Selective Enrichment and Quantitation of Phosphoproteins Involved in Cell Proliferation

Michael Major*, Douglas Hughes*, David Sarracino#, Bryan Krastins#, Sarah Feuillerat*, Michael Rosenblatt*, Navid Haghdoost*, Barbara Kaboord* Mary Lopez#, and John Rogers*
Thermo Fisher Scientific, *Rockford, IL 61101 and #Cambridge, MA 02139

www.thermo.com/pierce

Introduction

The serine/threonine kinase Akt has been shown to regulate multiple key proteins involved in apoptosis (Bad), cell proliferation (FOXO), translation (mTOR and its effector proteins), and glycogen synthesis (G SK3b).[1] To better understand the complex regulation of phosphorylation in cellular proliferation, phosphoproteins and phosphopeptides were enriched from insulin responsive 293T cells treated with or without wortmannin or rapamycin. Phosphoproteomes were quantitatively analyzed with TMT isobaric mass tags, interpreted by pathway analysis, and verified by Western blot analysis.

Overview of the PI3K-Akt Cell Survival Signal Transduction Cascade

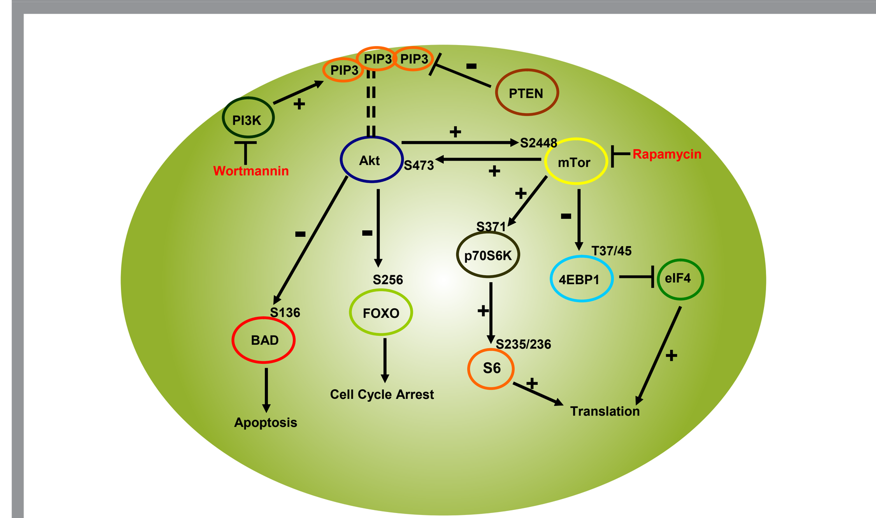


Figure 1: In response to growth factor signaling the PI3K-Akt pathway exerts its effects through phosphorylation of multiple downstream target proteins involved in apoptosis (BAD), cell cycle arrest (FOXO), and translational control (mTOR, 4E-BP1, p70S6K). The PI3K-Akt pathway is regulated by the tumor suppressor protein PTEN which functions as a phosphatase to regulate phosphorylation of phospholipids at the cell membrane. The pharmacological inhibitors wortmannin and rapamycin have been shown to be effective inhibitors of the PI3K-Akt and Akt-mTOR pathways, respectively. Deregulation of the PI3K-Akt pathway has been documented in numerous human cancers.

