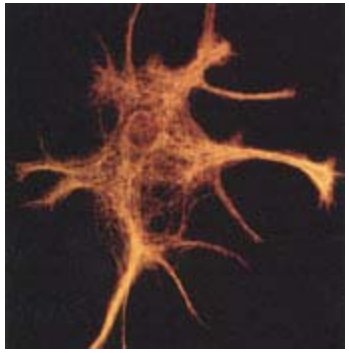
2D gel Western blotting using antibodies against ubiquitin, SUMO and acetyl PTM

Nancy Kendrick, Jon Johansen & Matt Hoelter,
Kendrick Labs Inc
www.kendricklabs.com

Talk Outline

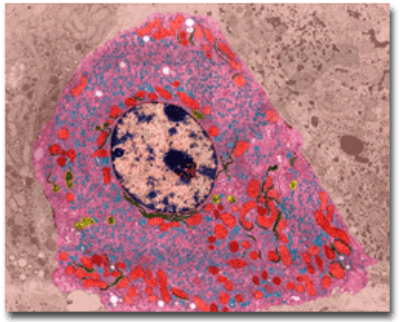
- Significance
- Method description
- Results for anti-ubiquitin, anti-Sumo, and anti-acetyl Western blotting
- Summary, future plans, collaborators

The 200+ different cell types in the human body contain the same DNA but are dramatically different.



Brain astrocyte

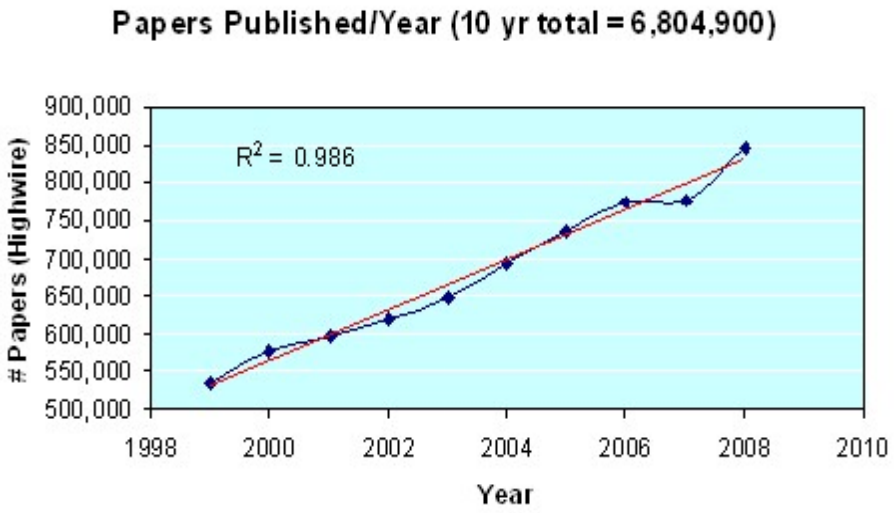
White blood cell (leukocyte)

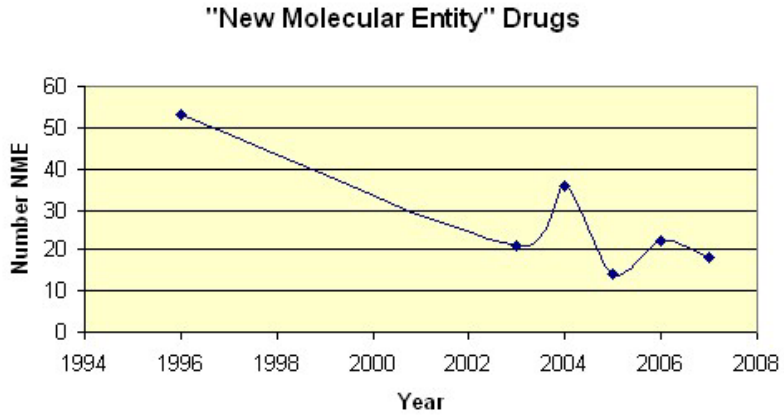


Liver cell

- Different sets of *proteins* are expressed during differentiation
- Continuous post-translational modification of proteins occurs. This is not well understood.

NIH has a problem: the research publication rate is up, but the new drug rate is down





What's going on?

