# Quantitative profiling of signaling pathways using immunoaffinity purification and LC-MS/MS

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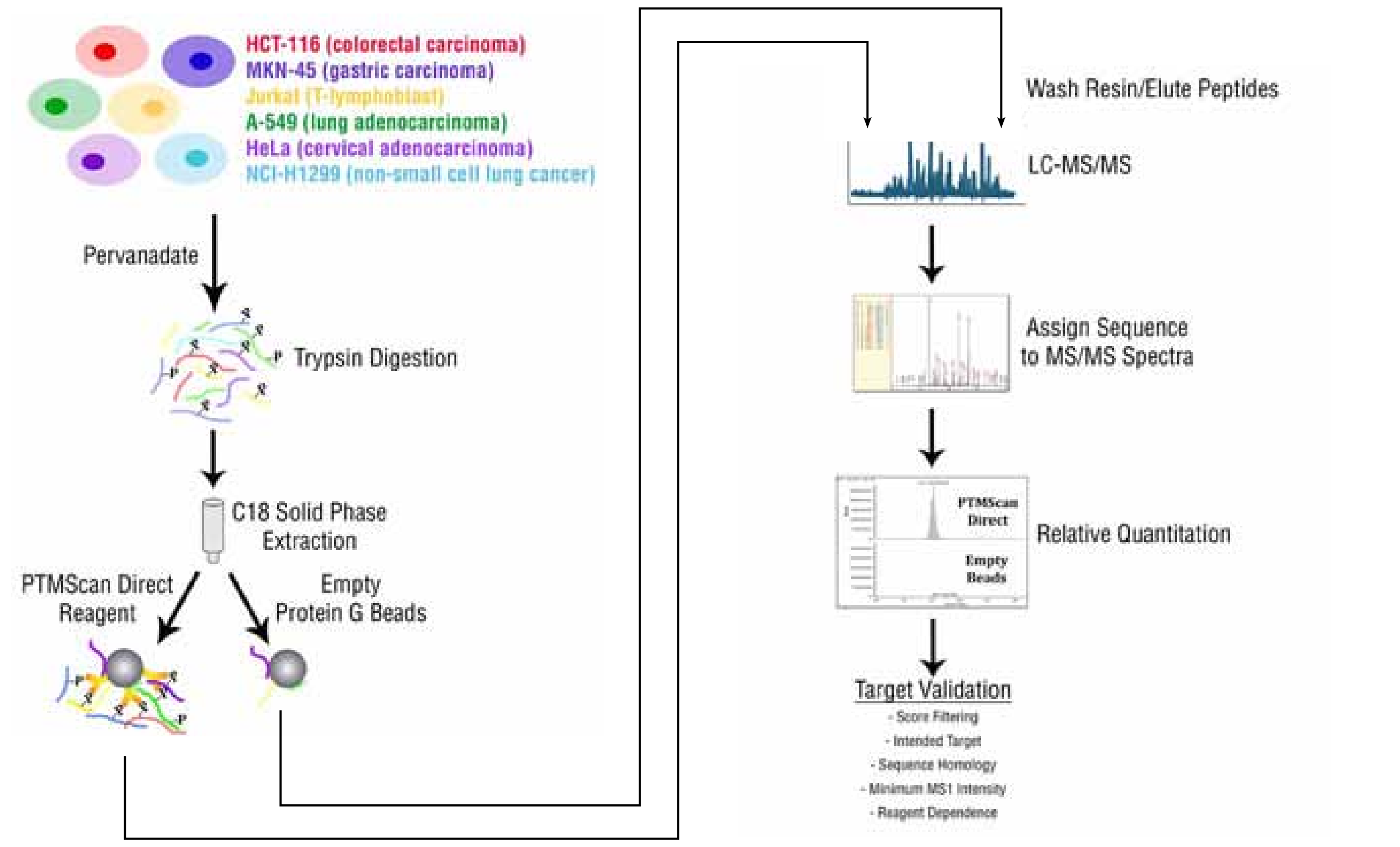
## Introduction

Proteomic analysis of post-translationally modified peptides has traditionally employed methods that broadly sample the proteome but are unfocused with respect to the sites identified. We have developed a novel immunoaffinity method for identification and quantitation of post-translationally modified peptides from proteins that reside in the same signaling pathway or pathways, allowing a global view of pathway activation from a single LC-MS/MS run.
Six different antibody reagents have been prepared that focus on diverse signaling areas: Ser/Thr Kinase activity, Tyr Kinase activity, PI3K/Akt signaling, Cell Cycle/DNA Damage signaling, Apoptotic/Autophagolytic pathways, and a Multipathway reagent for detection of critical signaling proteins across many different pathways. Reagents were validated using human and mouse samples with a variety of treatments (inhibitors, growth factors, etc.). This technology is broadly applicable to any experimental system in which quantitative profiling of specific critical signaling molecules is desirable.