Highly Specific Phosphoprotein Enrichment From Complex Biological Samples

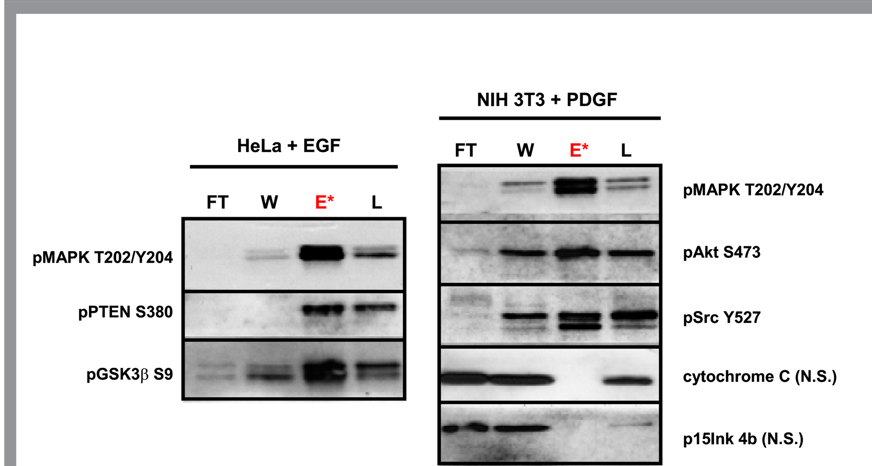


Figure 2: Serum starved HeLa and NIH 3T3 cells were stimulated with EGF and PDGF, respectively. Phosphoprotein enrichment was performed using 2 mg of total cell extract. Concentrated flow-through, wash and elution fractions were resolved by SDS-PAGE. Gel lanes were normalized by protein concentration (10 μg/lane). Western blot analysis was performed using antibodies that detect site-specific phosphorylation events. Cytochrome C (pI 9.6) and p15Ink4b (pI 5.5) served as negative controls for nonspecific (N.S.) binding of non-phosphorylated proteins. FT = flow-through fraction, W = pooled wash fractions, E = pooled elution fractions and L = non-enriched total cell extract.

Methods

Cell culture was performed by growing 293T cells at 37°C, 5% CO_2 in DMEM (HyClone #SH30243.02) media containing 10% fetal bovine serum (FBS; HyClone #SH30071.02). Cells were rendered quiescent by serum starvation (0.25%) for 48 hours. Serum-starved cells were either treated with wortmannin (1mM, 1 hour), rapamycin (100nM, 1 hour), or left untreated. Following drug treatment 293T cells were stimulated with insulin (50mg/ml, 30 minutes) or left unstimulated. After stimulation, cells were washed in 50 mM HEPES (pH 7.4). Cell extracts were prepared by adding 1 ml of Lysis/Binding/Wash buffer (Pierce Phosphoprotein Enrichment kit, Product #90003) supplemented with Halt™ Protease Inhibitor, EDTA-free (Product #78410) and Halt™ Phosphatase Inhibitor (Product #78420) to each 150mm cell culture dish. Cells were scraped into an appropriately sized collection tube and placed on ice for 45 minutes with periodic vortexing. Protein concentrations were determined by Pierce Coomassie Plus Bradford Assay (Product #23238). Phosphoproteins were enriched using a novel phosphoprotein isolation column followed by enzymatic digestion and labeling with TMTsixplex Tandem Mass Tags (Product #90064). Labeled peptides were combined and fractionated on a Biobasic Strong Cation Exchange column. Phosphopeptides were enriched with Thermo Scientific Pierce Magnetic TiO_2 Phosphopeptide Enrichment Kit beads (Product #88811) on a Kingfisher96 robot. Mass spectrometric analysis of unenriched and phosphopeptide enriched samples was performed with nanoLC-ESI-MS/MS on an LTQ Orbitrap XL™ with HCD fragmentation. Data analysis was performed with Thermo Scientific BioWorks™ 3.3.1 and GenMAPP (www.genmapp.org). Protein and phosphopeptide regulation was confirmed by Western blot analysis.

Selective Capture of Phosphoproteins Involved in PI3K-Akt Mediated Cell Survival Pathway

