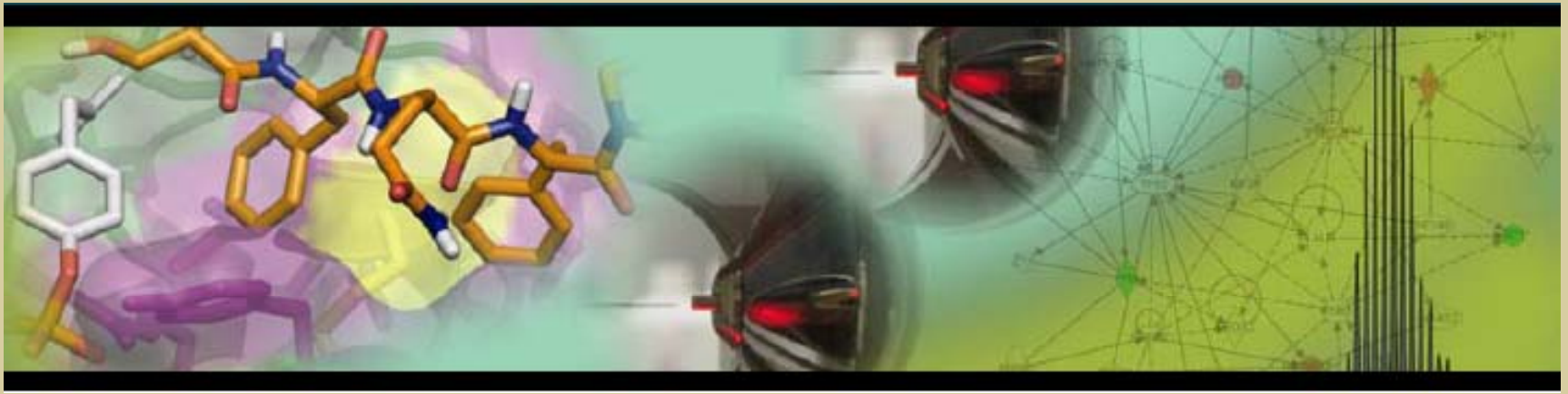

Phosphoproteomics: Mechanisms in Controlling Cell Volume and Neuronal Excitability

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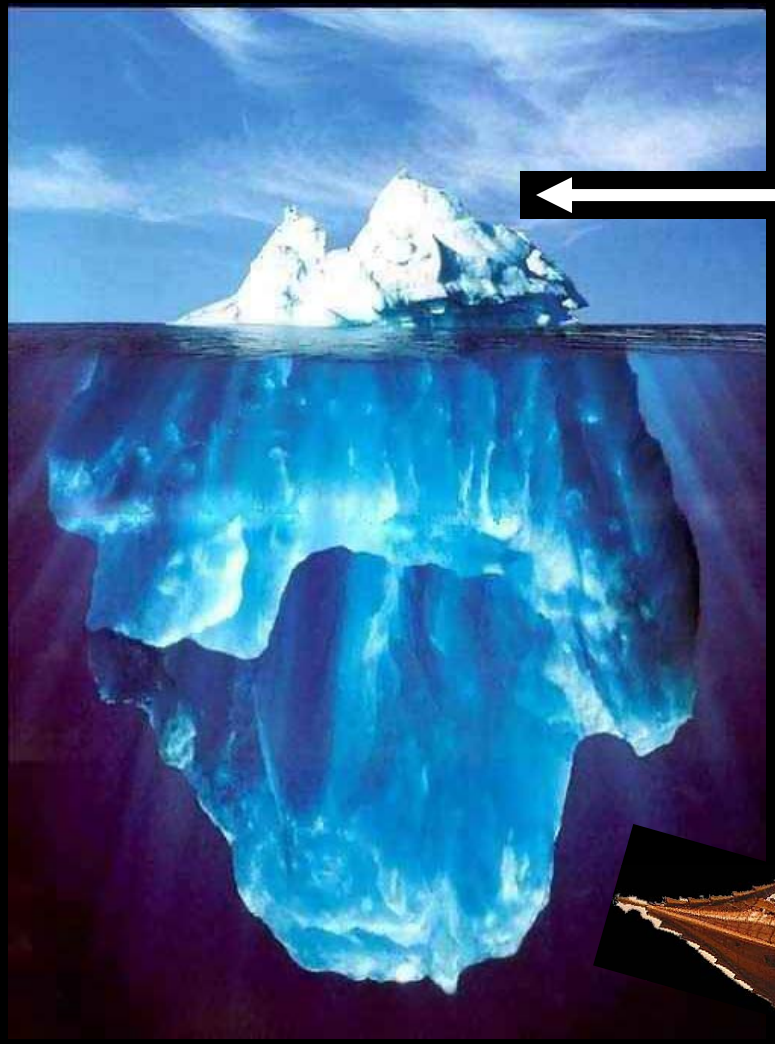
Department of Genetics, Howard Hughes Medical Institute,
Yale University School of Medicine

Proteomic tools for direct analysis of biological systems



Modern Proteomics

- Proteomics is in its infancy
- No complete proteome to date
  - Contrast to genomics
  - Splicing, PTMs, compartmentalization,..
- We have come a long way quickly.
- *New tools, techniques, and technology means we are gaining access faster!*

Targeted Proteomics

Challenges for Phosphoproteomics

Phosphopeptides are hard to detect due to:

- Low stoichiometry
- Heterogeneity of phosphorylation
- Low ionization efficiency
- Hardware/data analysis issues

