminidase
- Originally identified as a Menigioma Autoantigen (MGEA5)--multiple splice variants
- May have hyaluronidase but not HAT activity (???)
- Enzyme is a Substrate for the Executioner Protease of Apoptosis, Caspase-3
- PUGNAc--a GlcNAc analogue inhibitor (Ki~50nM)
- More specific (?) inhibitors recently developed (GlcNAcstatin and NButGT)
- 10q24--Implicated in Multiple Neurological Disorders
- SNP in O-GlcNAcase strongly correlated with type II diabetes in Mexican-American populations
- Mutant form of OGA in GK-rats (causal mutation??)
Features of O-GlcNAc

- NOT elongated
- Nucleocytoplasmic Proteins
- Dynamic & Inducible
- Enzymes for its Addition (OGT) and Removal (O-GlcNAcase) are Nucleocytoplasmic
- OGT Knock-Out is Lethal at the ES cell stage
  - X-linked OGT, Females Heterozygotes are NOT mosaic
- Reciprocity with Phosphorylation (Yin-Yang) on Some Proteins
Reciprocal Glycosylation & Phosphorylation of c-Myc:

Thr58 is Mutation Hot Spot In Human Lymphomas.

### Isolation of a Yin-Yang Complex

**OGT(AL28) Western**

- F. Wash
- Bound
- Microcystin Sepharose Purification
- PP-Ab
- OGT
- IP Antibody

**S/T Phosphatase (FL-18) Western**

- PP
- PreBleed
- OGT
- IP Antibody

---

### Classes of Proteins Modified By O-GlcNAc

~2,000 Proteins Identified to Date

- **Cytoskeletal Components**
  - Tau, Vinculin

- **Hormone Receptors**
  - ERα&β

- **Kinases & Other Signaling Molecules**
  - CKII, eNOS, IRS-2

- **Nuclear Pore Proteins**
  - NUP62, NUP155

- **Oncogenes & Tumor Suppresors**
  - p53, Rb

- **Transcription Factors**
  - c-myc, Sp1, Pax6

- **Metabolic Enzymes**
  - GAPDH, Pyruvate Kinase

- **Transcriptional and Translational Machinery**
  - RNA Pol II, EIF4A

- **Viral Proteins**
  - SV40 T Antigen, v-Erb-a

- **Heat-Shock Proteins**
  - HSP90, α-crystallin

---

More Proteins and Many Sites to Go
~2,000 sites mapped, some proteins
have as many as 50 sites

Silver stain of anti-O-GlcNAc immunopurified proteins

Detection of O-GlcNAc

- Antibodies (RL-2, 110.6, mAb3,10,14)
- GalT labeling
- Radiolabeled GlcN
- GlcNAc-azido followed by Staudinger Rxn
- Beta-elimination/Michael Addition
- Pilot Approaches--Sulfotranferases and Kinases
Figure 17: BEMAD Methodology. (A) Schematic of the β-elimination, Michael (conjugate) addition with DTT approach for replacing O-GlcNAc with a stable tag. (B) M+H of O-GlcNAc modified peptide before BEMAD and (C) M+H of peptide following chemistry by MALDI-TOF analysis. (D) DTT-modified peptide can be purified from a mixture using thiol-chromatography.
Figure 22: ECD Fragmentation of O-GlcNAc Modified Peptide. Fragmentation of doubly charged parent ion of O-GlcNAc modified peptide on a LTQ-FT (Finnigan) using ECD fragmentation. Note mass accuracy of less than 2 ppm and Z7-Z12 and C7 ions still contain O-GlcNAc modified serine.

Functions Postulated for O-GlcNAc

- Blocking Phosphorylation Sites (CTD of RNA Pol II, Tau)
- Regulation of Chromatin Structure (msin3A, Tet2/3, histones, demethylases)
- Regulation of Stability (TFs, Proteasome Regulation)
- Regulation of Transcription (Sp1, CRTC2, HCF-1)
- Regulation of Translational Initiation (gp67-eIF2A)
- Regulation of Protein:Protein Interactions (STAT5-CBP)
- Regulation of Signal Transduction (IRS/PI3K)
- Regulation of Secretion (Adipocytokines, Hormones)
- Protection from Stress (Upregulation of HSPs)

- GENERAL THEME???
  - Glucose Sensor and Regulator of Energy Homeostasis
A. Glycogen PPP -> Glycolysis -> Hexosamine Biosynthetic Pathway (HBP) -> UDP

B. Complex Glycosylation

Key:
- = Glc
= Fru
= GlcNAc
= GalNAc
= GlcN
= Neu5Ac

High-Energy Metabolism

Glucose Metabolism

Amino Acid Metabolism

UDP-GlcNAc

Nucleotide Metabolism

Fatty Acid Metabolism
Modulation of O-GlcNAc Levels By A Variety of Stimuli

- 60min T-Cell Activation
- 1-5 minute Neutrophil Stimulation
- GlcN+INS O/N Feeding
- Thermal Stress

- Hart

- Hart

- Hart

- Hart

- Hart
A Role for O-GlcNAc in Insulin Resistance
(the Hallmark of Type II Diabetes)

Insulin Resistance
Insulin-Stimulated Glucose Uptake
In Adipocytes Is Impaired By PUGNAc

Defective AKT Phosphorylation & Activation

Glucose FFA Glutamine Glucosamine Uridine

Glc-6-P Fruc-6-P GlcN-6-P UDP-GlcNAc

GS Glycogen Synthesis Glycolysis O-GlcNAc Modification Complex Glycosylation

GSK3β AKT PDK-1 PI3-K IRS Leptin

Glut4 PKC Insulin

3/28/17
Deletions in the O-GlcNAc Cycling Enzymes Modulate Median Lifespan in *C. elegans*