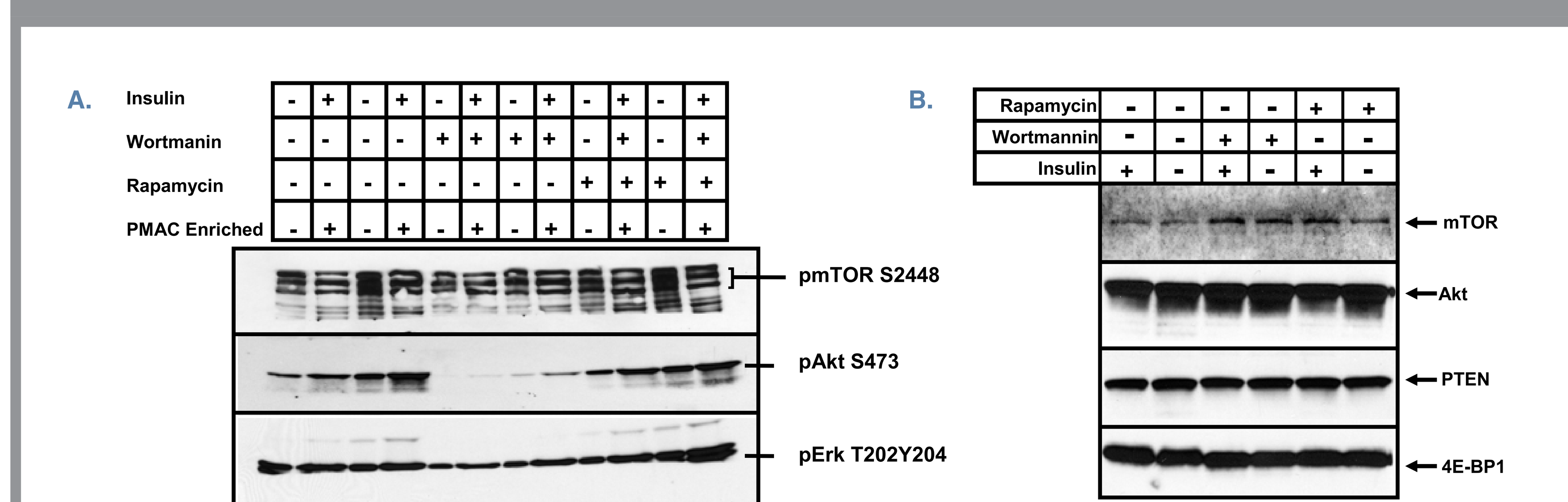


Figure 3: A. 293T cells were rendered quiescent in reduced serum (0.25%) media for 48 hours. Prior to insulin stimulation cells were treated with wortmannin and/or rapamycin or left untreated. Following drug treatment cells were either stimulated with insulin for 30 minutes or left unstimulated. Phosphoprotein enrichment was performed by loading 4 mg of total 293T cell extract onto a Pierce Phosphoprotein Enrichment column. Concentrated elution fractions were resolved by SDS-PAGE. Gel lanes were normalized by protein concentration (25μg/lane). Western blot analysis was performed using antibodies that detect site specific phosphorylation events. B. Protein abundance (75μg/lane) of non-PMAC enriched total cell extract was assessed with pan specific antibodies. No significant change in the expression level was observed indicating that changes in protein binding to the PMAC column reflect changes in phosphorylation status.