- The human genome sequence was first published in 2001. Since then there's been a tremendous research push to understand human diseases using genomic analysis, especially cDNA microarrays.
- But genomic analysis isn't working well, even for prediction. For example, two researchers at Mayo Clinic looked at 3 major array studies for lung cancer (Michigan, Harvard, and Stanford) and concluded that outcome prediction from gene arrays can be mostly explained by well-known conventional predictors. Cancer Epidemiol. Biomarkers Prev., Nov 2006; 15: 2063.

Proteomic analysis hasn’t worked well either

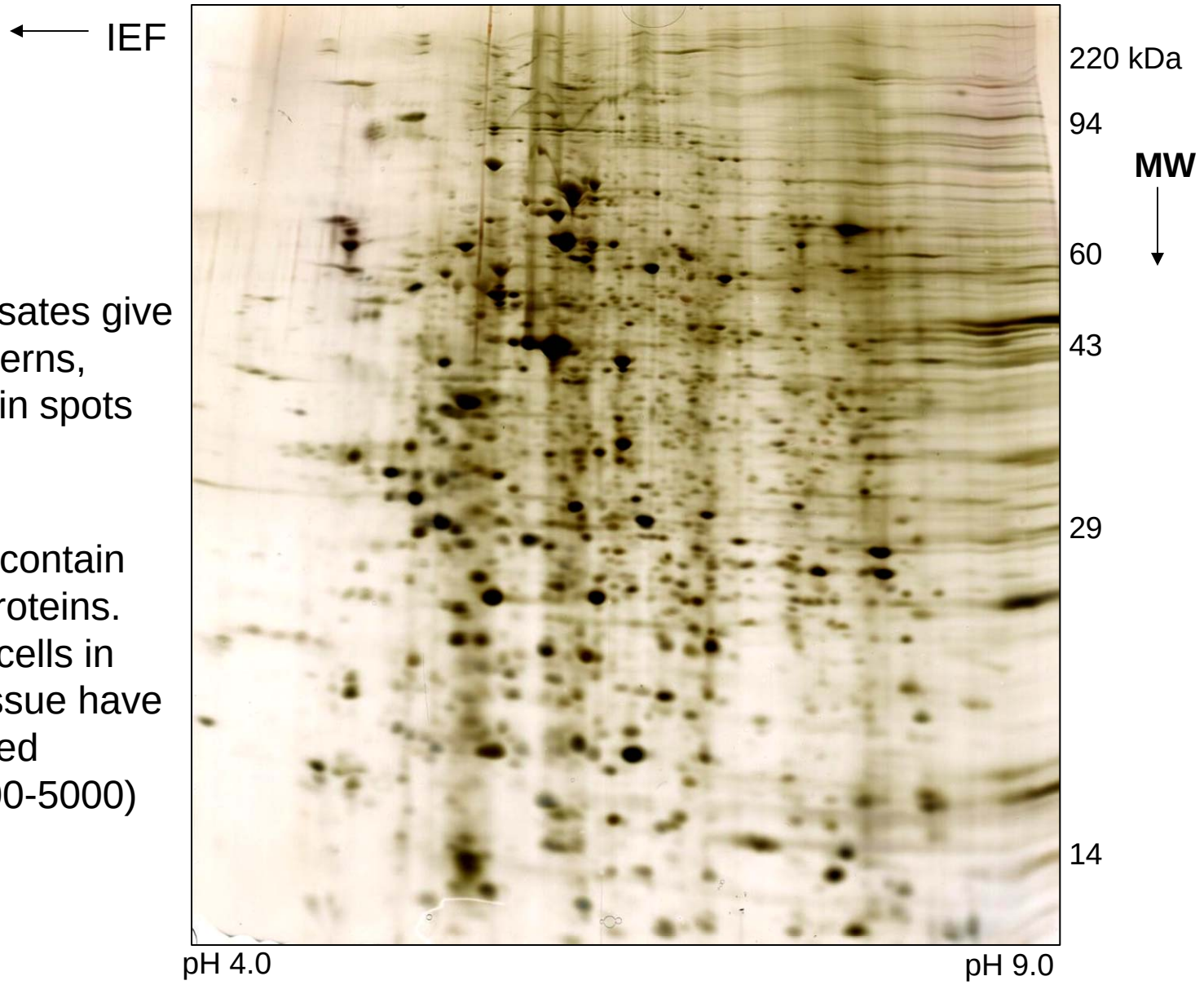
NIH funded ten mass spectrometry centers around the country in 2002, but the expected discovery of disease biomarkers hasn’t occurred.