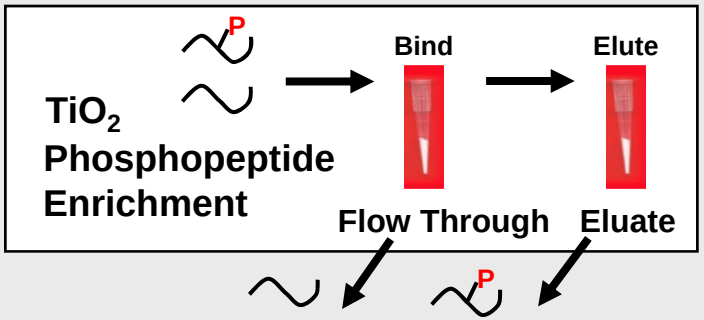
Overcoming the challenges:

Technology Tools:

- Acquired over 1.5 million $ in new MS equipment
- Phosphopeptide enrichment
- Accuracy of phosphoprotein ID and determination of phosphorylation sites

Target Functionally Important Proteins for Disease

Using Biomedical Technology Development for Understanding of Disease and Addiction

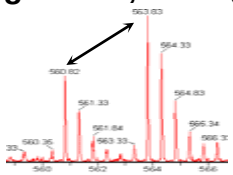


Protein Phosphorylation and Quantitation → Disease Mechanism

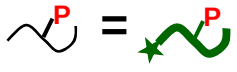
Proteomics Pipeline
from
Discovery to **Validation**

MS identification of phosphorylation sites Using phosphopeptide enrichment

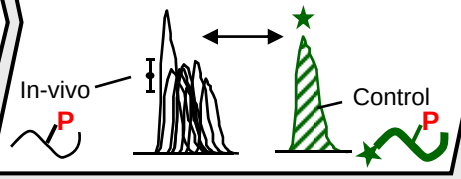
***In vitro* comparison of phosphorylation levels using SILAC, iTRAQ etc.**



Synthesize stable isotope labeled phosphopeptides

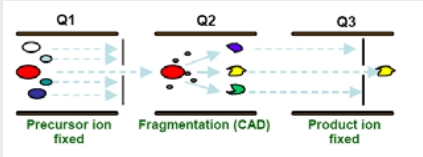


***in vivo* functional comparison in large sample sets by MRM**



n-UPLC LTQ-Orbitrap MS

Schematic of the MRM scan



QTRAP 4000
Triple Quadrupole MS

Phosphorylation dynamics in electrolyte homeostasis

-What are the phosphorylation sites involved?

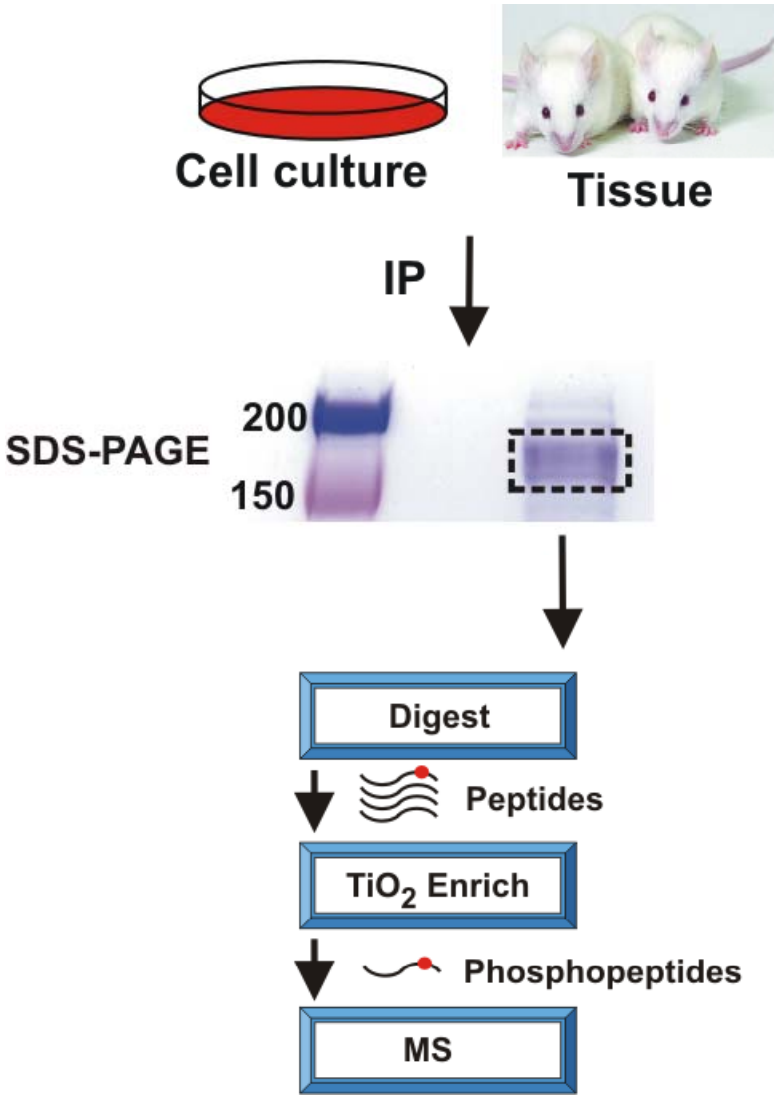
-What are the physiological mechanisms involved in their signaling pathways?