Phospho Enrichment and Quantification Workflow

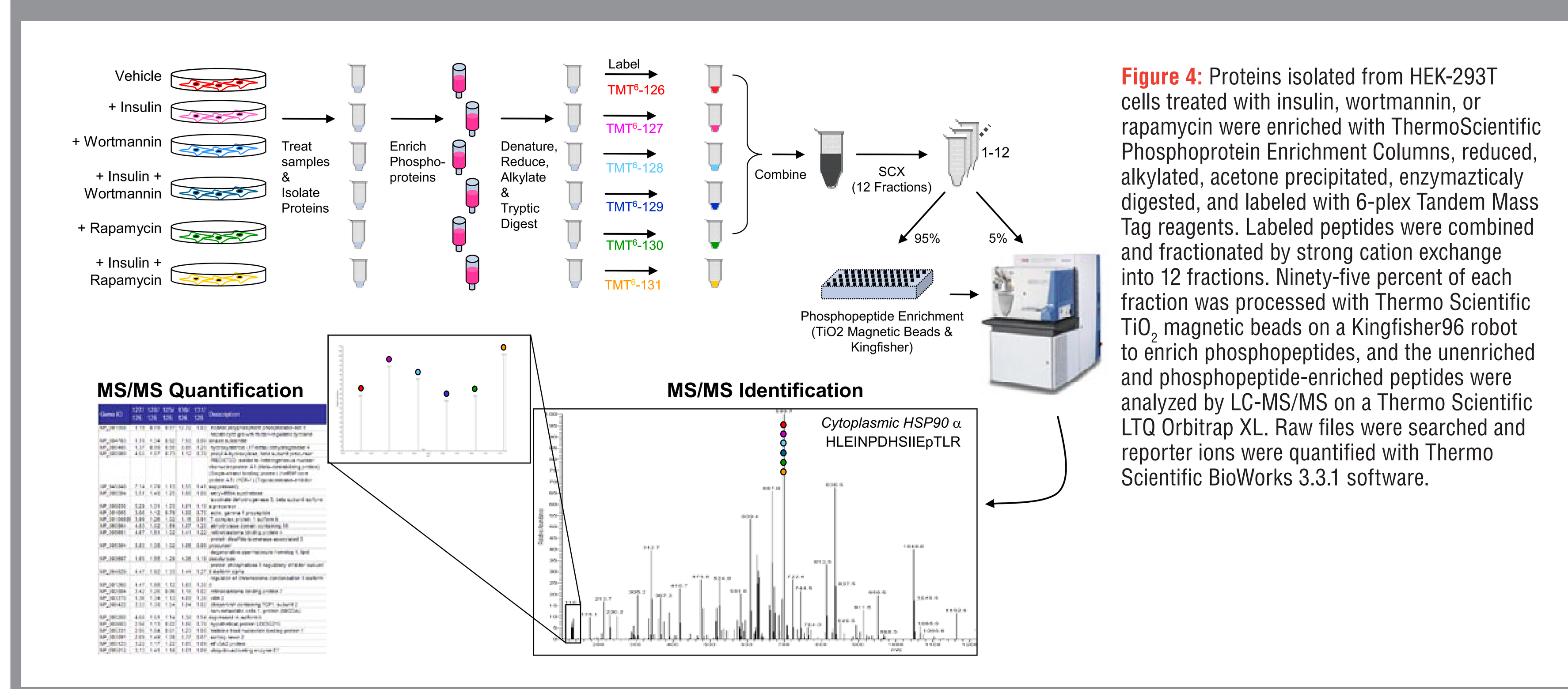


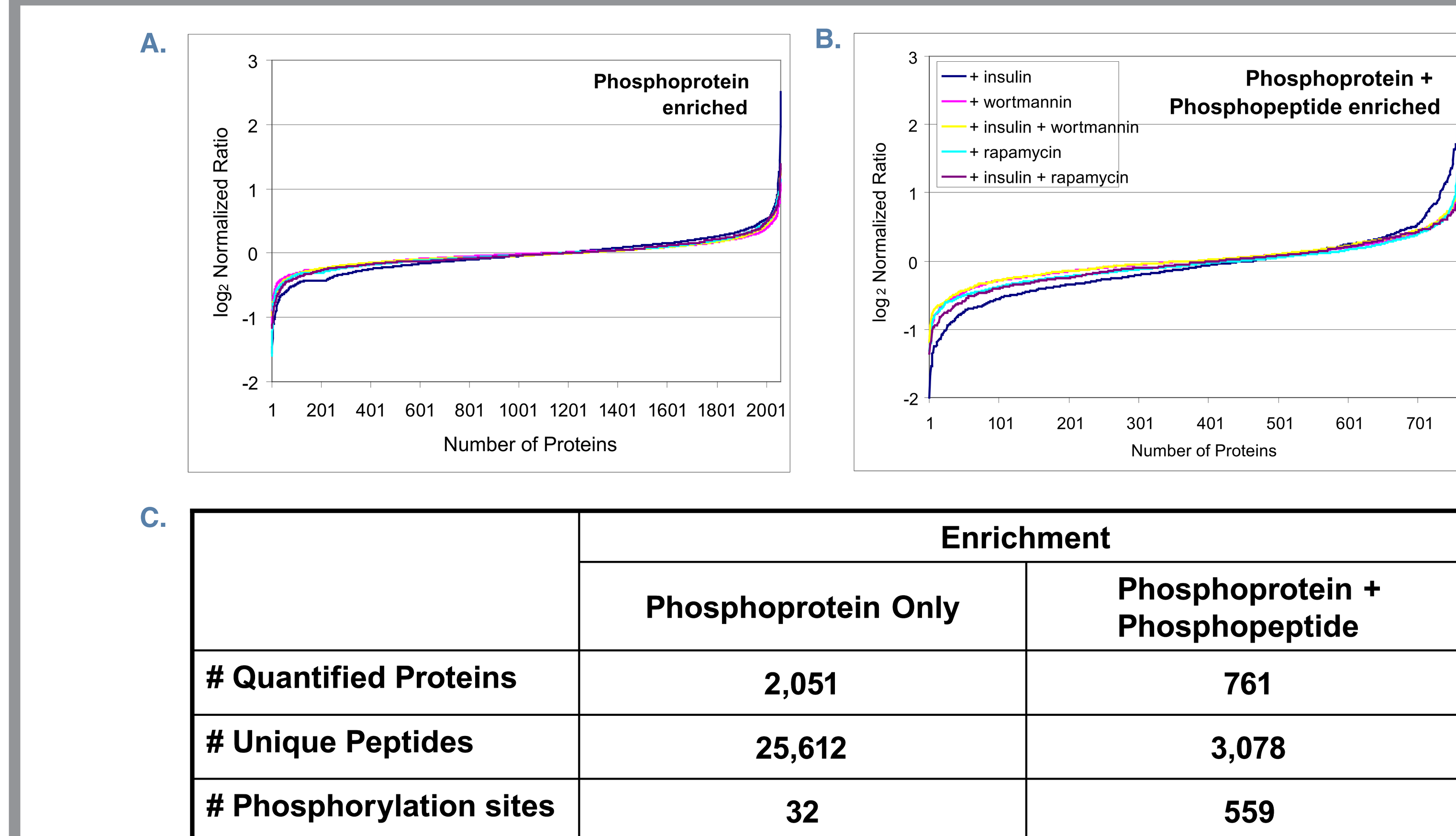
Figure 4: Proteins isolated from HEK-293T cells treated with insulin, wortmannin, or rapamycin were enriched with Thermo Scientific Phosphoprotein Enrichment Columns, reduced, alkylated, acetone precipitated, enzymatically digested, and labeled with 6-plex Tandem Mass Tag reagents. Labeled peptides were combined and fractionated by strong cation exchange into 12 fractions. Ninety-five percent of each fraction was processed with Thermo Scientific TiO_2 magnetic beads on a Kingfisher96 robot to enrich phosphopeptides, and the unenriched and phosphopeptide-enriched peptides were analyzed by LC-MS/MS on a Thermo Scientific LTQ Orbitrap XL. Raw files were searched and reporter ions were quantified with Thermo Scientific BioWorks 3.3.1 software.