## PTMScan® Direct Method

**Figure 1: PTMScan® Direct Reagent validation strategy.** PTMScan® Direct is a published method (Stokes et al. 2012) adapted from the original PhosphoScan® method (Rush et al. 2005) developed at Cell Signaling Technology. PTMScan Direct Reagents are validated using mixtures of pervanadate-treated human cancer cell lines digested with trypsin. Peptides are desalted over C18 columns and immunoprecipitated with either the PTMScan Direct reagent or empty Protein G beads. Immunoprecipitated peptide mixtures are analyzed by LC-MS/MS and relative quantitation is performed. Validated peptides meet several strict criteria: they are targeted by the reagent or are homologous to a target, they must pass score filtering (Lundgren et al. 2009) and signal intensity thresholds, and they must be present in higher abundance in the PTMScan Direct reagent immunoprecipitation than the empty Protein G beads immunoprecipitation (Stokes et al. 2012).



## PTMScan® Direct

1. Multipathway (*updated*)
2. Ser/Thr Kinases
3. Tyr Kinases
4. Akt/PI3K
5. DNA Damage/Cell Cycle
6. Apoptosis/Autophagy

Interaction maps were generated from IPA® pathway analysis or the STRING database (string.embl.de) using high confidence scores (>0.700) from experimental, database, and text mining lines of evidence. Interactions were also defined from the substrate search page of PhosphoSitePlus® (www.phosphosite.org). Node colors and shapes denote different protein classes. Edge color denotes interaction type.

## Multipathway

Monitoring more than **1,000** phosphorylation sites on over **400** critical signaling proteins.