-Phosphorylation controls Cl^- homeostasis in neurons
- -Fundamental to GABA signaling in healthy and addicted individuals
- -Mechanisms *via* phosphorylation is poorly understood

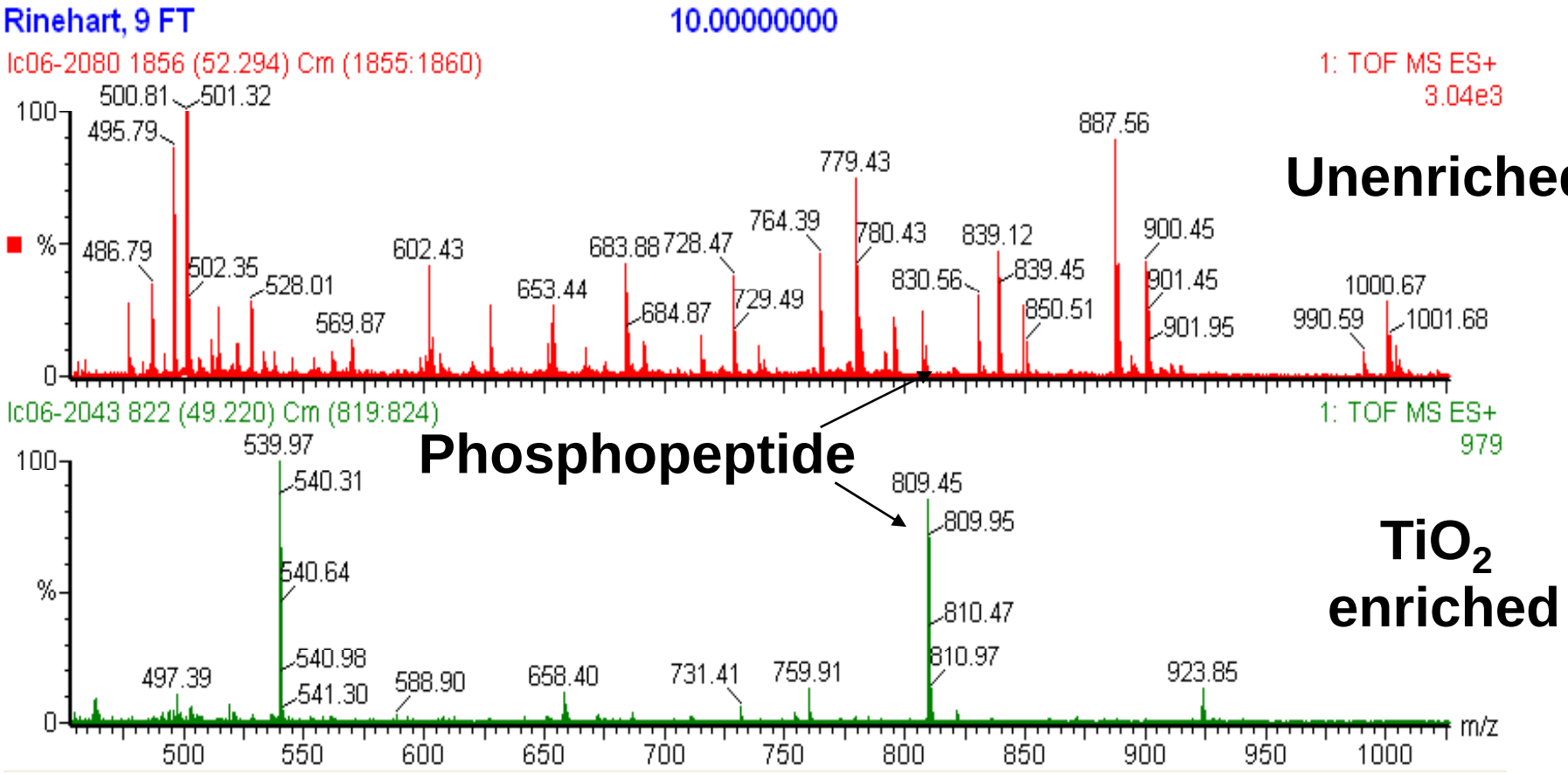
Approach

- Use proteomic tools to target phosphoproteins involved in electrolyte homeostasis.
- Develop new techniques to address complex phosphorylation dynamics in vivo.