Results

We have studied the phosphoproteomic response of insulin treated 293T human embryonic kidney cells to inhibitors of cell proliferation (wortmannin) and translation (rapamycin, Figure 1). Since the regulation of protein phosphorylation is so important to these processes, we utilized a phosphoprotein enrichment resin to enrich phosphoproteins from treated cells. This resin selectively and effectively enriched relevant phosphoproteins from complex lysates, as determined by Western blotting (Figure 2, 3). While this antibody-based strategy is effective for monitoring known proteins of interest, it is limited by throughput, by our knowledge and understanding of complex regulatory networks, and by antibody availability (Figure 3).

We addressed these problems with parallel protein identification and multiplexed quantitation by mass spectrometry (Figure 4). Furthermore, we integrated peptide enrichment into this workflow, as described recently,[2] to improve the detection and quantitation of phosphopeptides. The combined phosphoprotein and phosphopeptide enrichment strategy decreased the number of quantified proteins 3-fold, but enhanced phosphopeptide detection nearly 130-fold (Figure 5). This allowed us to monitor and confirm the regulation of multiple important sites of phosphorylation, including Erk1/2 by both Western blotting and MS (Figure 3A and 6B).

Distribution of Protein Regulation



	Enrichment	
	Phosphoprotein Only	Phosphoprotein + Phosphopeptide
# Quantified Proteins	2,051	761
# Unique Peptides	25,612	3,078
# Phosphorylation sites	32	559

Figure 5: Protein expression changes were calculated relative to vehicle treatment using TMT reporter ion intensity ratios. Log2 normalized ratios were analyzed to evaluate the distribution of protein expression changes after phosphoprotein enrichment alone (A) or after phosphoprotein and phosphopeptide enrichment (B).