IPA® - Molecular Mechanisms of Cancer

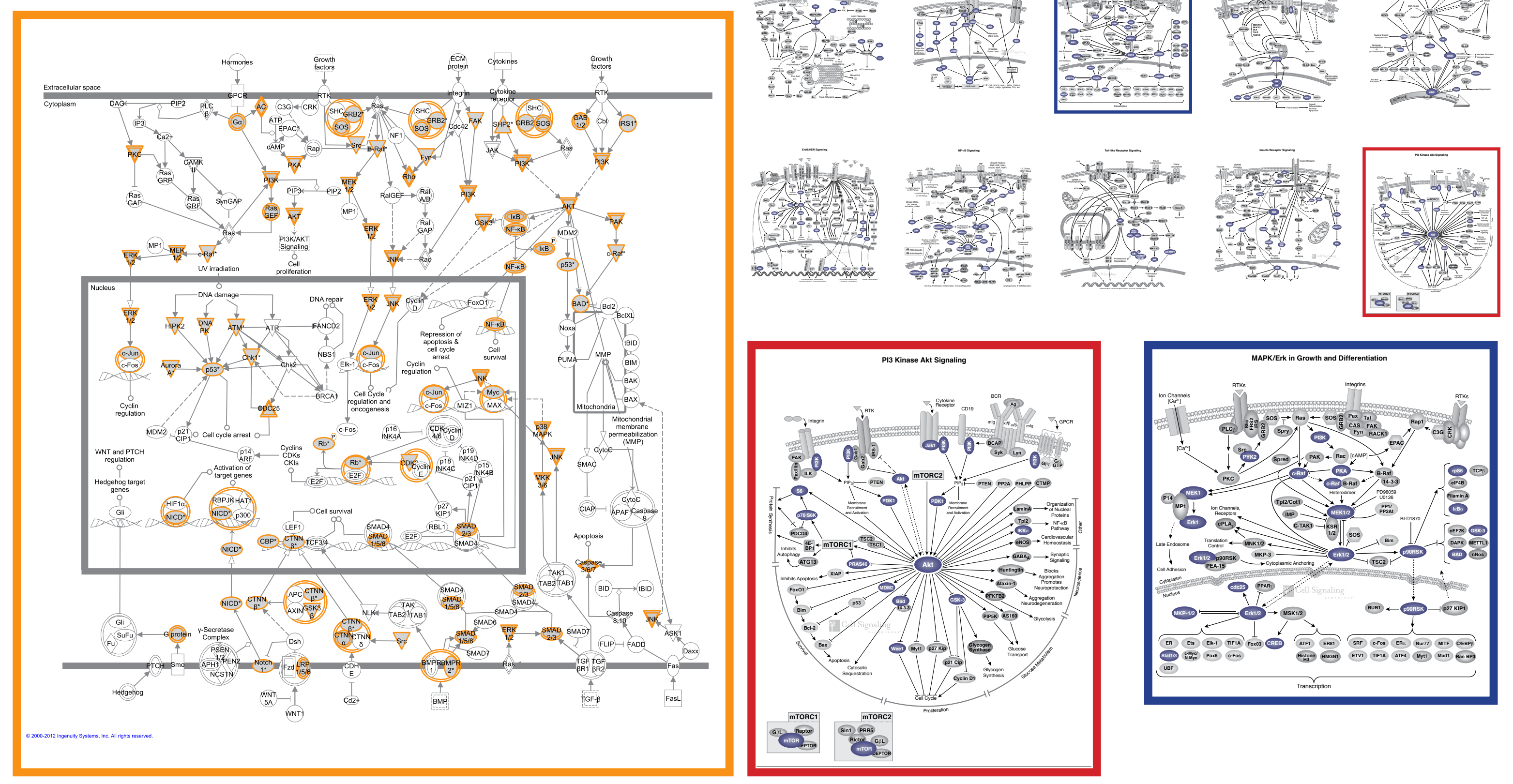