Phosphorylation mapping with TiO_2 phosphopeptide affinity matrix

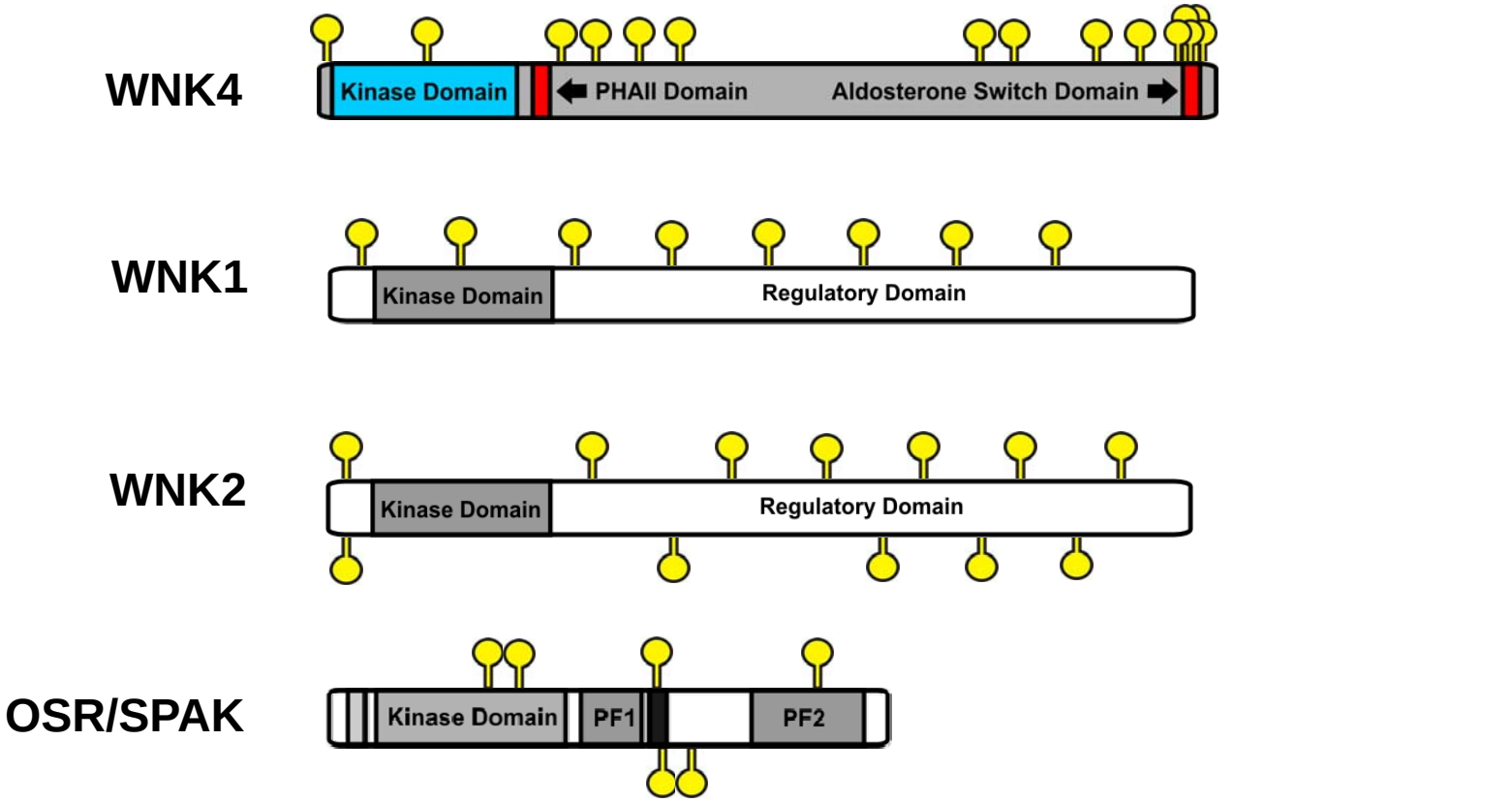


Phosphorylation mapping with TiO_2



Enrichment reduces complexity

Kinases that control electrolyte homeostasis

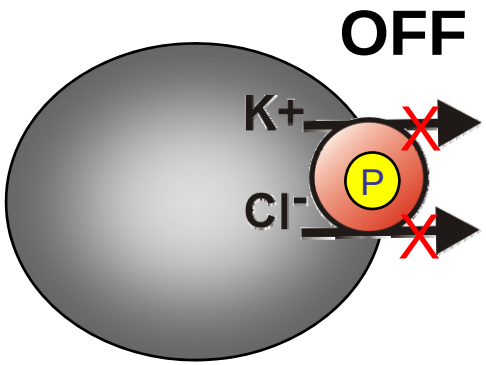


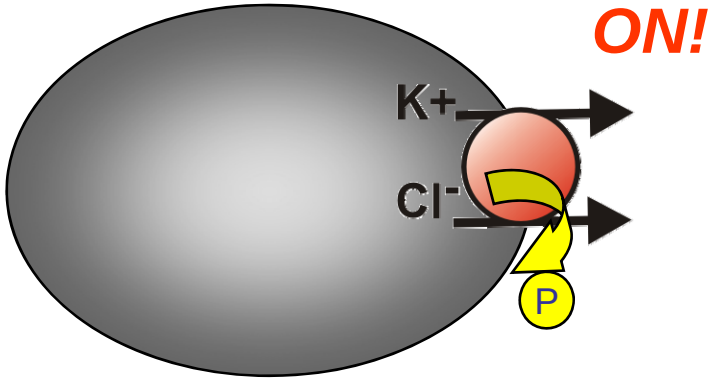
•Potassium (K+) and Chloride (Cl-) cotransporters (KCC) are integral membrane proteins that couple K-Cl transport out of the cell.

•All KCCs are activated by cell swelling, and their phosphorylation state controls KCC activity.

• *However*, <u>no direct evidence</u> for phosphorylation that drives KCC activity

•Insight into KCC regulation would help understand and potentially treat diseases like Hypertension, Sickle Cell Disease, & Epilepsy

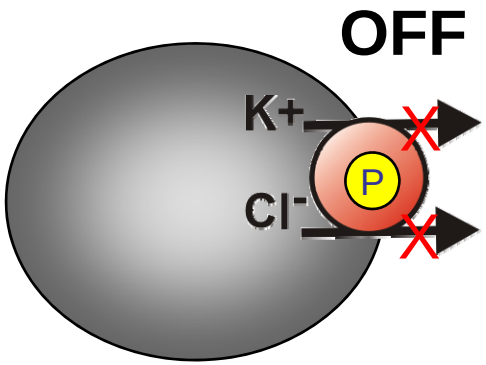




When phosphorylated, KCC is *off*
Isotonic Conditions

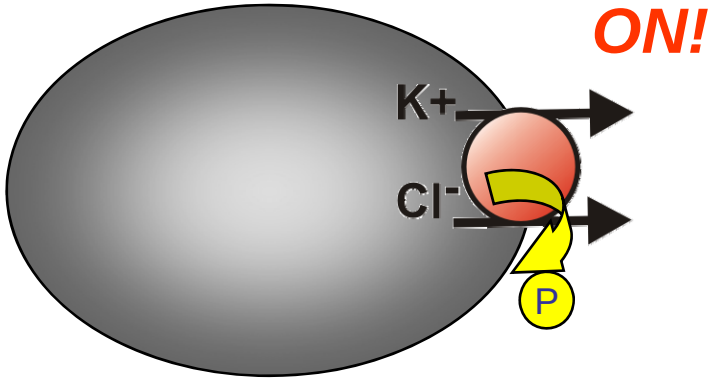
De-phosphorylation *activates* KCC
Hypotonic Conditions

How can we determine the functional phosphorylation sites in KCC that control co-transporter activation?