Kendrick Labs is a proteomics service company specializing in 2D electrophoresis. What approach could we take that might improve the success rate?

- Experience taught us it's easier to sort out proteomic problems when clients look at protein subsets – comparing control with experimental.
- We decided to focus on PTM subsets that 2DE could easily detect, starting with phosphoprotein WB. That worked well so we're expanding the approach.

Whole cell lysates give complex patterns, ≥ 1000 protein spots

Cultured cells contain 4,000-8,000 proteins. Differentiated cells in mammalian tissue have fewer expressed proteins (~3000-5000) but still a lot.

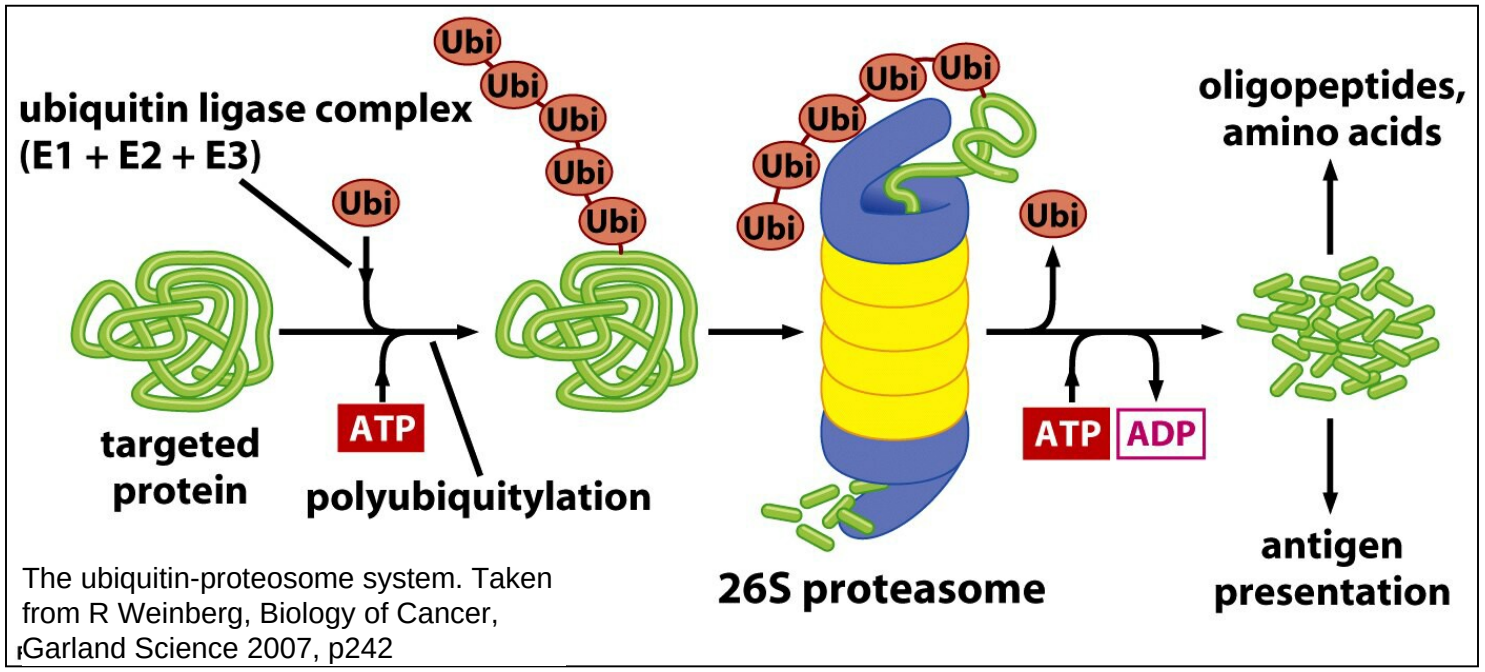


Acute Lymphoblastic Leukemia (ALL) cell line. Shown with permission of Dr. Terzah Horton, Baylor College Medicine.