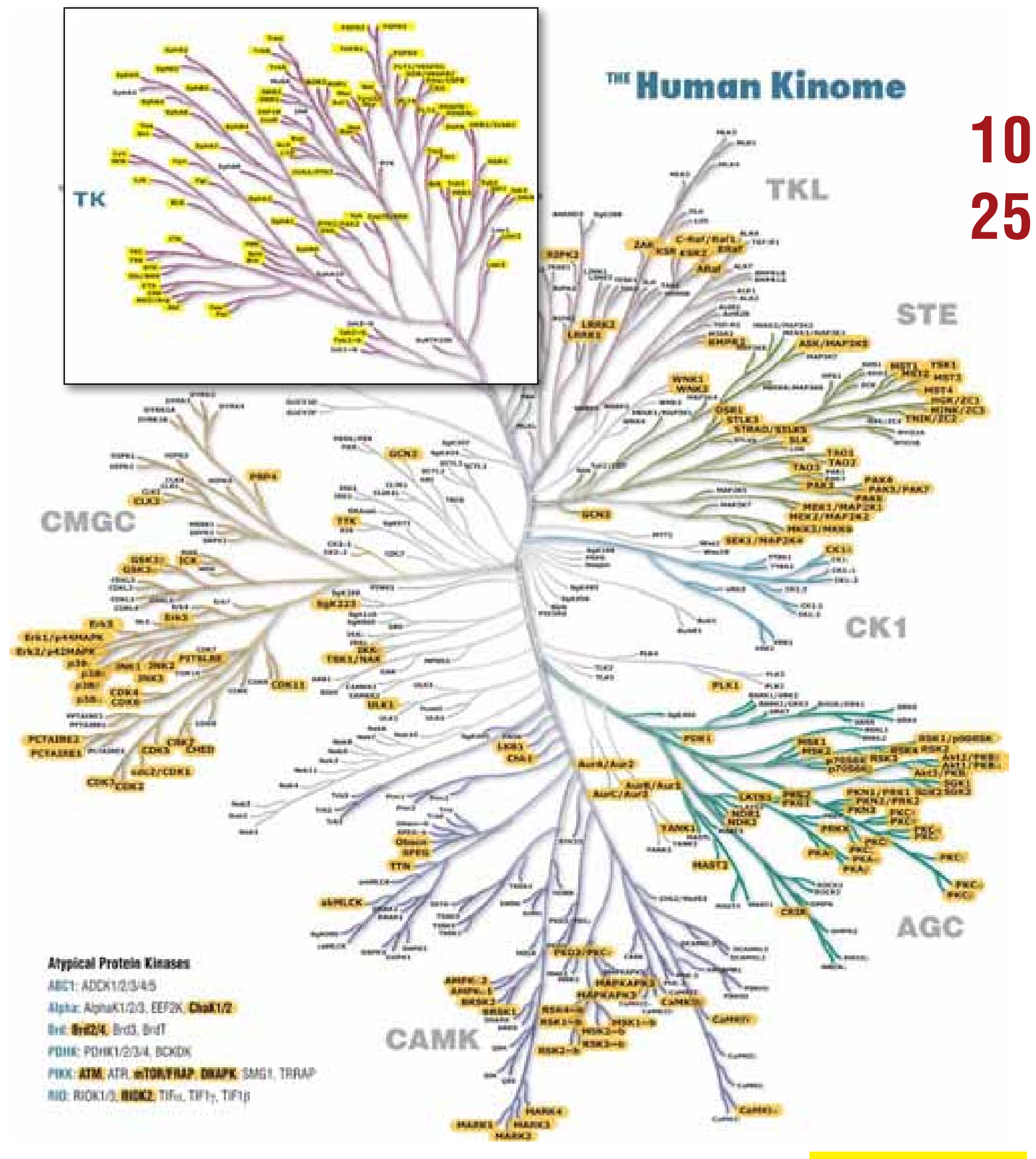