Representative Phosphopeptide Identification and Quantification

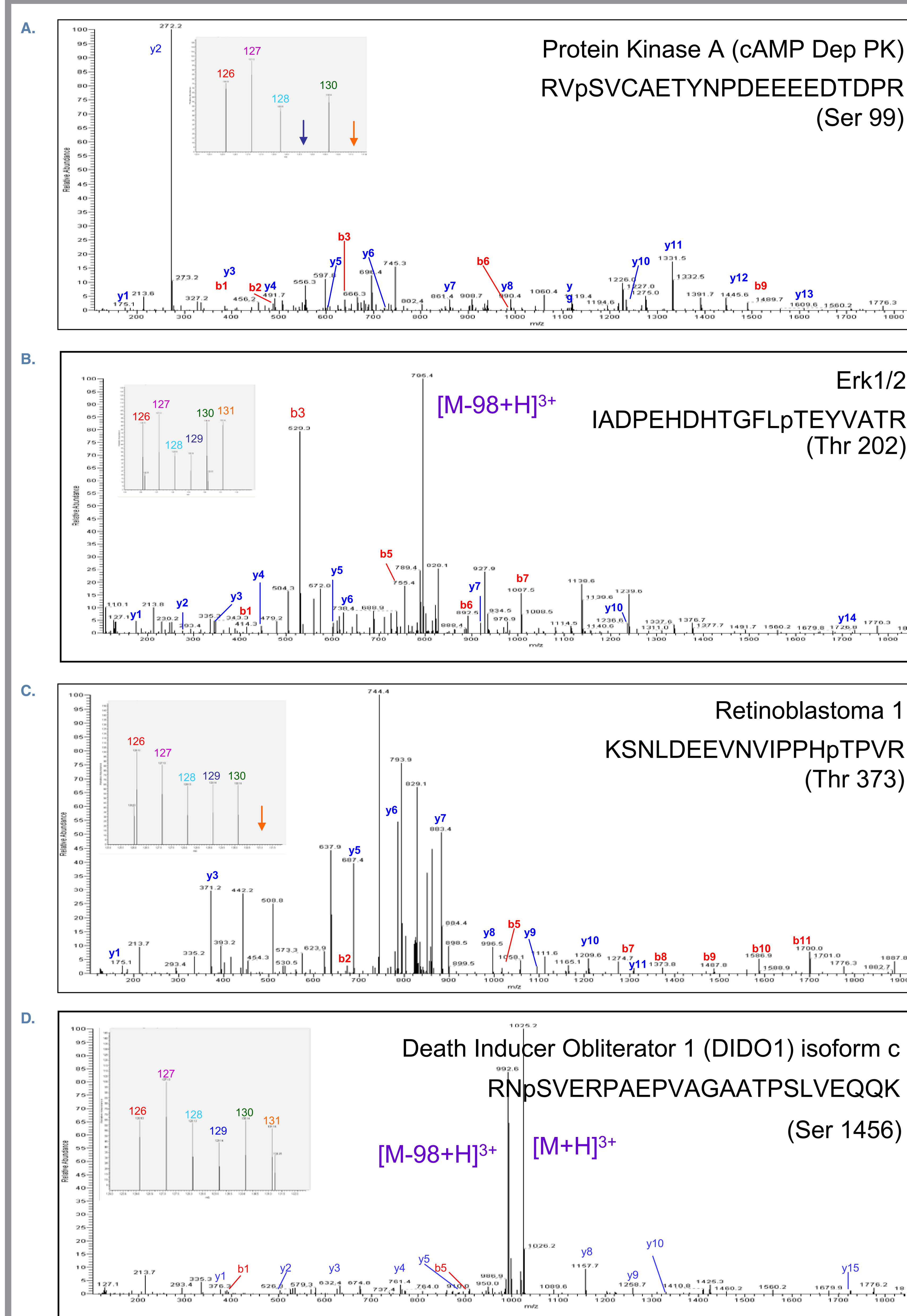


Figure 6: Representative phosphopeptide identification and quantification from phosphoprotein and phosphopeptide enriched samples.

Conclusions

- Phosphoprotein enrichment using PMAC technology is a viable, scalable alternative to traditional antibody immunoprecipitation for selective phosphoprotein enrichment with negligible non-specific binding.
- Multiplexed identification and quantification by mass spectrometry provides parallel quantification of thousands of peptides and proteins, and results that are consistent with Western blotting.
- Integration of strong cation exchange HPLC and TiO_2 magnetic bead enrichment reduces sample complexity and improves sensitivity, greatly assisting quantitation efforts.

References

1. Hennessy, B.T., et al (2005). Exploiting the PI3K/AKT Pathway for Cancer Drug Discovery. *Nature Reviews in Drug Discovery* 4, 988-1004.
2. Cantin, G.T., Yi, W., Lu, B., Park, S.K., Xu, T., Lee, J-D. and Yates, J.R. III (2008). Combining protein-based IMAC, peptide-based IMAC, and MudPIT for efficient phosphoproteomic analysis. *J. Proteome Res.* 7:1346-1351.

All trademarks are the property of Thermo Fisher Scientific Inc. and its subsidiaries.