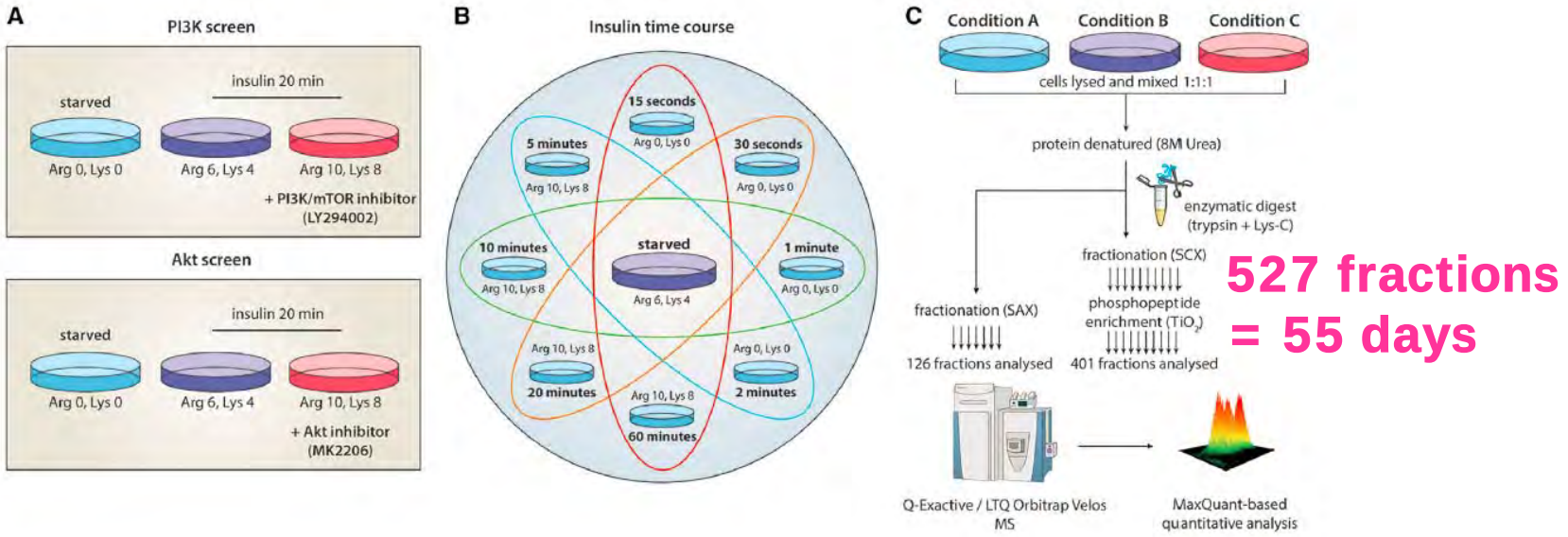
Discovery and targeted phosphoproteomics in under a week: Phosphorylation analysis using data-independent acquisition (DIA)