**Figure 2: Multipathway coverage of selected signaling pathways.**

## Ser/Thr Kinases and Tyr Kinases

Profiling sites of activation and inhibition on protein kinases.



**1056** unique sites on
**250** kinases

**Figure 3: Ser/Thr Kinase and Tyr Kinase targets mapped onto the human kinome tree.** Yellow highlighting indicates kinases for which peptides are identified using the reagents. Tyr Kinase coverage is shown in the inset ("TK").

## Akt/PI3K Pathway

In-depth analysis of PI3K/Akt and associated signaling pathways.

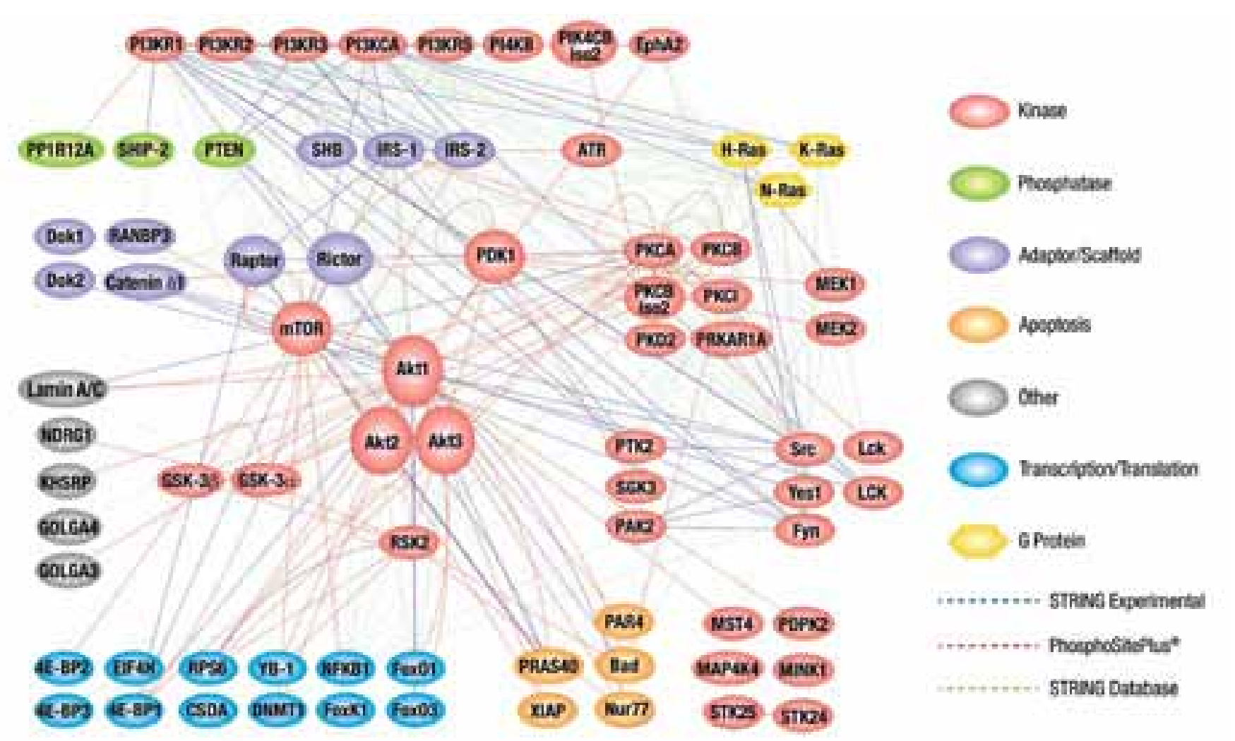


**Figure 4: Akt/PI3K Pathway - interaction map of validated targets.**

**296** unique sites on
**105** proteins

MOUSE TISSUE STUDY

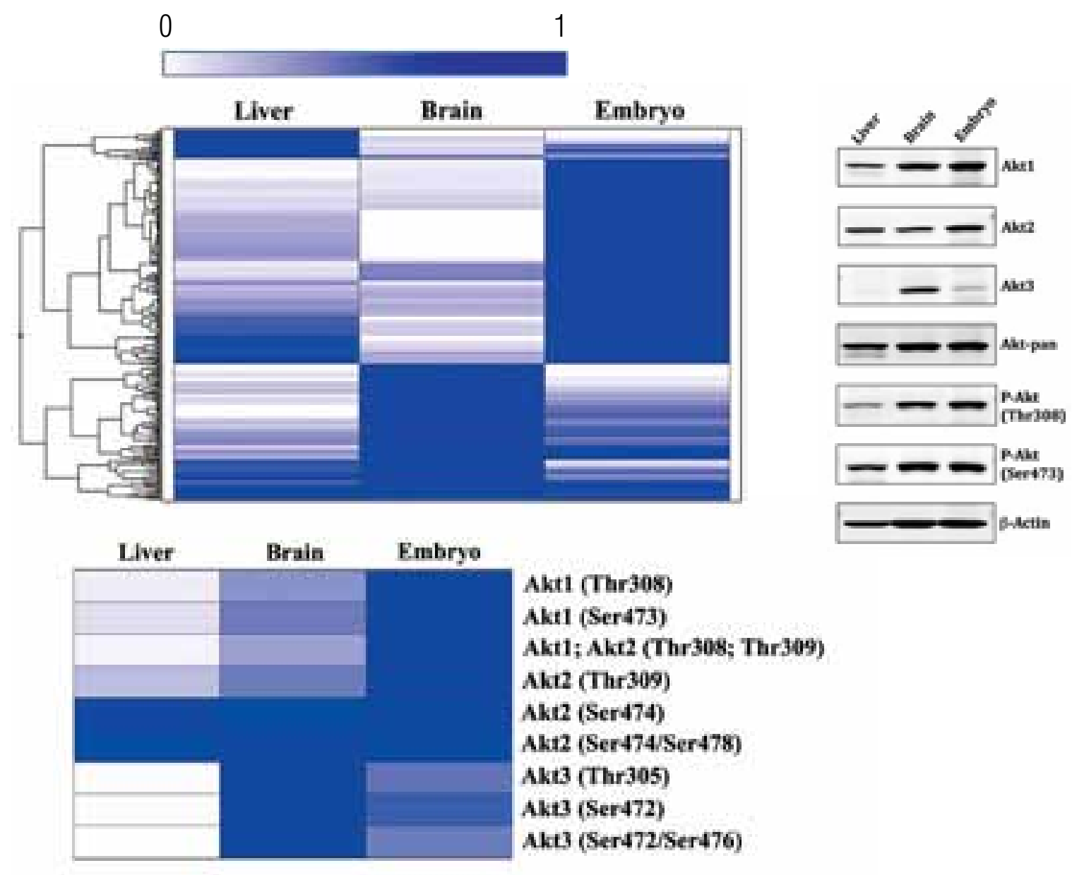


**Figure 5: Akt/PI3K Pathway mouse tissue profiling.** Hierarchical clustering of relative intensities for mouse liver, brain, and embryo. Each row represents a different validated Akt/PI3K pathway peptide. The maximum intensity across the three tissues was set to 1 and the other two intensities normalized to the max. Blue indicates higher intensity. Selected kinase peptides are shown in detail with accompanying western blots.

## DNA Damage/Cell Cycle

A comprehensive view of the DNA damage response.