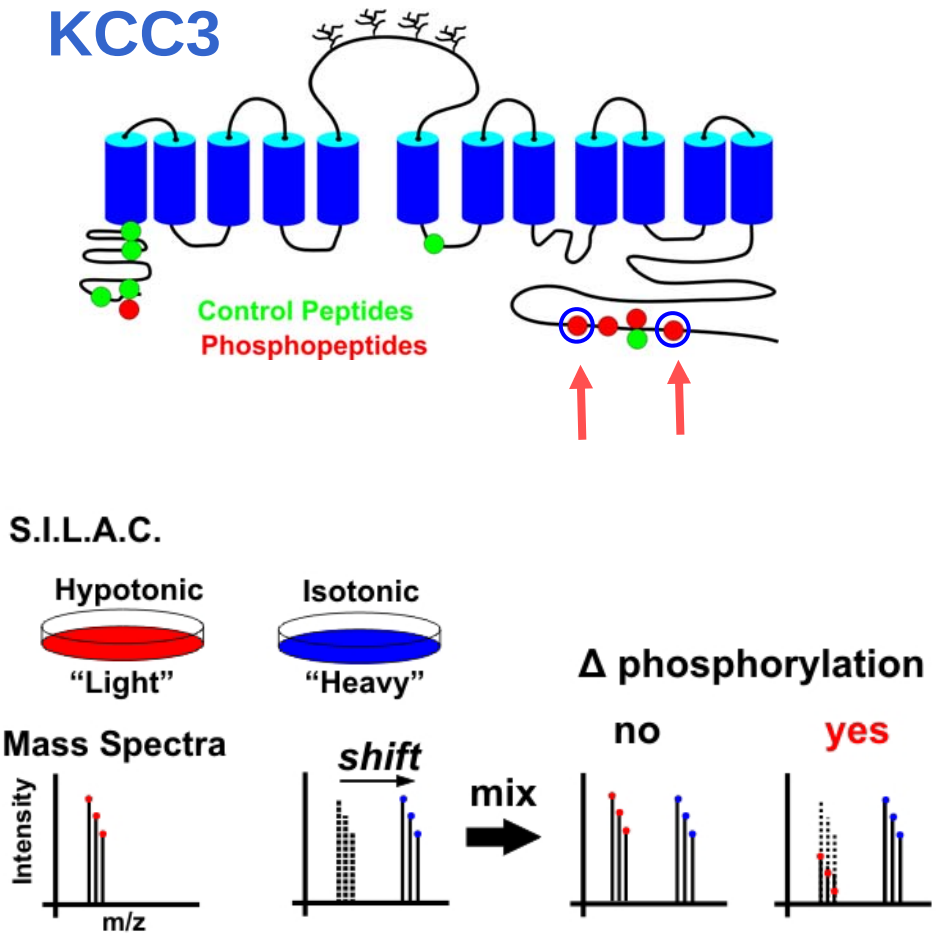
When phosphorylated, KCC is *off*

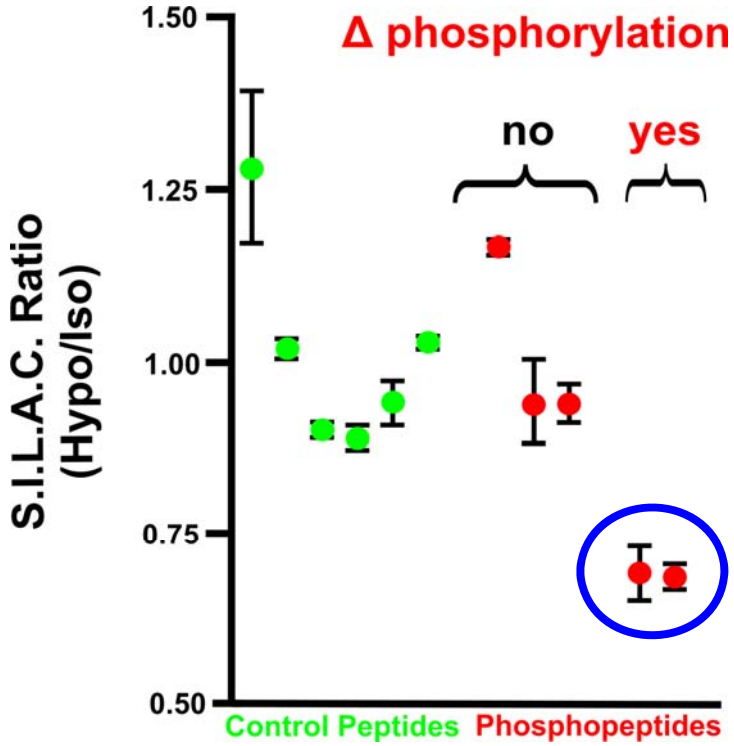
Isotonic Conditions



De-phosphorylation *activates* KCC

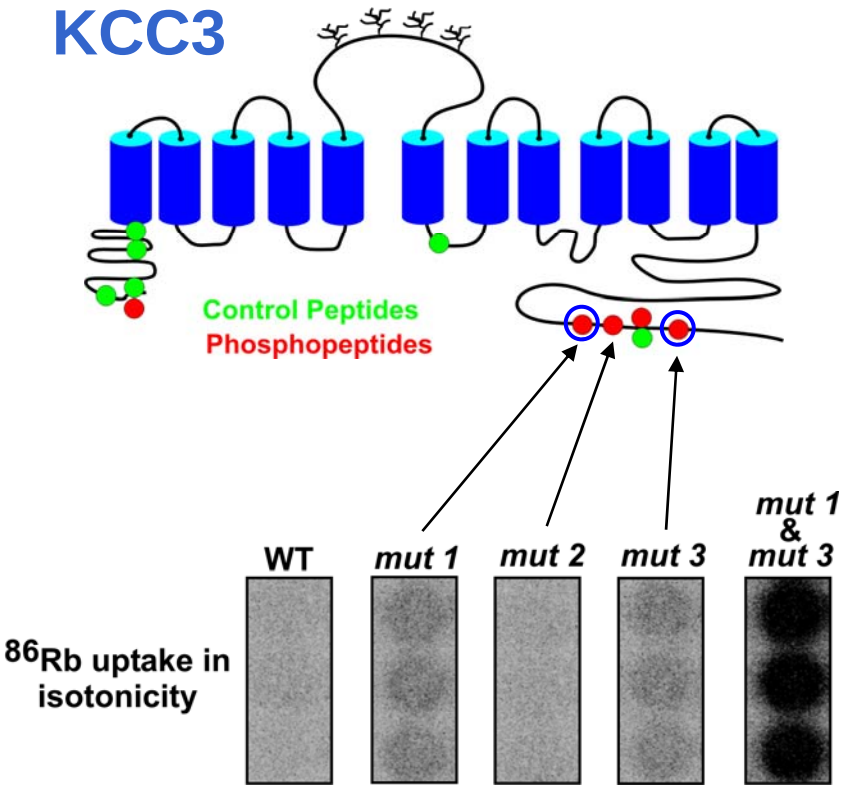
Hypotonic Conditions

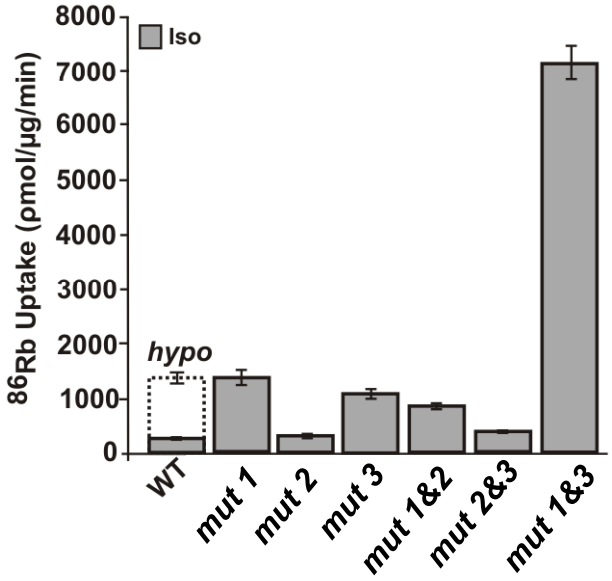




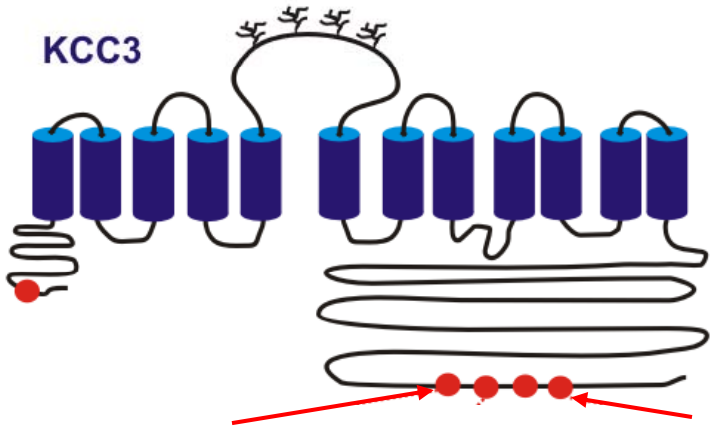
Two phosphorylation sites within KCC respond to altered cell volume

Two phosphorylation sites control KCC activity
⇒ A double alanine mutation ablates normal regulation





Two regulatory phosphorylation sites are strictly conserved in KCCs:



Essential KCC functions

KCC1: cell volume, house keeping function

KCC2: neuronal function (neuron specific isoform)