Benjamin L. Parker

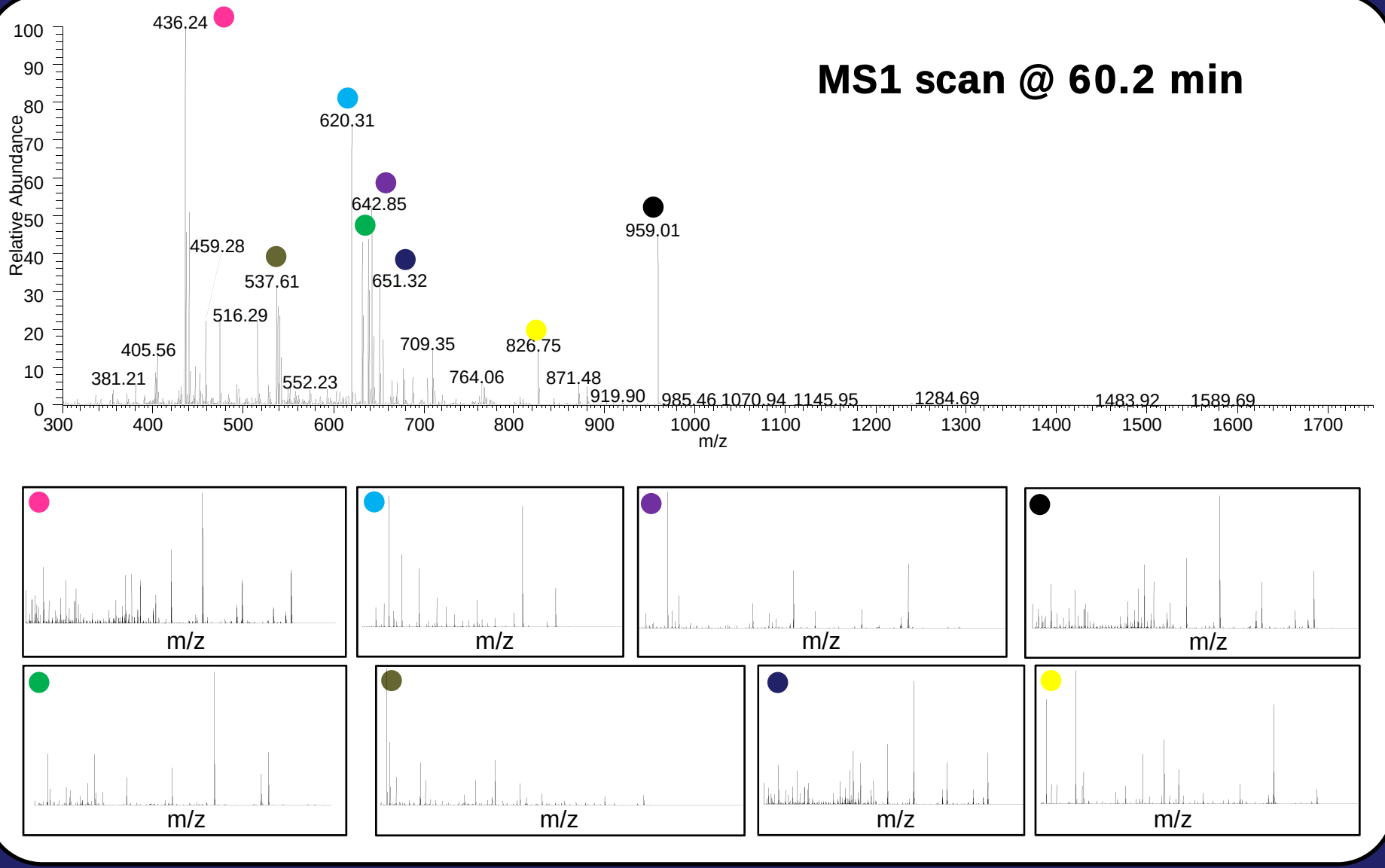
NHMRC Early Career Fellow
Diabetes & Obesity Program
Biological Mass Spectrometry Unit Manager
David James Laboratory
Garvan Institute of Medical Research, Sydney, Australia

Case study: Insulin stimulated phosphoproteome Humphrey *et. al. Cell Metab.* 2013

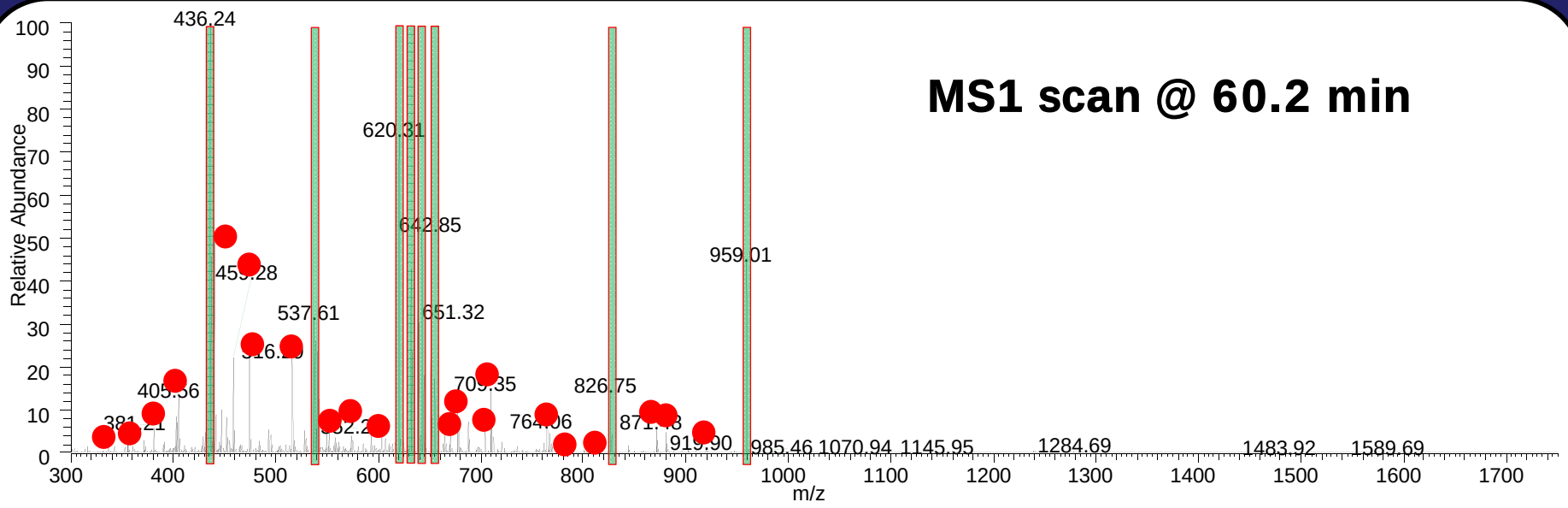


527 fractions = 55 days

Data-Dependent Acquisition (DDA)