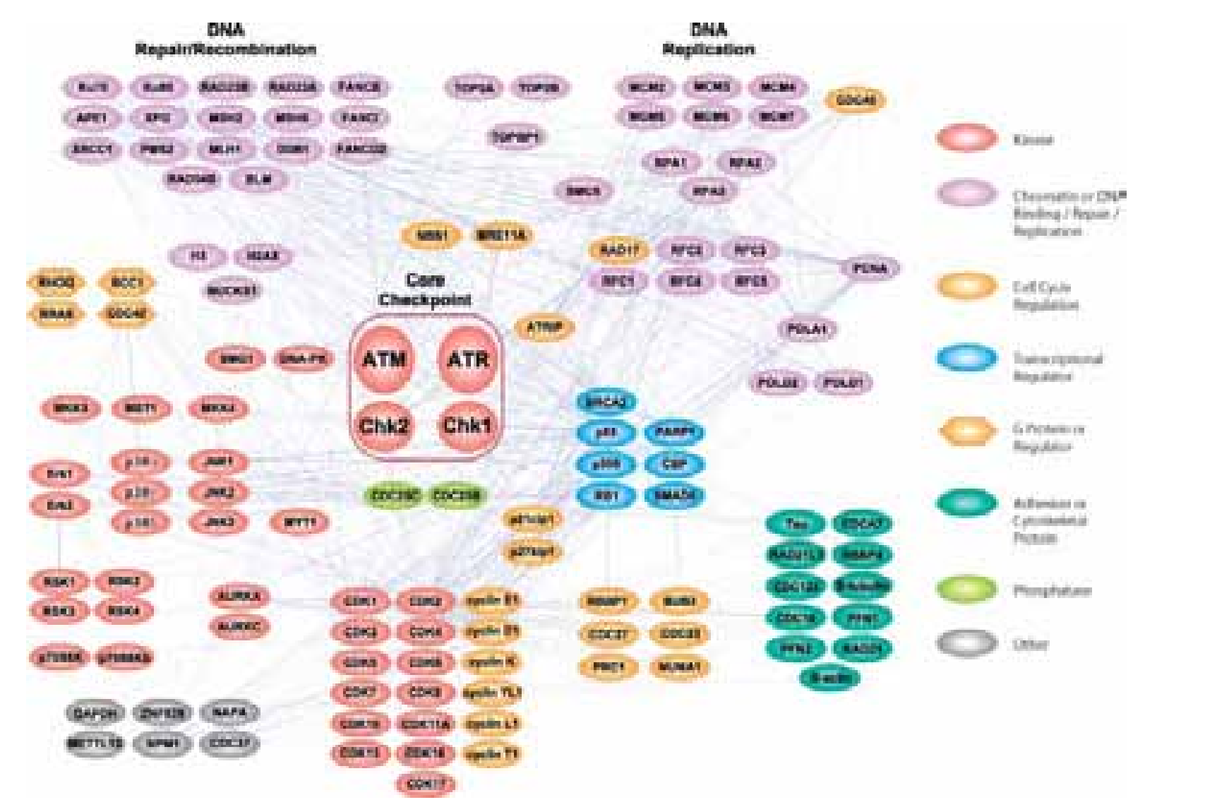
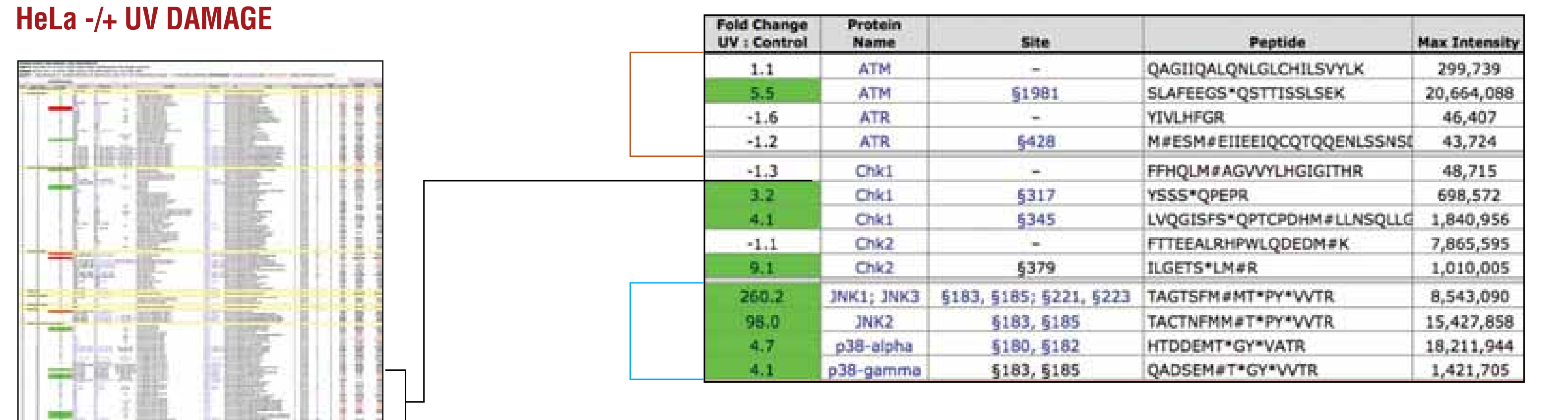