O-GlcNAc regulates numerous cellular processes involved in transcription:

1) Chromatin remodeling
2) Activation/Initiation Complex
3) Stability
4) Localization
5) DNA binding
6) Transcriptional activation
7) Protein-protein interaction

Adapted from Brimble et al 2009
O-GlcNAc modifies histones and members of chromatin complexes

OGT and O-GlcNAc Influence DNA Availability and Gene Transcription

O-GlcNAc is Part of the “Histone Code” and Adds to Epigenetic complexity

Testing the O-GlcNAc is a Glucose Sensor Model: Where better than a Mammalian β-cell
OGA Inhibition Elevates Nuclear O-GlcNAc Levels in Min6 Cells

Nuclear Extract

O-GlcNAc (Mab10)

α Tubulin

P value: * < 0.05

n=3

O-GlcNAc Inhibits Desensitization of Insulin Secretion under Prolonged Hyperglycemia Exposure

P values: * < 0.05 vs. LG; ** < 0.01 vs. LG; # < 0.03 vs. HG

n=3
Elevating O-GlcNAc Increases Intracellular Insulin in Min6 cells

1 hr Stimulation: RIA

Green: Insulin (all forms); Blue: Toto3 nuclear

P values: * < 0.05 vs. LG; ** < 0.01 vs. LG

Increasing O-GlcNAc Augments Mouse Insulin 1 and 2 Steady State mRNA in Min6 cells

Ins2 mRNA half-life in Min6 cells is > 24 hr (Ritz-Laser et al 1999)

P value: * < 0.05 vs. LG; ** < 0.01 vs. LG; *** < 0.001; & < 0.05 vs. HG

Both n=10
Elevating O-GlcNAc Leads to Increased Histone H3 Epigenetic Activating Marks at the *Ins2* Promoter

Epigenetic Marks on Total Histone H3 are Unchanged Between Conditions

- Acid extracted histone samples blotted with histone H3 activating marks and protein antibodies
- All samples normalized to protein input as determined by Ponceau S stain
O-GlcNAc and Hyperglycemia Regulate a Similar Subset of Genes in Min6 cells

- RNA-sequencing results:
  - >50% of O-GlcNAc affected genes are similarly influenced by HG
  - Approx. one-third of HG altered genes are similarly affected by O-GlcNAc
  - Increasing O-GlcNAc heavily contributes to the impact of hyperglycemia on Min6 gene transcription

What will the 4th Decade of O-GlcNAc Bring?

Role in Pathophysiology
Metabolic Syndrome X
Diabetes
Cardiac Disease
Tauopathies
Etc.

Mutations in OGT causal for XLID
X-linked Intellectual Disability (XLID)

- 1-3% of total population is affected by ID
  - IQ <70
- 5-10% of ID in males is X-linked (~1 in 1,000 males affected)
  - impaired cognitive function by 5 years of age
- Fragile X syndrome was the first marker identified
  - most common ID syndrome
- Many genes identified to date but 30-50% unidentified genetic etiology
- Varying phenotypes other than IQ deficit in Syndromal Patients

Novel mutation in OGT identified by X-chromosome exome sequencing of XLID patients with Charles Schwartz
Steady state OGT protein levels are decreased in L254F XLID patients

L254F OGT has a half life that is ~ half that of WT

Vaidyanathan et al., JBC. In Press, Accepted 3.16.17
Recombinant L254F Active as GT and HCF1-Protease

O-GlcNAc Levels Unaltered by WB
Does the O-GlcNAcome of the L254F variant differ from the WT?

The O-GlcNAcome of the avg. controls vs. avg. patients do not significantly differ

Surprisingly, OGA levels decreased
Global transcriptomes of XLID and unaffected males cluster separately

Differential expression of genes in XLID and WT

<table>
<thead>
<tr>
<th>Total no of genes</th>
<th>2-fold</th>
<th>3-fold</th>
<th>4-fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>8800</td>
<td>349</td>
<td>89</td>
<td>38</td>
</tr>
<tr>
<td>Disease/natural variation</td>
<td>4.8 fold</td>
<td>3.9 fold</td>
<td>3.3 fold</td>
</tr>
</tbody>
</table>

Up 3-fold in XLID
58

Down 3-fold in XLID
8711

31

~ 1% of the genes are differentially regulated in XLID
**Observation:**
Missense Mutations in OGT Segregate with XLID

**Findings:**
L254F-OGT produces an active enzyme with reduced stability
Global O-GlcNAc levels are unaltered in lymphoblastoids
OGA transcript and protein levels are reduced in L254F cells
OGT is enriched at OGA promoter in affected L254F cells

**Significance:**
A compensation mechanism, albeit imperfect given the phenotype of the patients, exists whereby OGT regulates OGA to maintain steady state O-GlcNAc levels in L254F lymphoblastoids.

**Why do mutations in OGT cause XLID?**

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### O-GlcNAc Challenges

- Assigning function to specific site on particular protein (mapping sites)
  - Recent data has demonstrated this
  - Great example CAMKIV (O-GlcNAc in active site)
  - Another Example: Fox01 repression of gluconeogenic enzymes in liver
  - Another Example: O-GlcNAc of Histone required for mono-Ub modification
- Regulation of OGT/OGA (PTM, protein:protein associations)