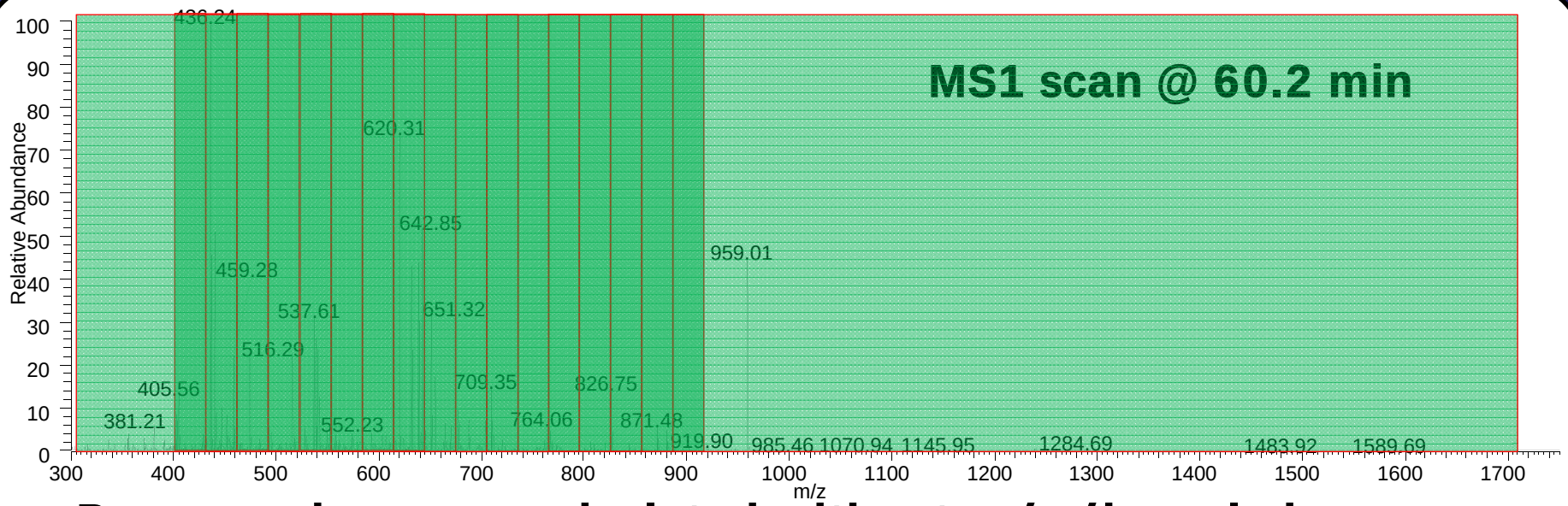
Data-Dependent Acquisition (DDA)



- Precursor ions trigger an isolation in a 1-3 *m/z* window and are subjected to MS/MS.
- *Simplified* MS/MS spectra for characterization of the isolated analyte(s).

- Many precursor ions are not subjected to MS/MS and cannot be characterized = missing values between replicates

Data-Independent Acquisition (DIA)



- Precursor ions are co-isolated without *priori* knowledge

- the entire mass range (all ion fragmentation: AIF)