KCC3: cell volume control, blood pressure

KCC4: cell volume control, hearing, blood pressure

Essential KCC functions

KCC1: cell volume, house keeping function

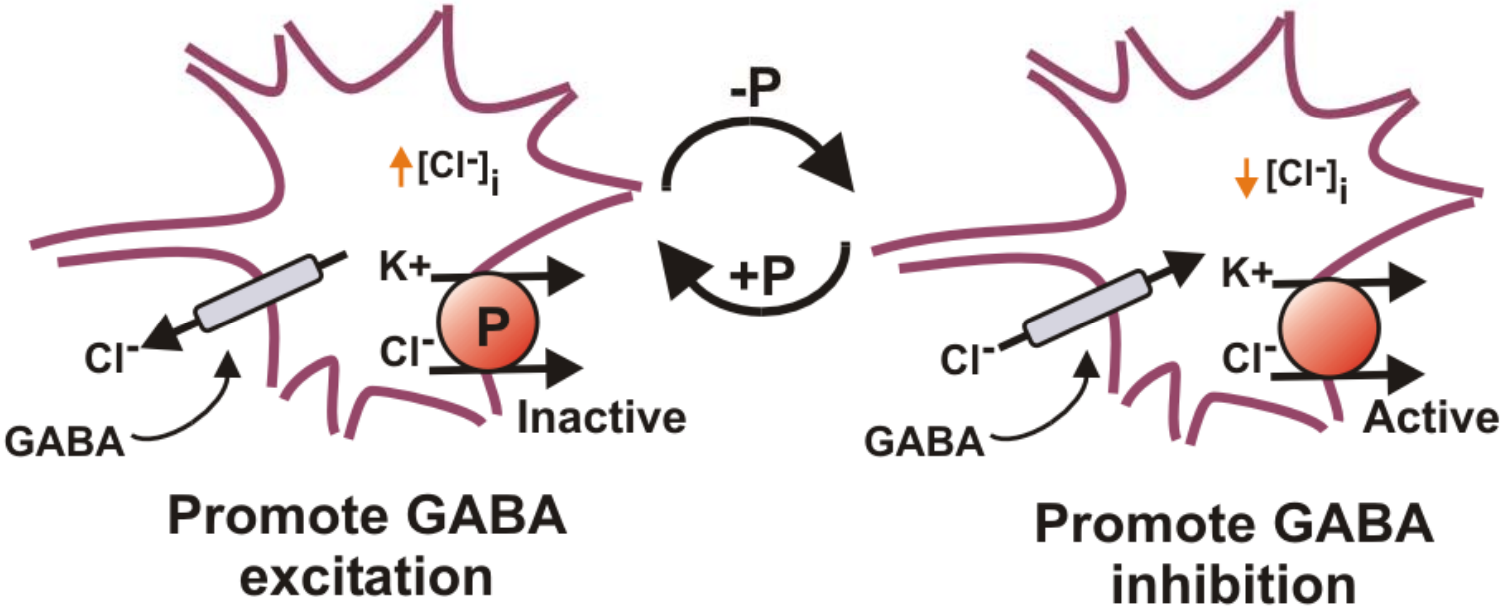
KCC2: neuronal function (neuron specific isoform)

KCC3: cell volume control, blood pressure

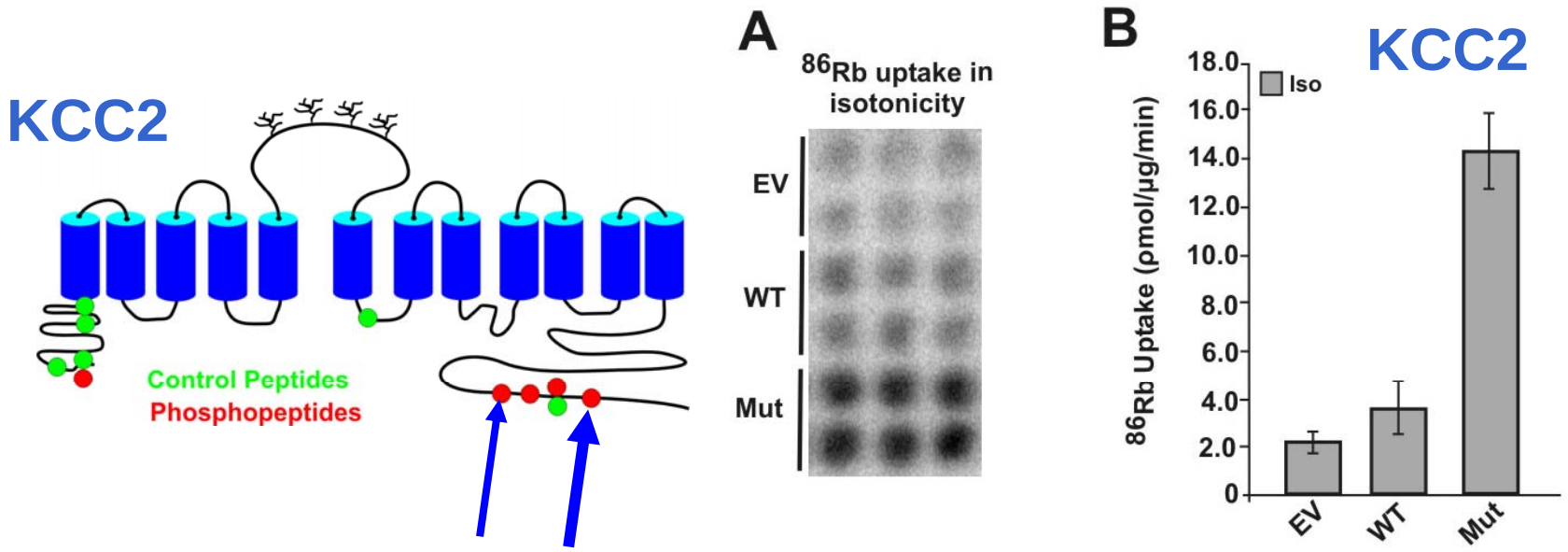
KCC4: cell volume control, hearing, blood pressure

Cell volume control and GABA signaling are linked
By a common mechanisim that drive Cl- balance via
Activation of the K-Cl cotransporters

Hypothesis: If KCC function is conserved,
KCC2 should share these regulatory sites.