2D gel Western blotting of PTMs is a way to look at protein subsets with high sensitivity, 10-100 X greater than staining

1. Transfer all the 2D gel proteins to a membrane called PVDF
2. Incubate with an anti-PTM antibody either 2 hr or overnight
3. Visualize the proteins with a secondary HRP-ab that induces ECL fluorescence; expose to x-ray film.

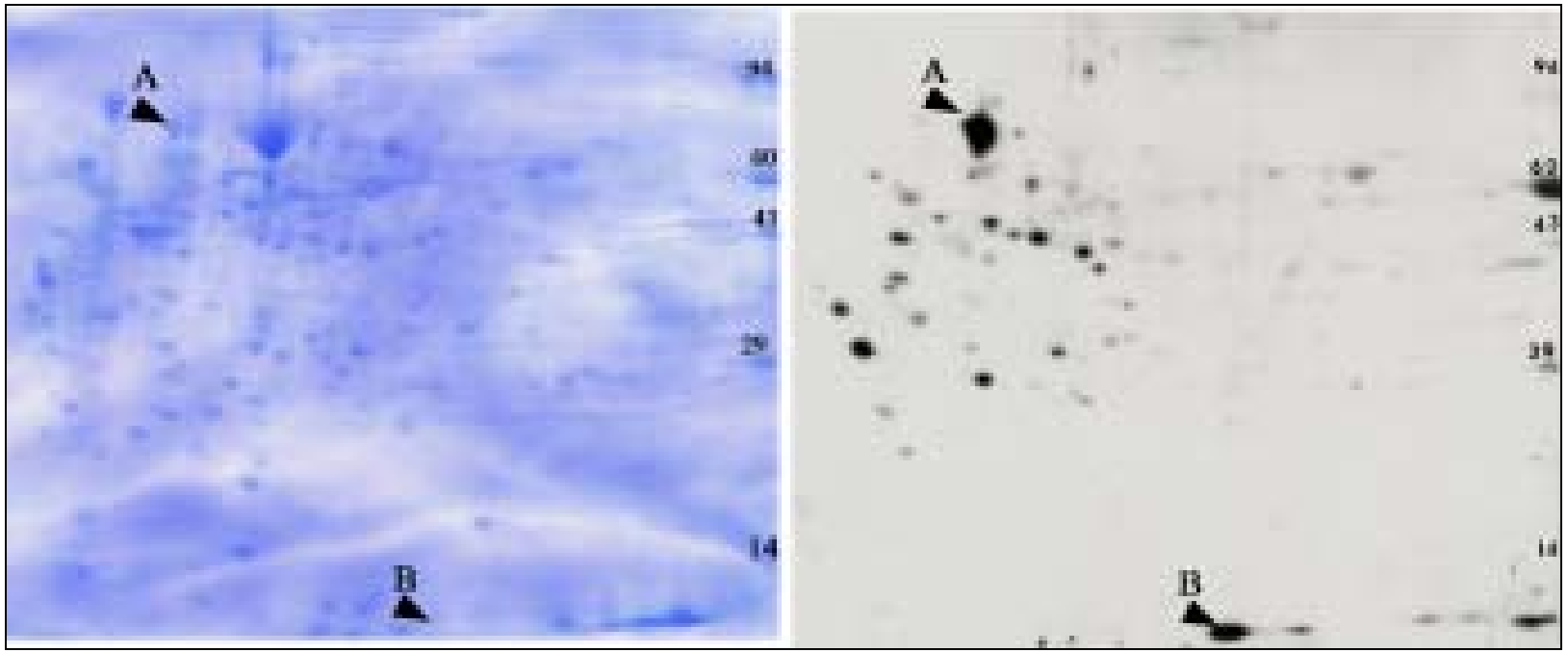
Anti-ubiquitin Western blotting



The ubiquitin-proteosome system. Taken from R Weinberg, Biology of Cancer, Garland Science 2007, p242

- The ubiquitin-proteosome system functions universally in eukaryotes to rapidly break down proteins. Ubiquitin, an 8.5 kDa protein, is highly conserved between species. The human genome contains over 500 ubiquitin E3 ligases that target individual proteins. Polyubiquitylation is known to be responsible for the fast turnover of P53 (20-min half life), Myc, Jun, and some cyclins.