**Figure 6: DNA Damage/Cell Cycle - interaction map of validated targets.**

**263** unique sites on
**137** proteins

HeLa -/+ UV DAMAGE



| Fold Change UV : Control | Protein Name | Site | Peptide | Max Intensity |
|---|---|---|---|---|
| 1.1 | ATM | – | QAGIIQALQNLGLCHILSVYLK | 299,739 |
| 5.5 | ATM | §1981 | SLAFEEGS*QSTTISSLSEK | 20,664,088 |
| -1.6 | ATR | – | YIVLHFGR | 46,407 |
| -1.2 | ATR | §428 | M#ESM#EIIEEIQCQTQQENLSSNS | 43,724 |
| -1.3 | Chk1 | – | FFHQLM#AGVVYLHGIGITHR | 48,715 |
| 3.2 | Chk1 | §317 | YSSS*QPEPR | 698,572 |
| 4.1 | Chk1 | §345 | LVQGISFS*QPTCPDHM#LLNSQLLG | 1,840,956 |
| -1.1 | Chk2 | – | FTTEEALRHPWLQDEDM#K | 7,865,595 |
| 9.1 | Chk2 | §379 | ILGETS*LM#R | 1,010,005 |
| 260.2 | JNK1; JNK3 | §183, §185; §221, §223 | TAGTSFM#MT*PY*VVTR | 8,543,090 |
| 98.0 | JNK2 | §183, §185 | TACTNFMM#T*PY*VVTR | 15,427,858 |
| 4.7 | p38-alpha | §180, §182 | HTDDEMT*GY*VATR | 18,211,944 |
| 4.1 | p38-gamma | §183, §185 | QADSEM#T*GY*VVTR | 1,421,705 |

**Figure 7: DNA Damage/Cell Cycle profiling of response to UV damage.** Hela cells were untreated or treated with 500 mJ/cm² UV light and harvested at 2 hr post treatment. Fold changes were calculated from chromatographic peak heights/areas. Green cells indicate peptides more abundant with UV damage; Red cells indicate peptides less abundant. Selected DNA damage response proteins are shown in detail with accompanying western blots.

## Apoptosis/Autophagy

Monitoring activity of apoptotic proteins and autophagolytic pathways.