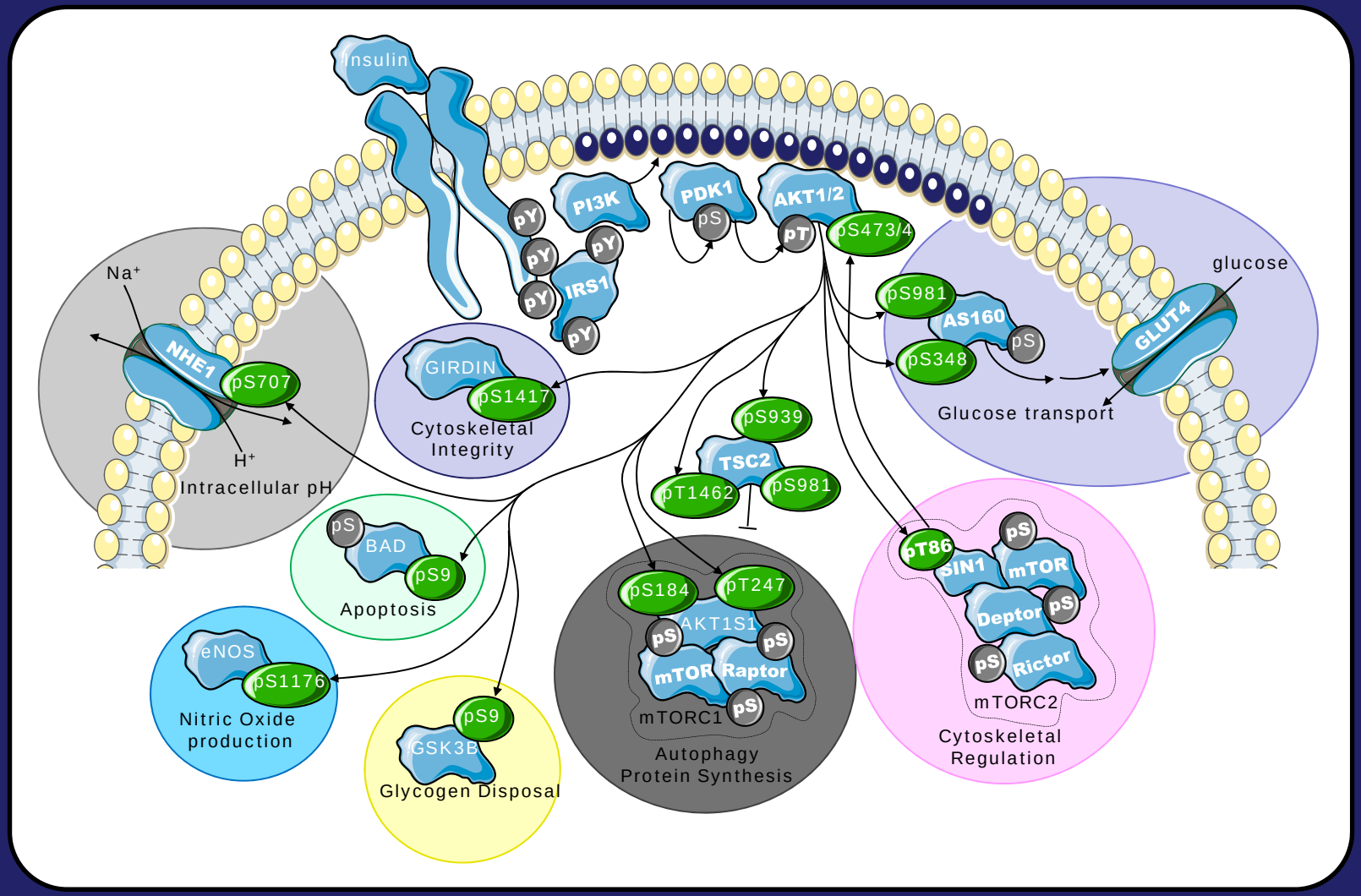
Masselon, *Anal. Chem* (2000); Purvine, *Proteomics* (2003); Plumb, *Rapid Commun. Mass Spectrum.* (2006); Geiger, *Mol. Cell. Proteomics* (2010)

- 1-30 *m/z* (SWATH)

Venable, *Nat. Methods* (2004); Panchaud, *Anal. Chem.* (2009); Gillet, *Mol. Cell. Proteomics* (2012); Egertson, *Nat. Methods* (2013)

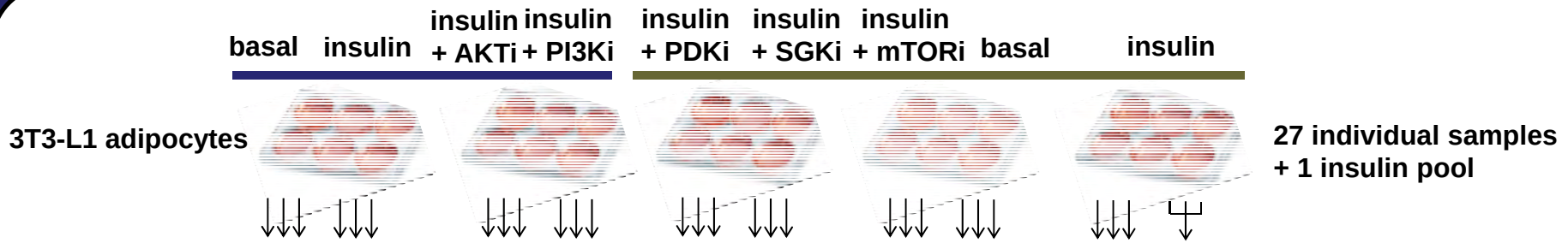
- Every precursor ion is subjected to MS/MS across the desired mass range = improved repeatability between replicates

1- Targeted quantification of AKT substrate phosphorylation using SID-DIA



15 synthetic heavy labelled phosphopeptides

1- Targeted quantification of AKT substrates using SID-DIA



260 µg protein digest Lys-C/trypsin & desalt

Peptides resuspended in TiO_2 Loading Buffer containing a normalized amount of mixed heavy labelled phosphopeptides. TiO_2 enrichment

Single-shot nanoUHPLC over 120 min DIA acquisition on a Q-Exactive: 450-850 m/z; 25 m/z isolation; loop count 8 with intermittent MS1 scans. Analysis in Skyline.