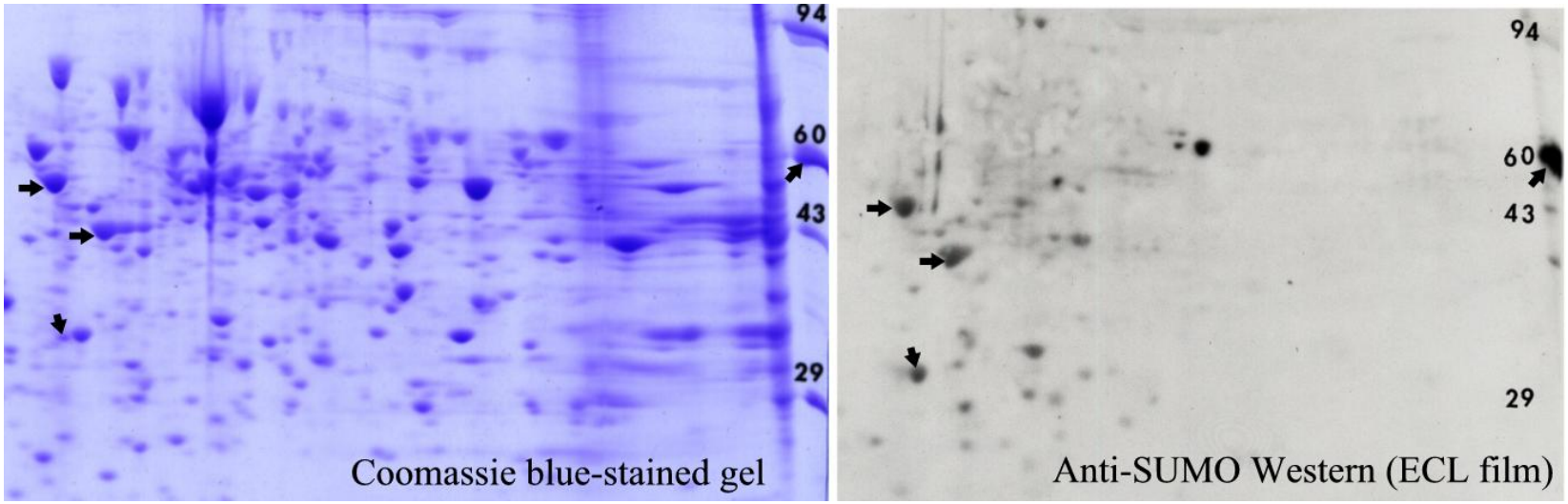
Anti-ubiquitin Western blotting



Left above shows a Coomassie stained 2D PVDF blot from rat liver homogenate diluted with SDS/Urea buffer. Right: ECL film obtained after overnight incubation of the left-hand blot with anti-ubiquitin antibody from Bethyl Labs diluted 1:2000. The film and blot are superimposable. The major proteins don't match between the patterns (see arrows), suggesting that the anti-ubiquitin ab is binding specifically. The 60 kDa marker lights up so probably some non-specific binding is present.