- KCC2 is inactive early in neuronal development and promotes GABA excitation
- As neurons develop, KCC2 activation promotes GABA inhibition
- KCC2 phosphorylation mirrors the progression of the developmental switch to GABA inhibition
- First evidence of KCC regulation in vivo



Two phosphorylation sites control KCC2 activity as well
⇒ A double alanine mutation ablates normal regulation

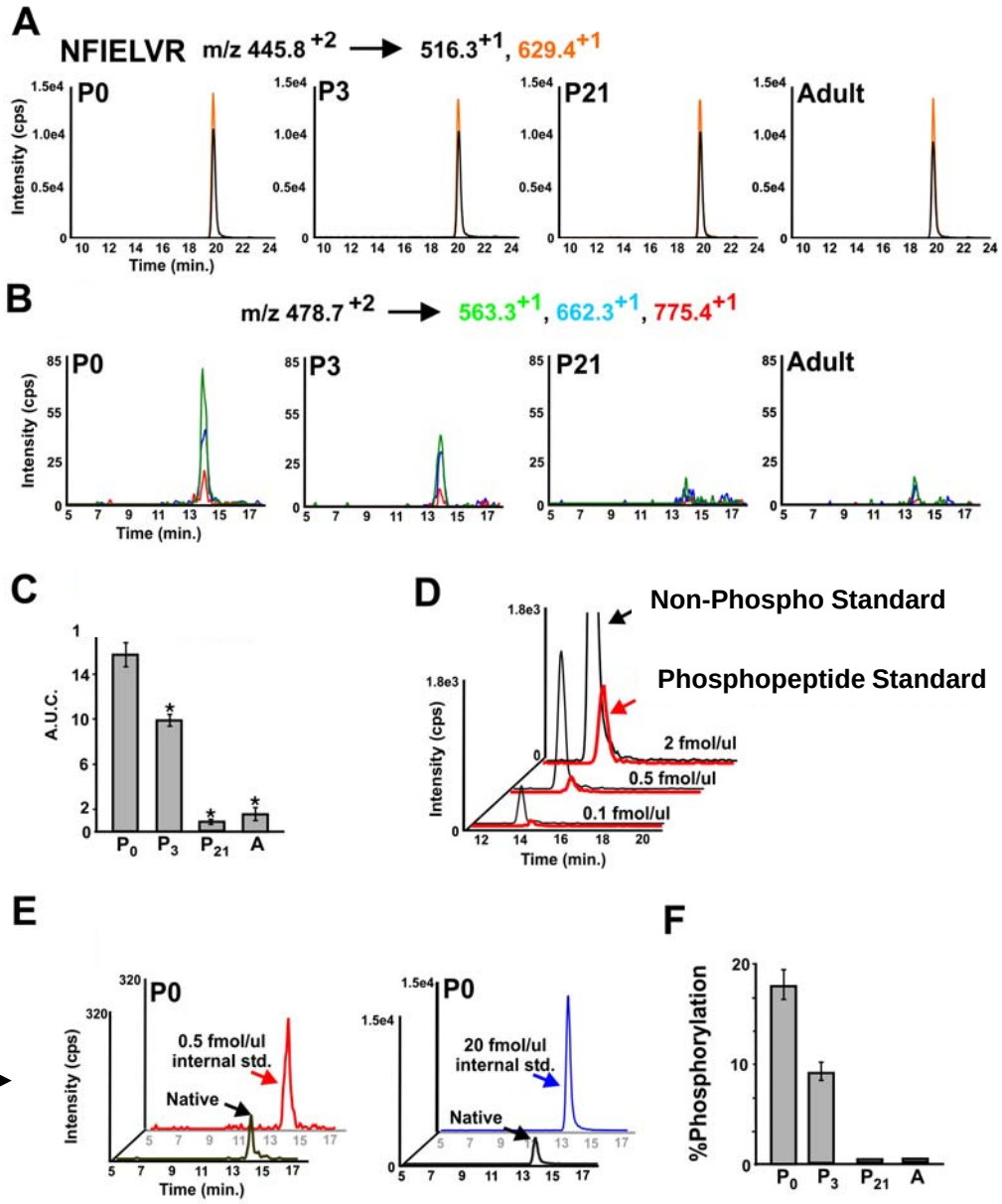
KCC2 is phosphorylated early in neuronal development

Non-phosphopeptide transition →

Phosphopeptide transition →

Relative Phosphorylation of in-vivo KCC2 →

Absolute Percent Phosphorylation of in-vivo KCC2 →



Phosphorylation dynamics in electrolyte homeostasis:

Achievements and Paths forward

Conclusions:

- **Quantitative proteomics can be used to target specific proteins to elucidate their function**
- **Mass spectrometry is highly adaptable**
- **Techniques and reagents can be developed, post-discovery, to address function *in vivo***
  - **Relevant to development of clinical proteomics**
- **Study both the transporter and putative upstream kinases in drug treated animals**

Acknowledgements

Collaborators:

Patrick G. Gallagher
- Department of Pediatrics, Yale University School of Medicine

Biff Forbush
- Department of Cellular and Molecular Physiology, Yale University School of Medicine

Clinton H. Joiner
- Cincinnati Comprehensive Sickle Cell Center and Department of Pediatrics, University of Cincinnati College of Medicine & Cincinnati Children's Hospital Medical Center.

NIDA Neuroproteomics Center:

Ken Williams
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Jean Kanyo
TuKiet Lam
Ted Voss
Kathrin Wilczak-Havill

YCMI:

Mark Shifman
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NIDA Investigators:

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