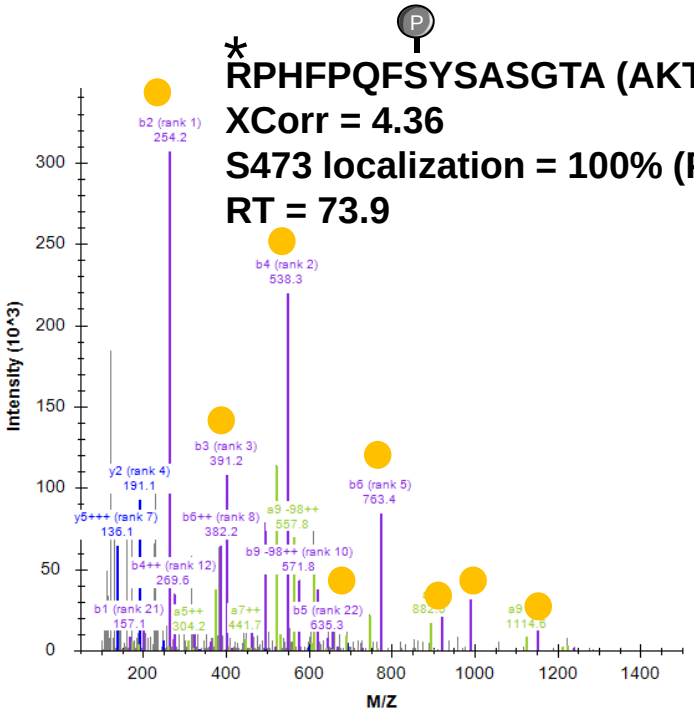
Skyline

Fractionated Accucore Amide-HILIC over 40 min; 8 fractions. Each fractionation DDA acquisition. Searched with Sequest HT/Percolator in Proteome Discoverer v1.4 and spectral library created in Skyline.

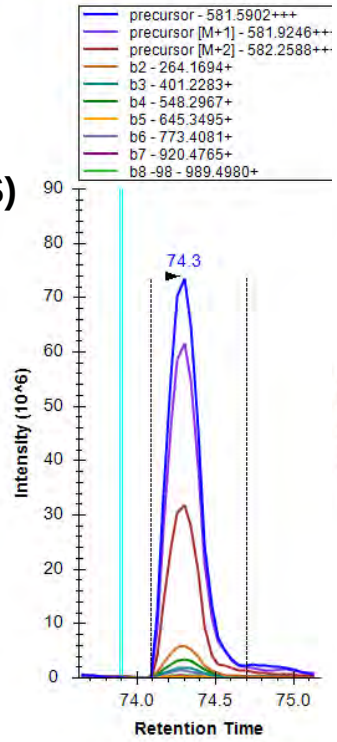
1- Targeted quantification of AKT substrate phosphorylation using SID-DIA

Targeted Data Extraction Gillet, *Mol. Cell. Proteomics* (2012)

DDA Spectral Library (Heavy)

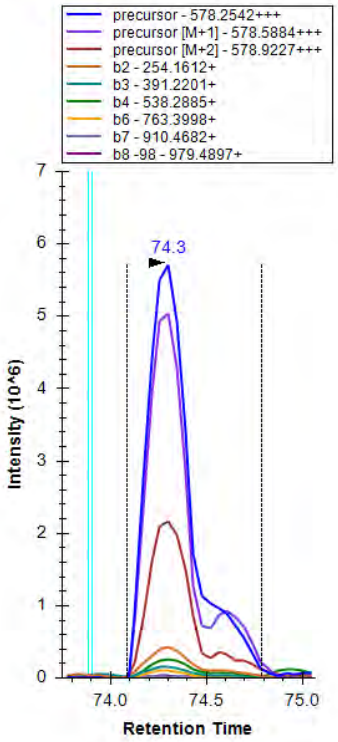


DIA (Heavy)