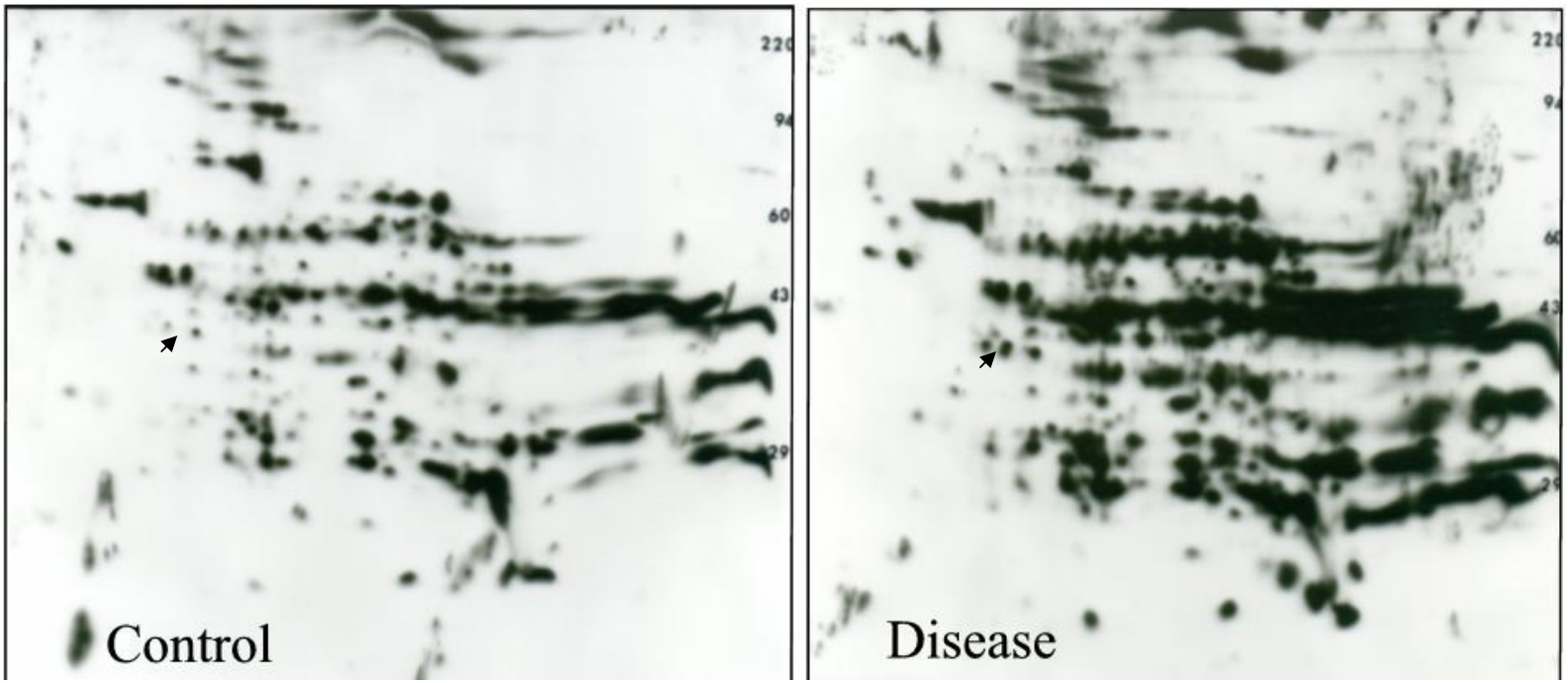
Anti-SUMO (small ubiquitin-like modifier) WB

SUMO attaches to lysines of target proteins like ubiquitin but doesn't promote protein degradation. Rather it alters protein subcellular location, protein partnering and DNA binding. (Hilgarth et. al. JBC, 279: 53899, 2004)



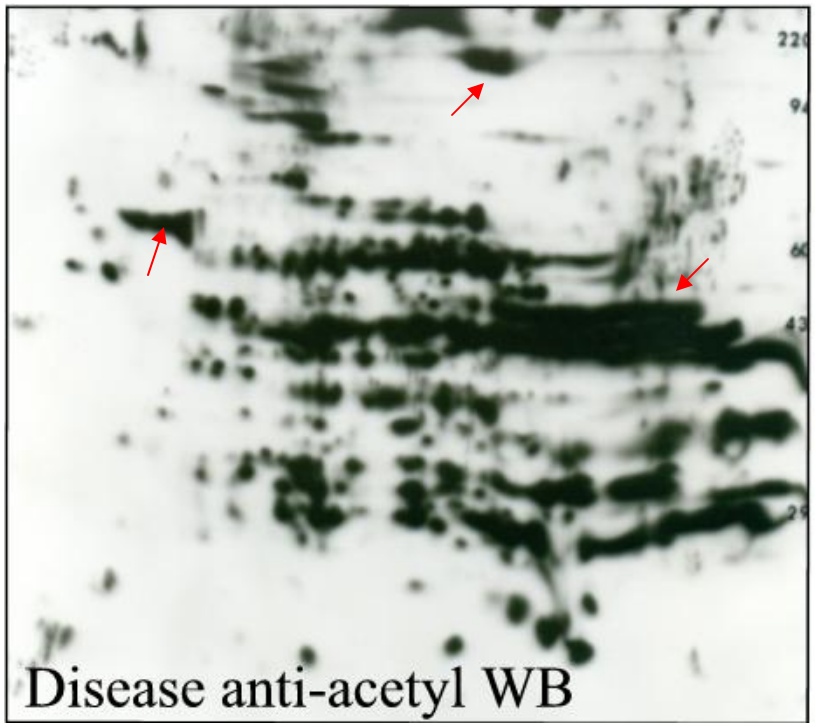
Anti-SUMO WB is shown above using a rabbit anti-SUMO antibody from Rockland Immunochemicals Inc diluted 1:2000 with a 2 hr incubation time. Left: 2D gel from rat liver homogenate stained with Coomassie blue. Right: ECL film showing putative SUMO-labeled proteins. Again, the major proteins don't match between the patterns (see arrows) suggesting that most of the ab binding is specific.