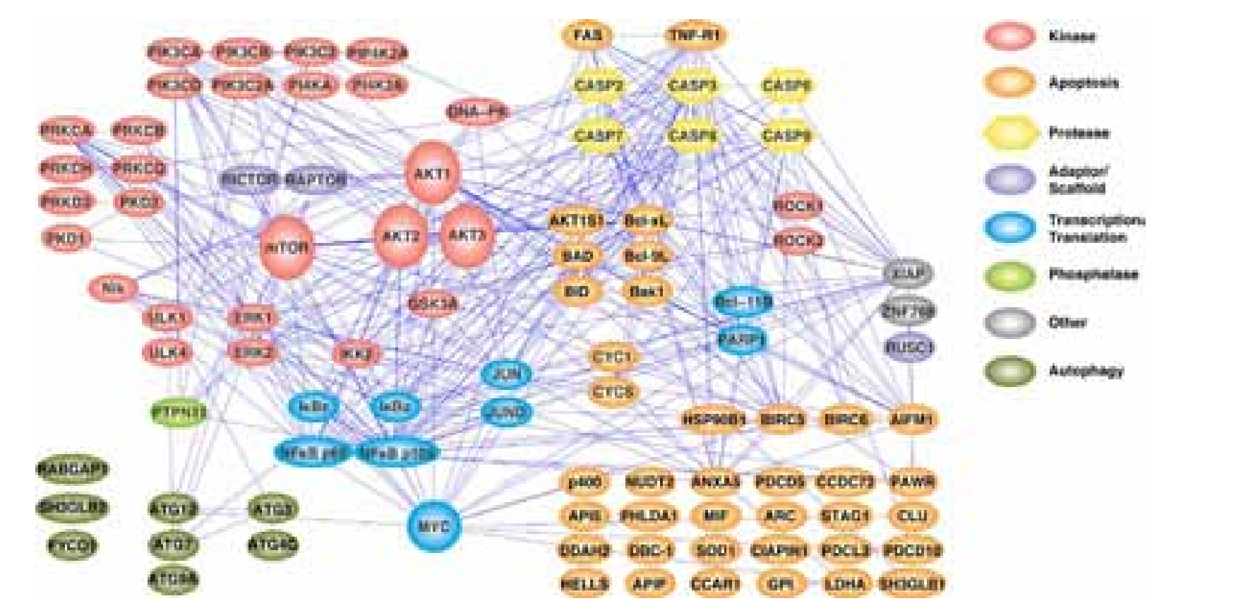


**Figure 8: Apoptosis/Autophagy - interaction map of validated targets.**

**175** unique sites on
**100** proteins

HeLa -/+ UV DAMAGE

| Fold Change UV : Control | Protein Name | Site | Peptide | Max Intensity |
|---|---|---|---|---|
| -1.3 | Casp3 | | GTELDCGIETDSGVDDDMACHK | 27,685,445 |
| 3.2 | Casp3 | 175 | GTELDCGIETD* | 151,336,865 |
| – | Casp3 | 176 | GTELDCGIETDS*GVDDDMACHK | 0 |
| 2.5 | Casp6 | 179 | GNQHDVPVIPLDVVD* | 12,209,365 |
| -1.3 | Casp7 | | GTELDDGIQADSGPINDTDANPR | 58,709,894 |
| 2.8 | Casp7 | 198 | GTELDDGIQAD* | 135,291,676 |
| 29.0 | Casp7 | 199 | GTELDDGIQADS*GPINDTDANPR | 2,070,506 |
| -1.0 | Casp8 | | GYCLIINNHNFAK | 48,868 |
| 1.8 | Casp8 | 374 | GIPVETD* | 12,668,813 |
| -1.8 | Casp9 | | DHGFEVASTSPEDESPGSNPEPDATPFQEGLR | 2,312,359 |
| 4.1 | Casp9 | §302 | DHGFEVASTS*PEDESPGSNPEPDATPFQEGLR | 4,466,555 |
| 5.2 | Casp9 | §302, 315 | DHGFEVASTS*PEDESPGSNPEPD* | 1,286,272 |
| 3.5 | Casp9 | §307 | DHGFEVASTSPEDES*PGSNPEPDATPFQEGLR | 101,500 |
| 2.2 | Casp9 | 315 | DHGFEVASTSPEDESPGSNPEPD* | 8,621,740 |

**Figure 9: Apoptosis/Autophagy profiling of response to UV damage.** Hela cells were untreated or treated with 500 mJ/cm² UV light and harvested at 2 hr post treatment. Fold changes were calculated from chromatographic peak heights/areas. Green cells indicate peptides more abundant with UV damage; Red cells indicate peptides less abundant.

## Conclusion

**PTMScan® Direct is a recently published method that allows identification and quantification of hundreds of peptides from selected protein types or signaling pathways. This approach allows focus on proteins of interest instead of the random sampling of peptides that occurs in traditional data-dependent proteomic analysis. PTMScan® Direct is widely applicable in drug development and discovery, as well as in any application where monitoring of known signaling pathways is desired.**

## References

1. Stokes, M.P. et al. (2012) *Mol. Cell Proteomics* 11, 187–201.
2. Rush, J. et al. (2005) *Nat. Biotechnol.* 23, 94–101.
3. Lundgren, D.H. et al. (2009) *Curr. Protoc. Bioinformatics* 13, 13.3.1–13.3.21.
4. Stokes, M.P. et al. (2012) *Int. J. Mol. Sci.* 14, 286–307.

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