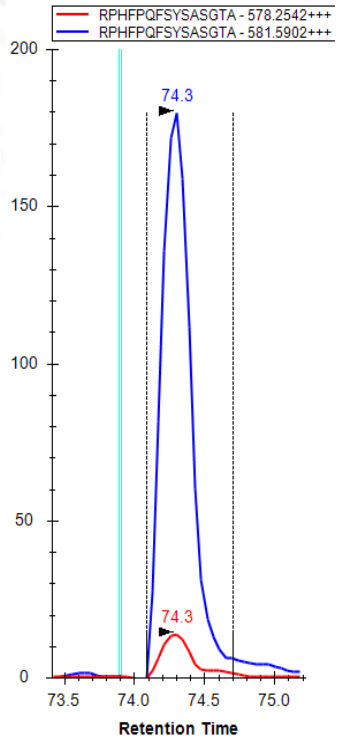
idotp = 0.99
dotp = 0.92

DIA (Light)



idotp = 0.98
dotp = 0.93

DIA (L/H)



Targeted Data Extraction
Identification Criteria

1- Retention time matching of spectral library to experimentally observed within 5% of the gradient length (i.e a 5 min window).

2- Co-elution of endogenous (L) and heavy (H) phosphopeptides.

3- Dot-product matching of theoretical to experimentally observed precursor-ion isotope distribution > 0.95.

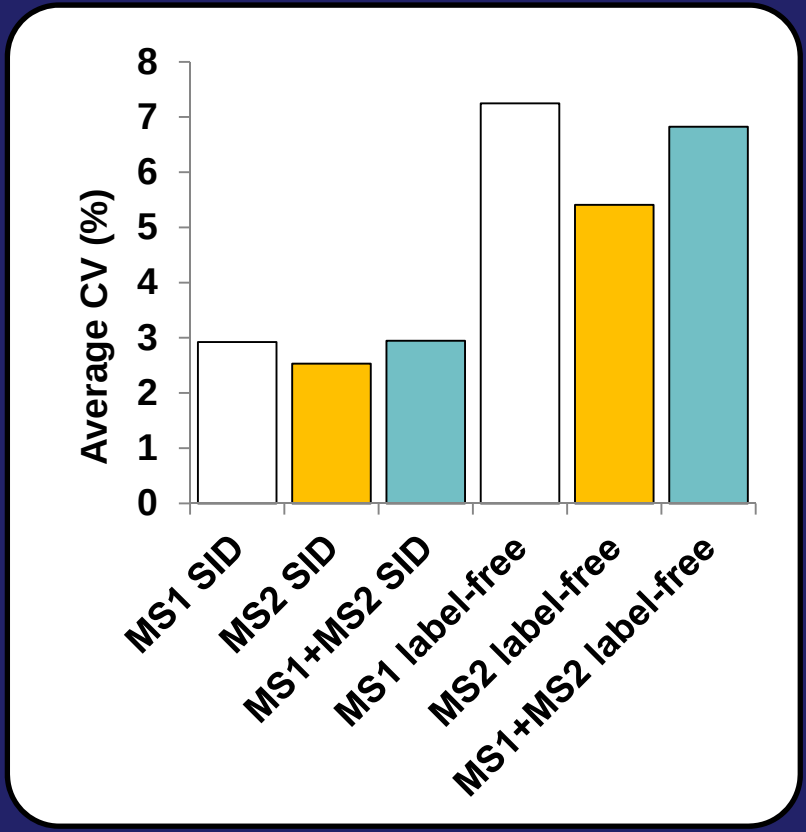
4- Dot-product matching of spectral library to experimentally observed product-ion distribution > 0.90.

5- Matching peak shape for precursor and product ions from both L and H phosphopeptides.

13 out of 15 phosphopeptides were successfully identified
11 out of 13 phosphopeptides had L:H within 10-fold

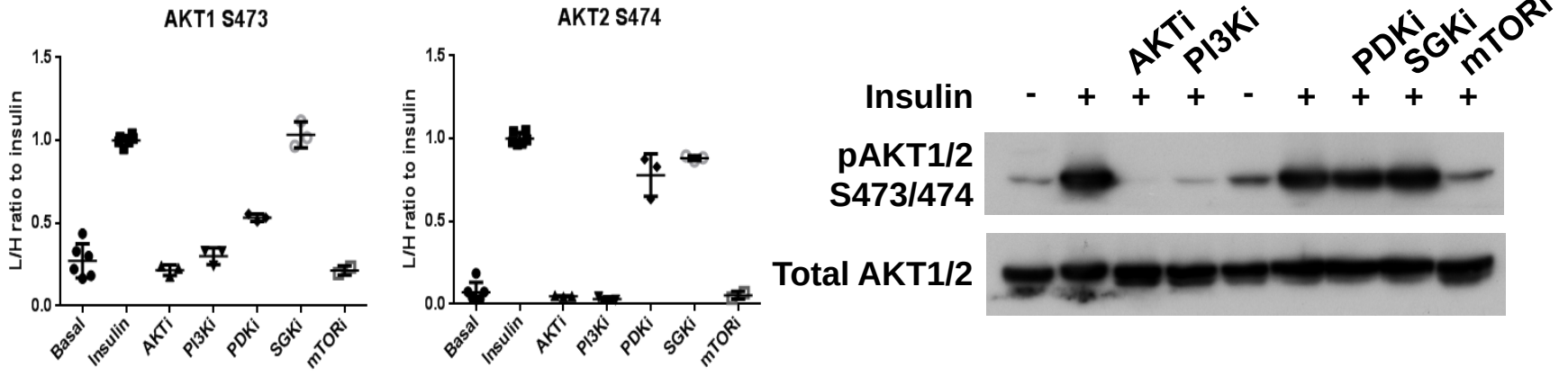
Quantification strategy and reproducibility