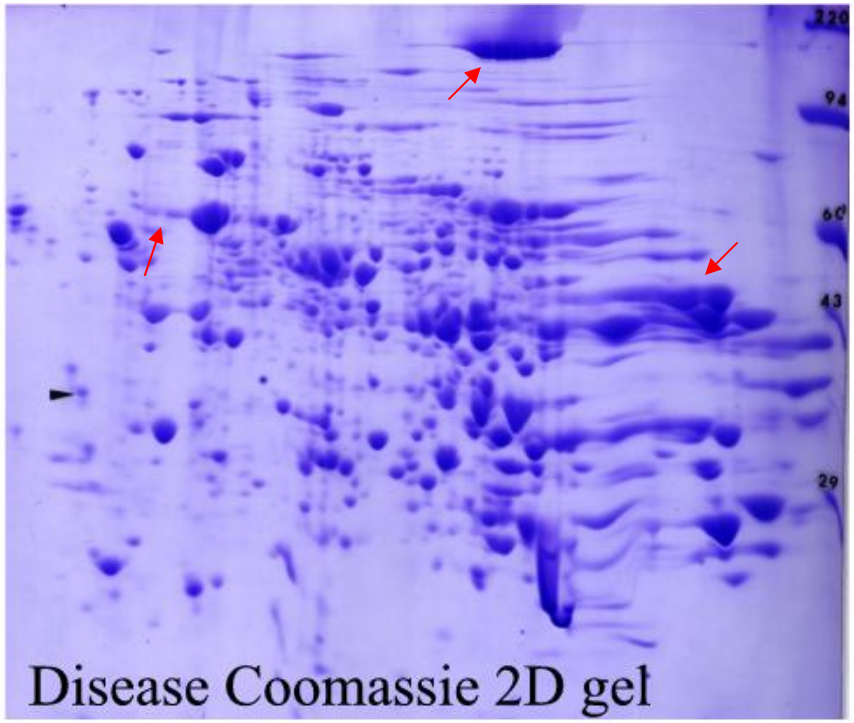
Anti-acetyl Western blotting



2D ECL films showing increased protein acetylation in rat liver associated with a disease state. 2D gels were loaded with equal amounts of protein from control and disease liver cytosol. An anti-acetylated-lysine antibody from Cell Signaling was used for WB at a 1:1000 dilution with overnight incubation. *The images are shown with permission of Blythe Shepard and Dr. Pamela Tuma, Catholic University of America, Washington, DC.*

Interesting acetylated proteins may be indentified by mass spectrometry





Each ECL film is superimposable with an image of the stained blot. Protein spots of interest found on the image are easily matched to a duplicate Coomassie blue stained gel for spot-cutting. Any CB spot, no matter how faint, is within range for MS.

Summary and Future Plans

- 2D gel Western blotting using antibodies against PTMs works well.
- This approach has the potential to elucidate disease-related protein changes that would be difficult to detect by other means.
- We’re now soliciting test samples that would further validate the method.

Collaborators:

Jon Johansen, Lab Manager

Matt Hoelter, Biochemist, AES Executive Director