Technical coefficient of variation (CV) of the entire protocol (insulin stimulated group) using precursor-ion XICs (MS1), product-ion XICs (MS2) and summed precursor- and product-ion XICs (MS1+MS2) with and without SID normalisation.

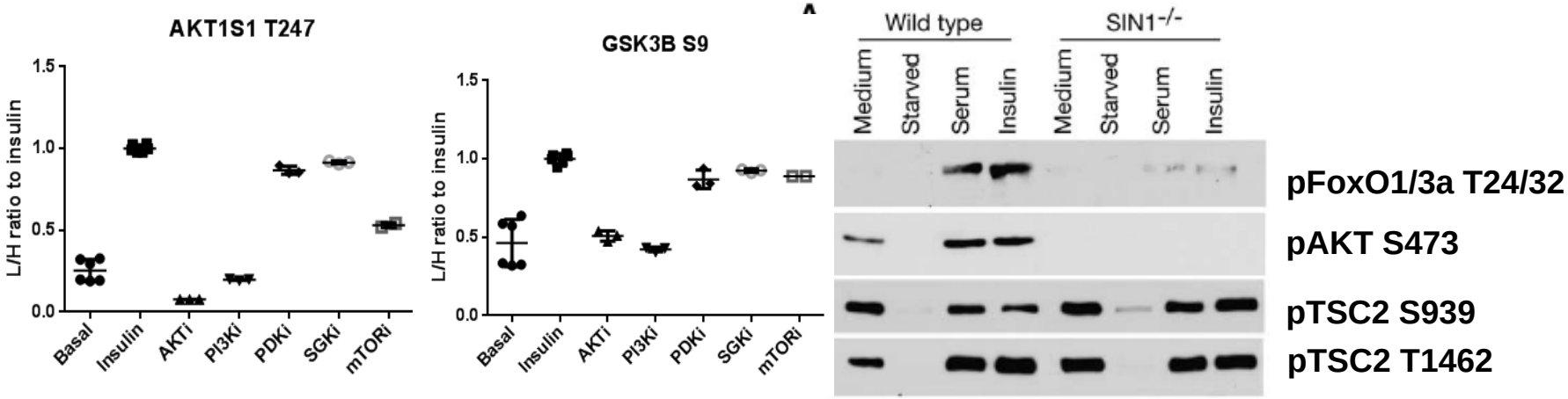


All 13 phosphopeptides were quantified with <20% CV regardless of the quantification strategy.

1- Targeted quantification of AKT substrate phosphorylation using SID-DIA



Potent inhibition (>80%) of AKT S473/4 was observed with AKTi, PI3Ki and mTOR while PDKi showed partial attenuation on AKT1 but not AKT2. SGKi had no effect on AKT phosphorylation.



Jacinto, E., *et. al., Cell* (2006)

2- Targeted quantification of predicted AKT and mTOR substrate phosphorylation using label-free DIA

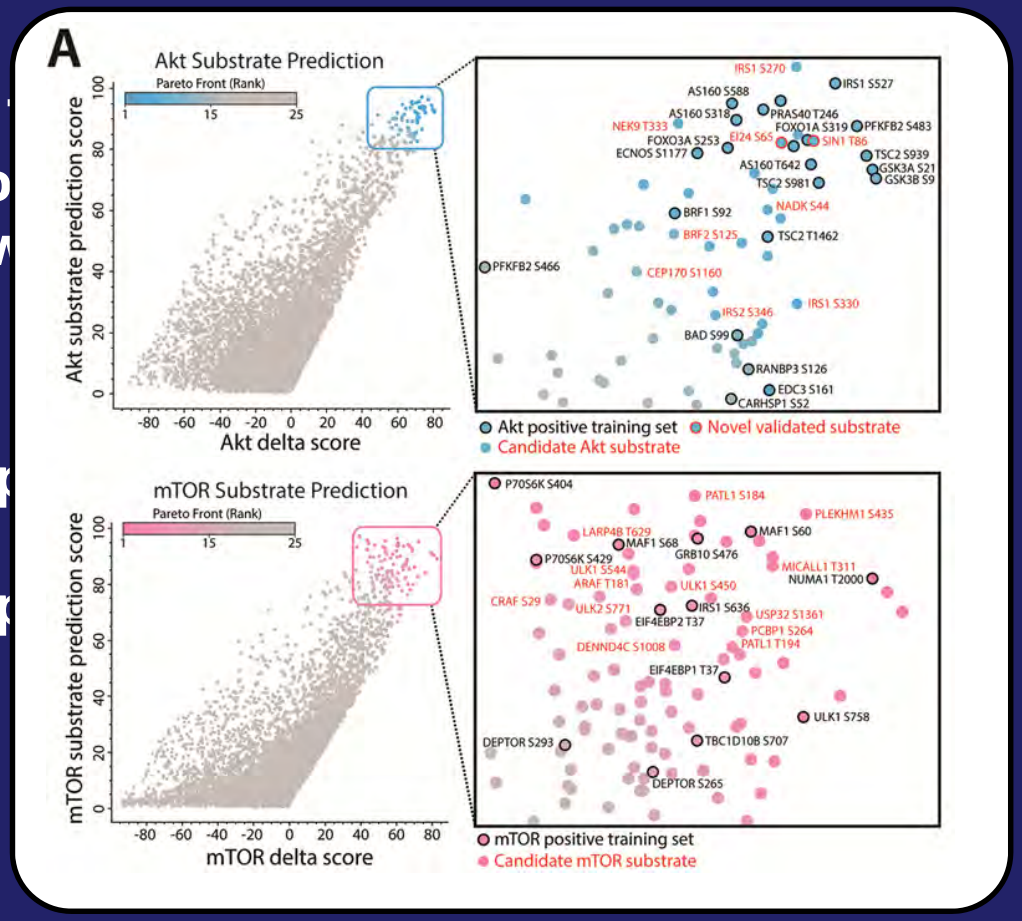
60 top predicted AKT and 60 top predicted mTOR substrates were selected from a recent large-scale SILAC experiment (Humphrey, *et. al., Cell Metab.* (2013). Machine learning:

1- phosphorylation motif
2- response to AKT and PI3K/mTOR inhibition
3- temporal profile
=Pareto Rankings

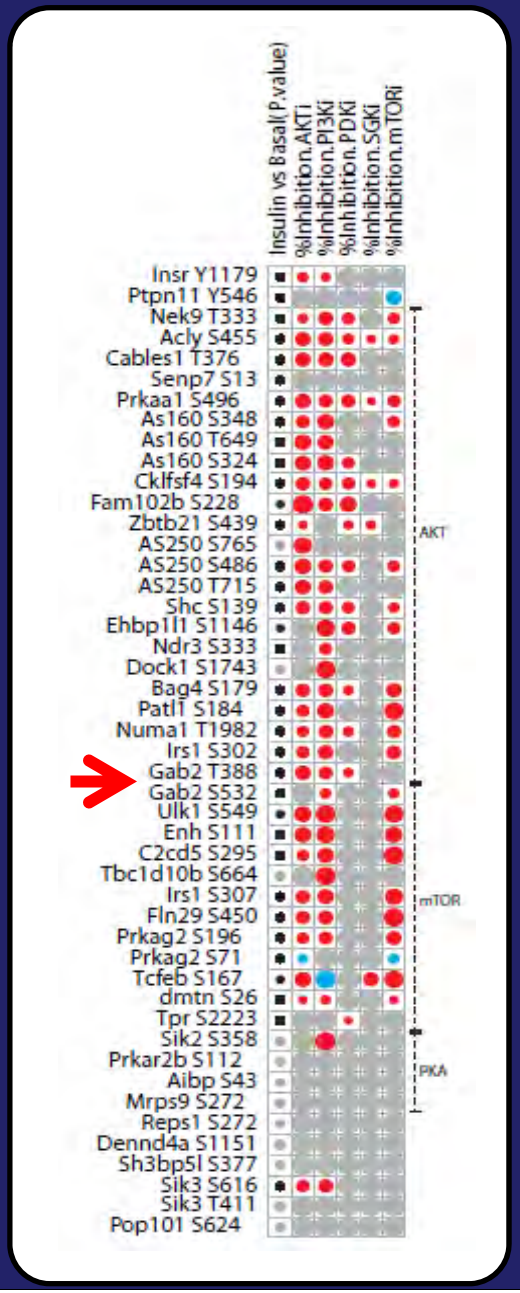
Spectral library containing 10,
(8,561 phosphosites >99% lo
98 phosphopeptides w
targeted list.

Targeted data extraction:

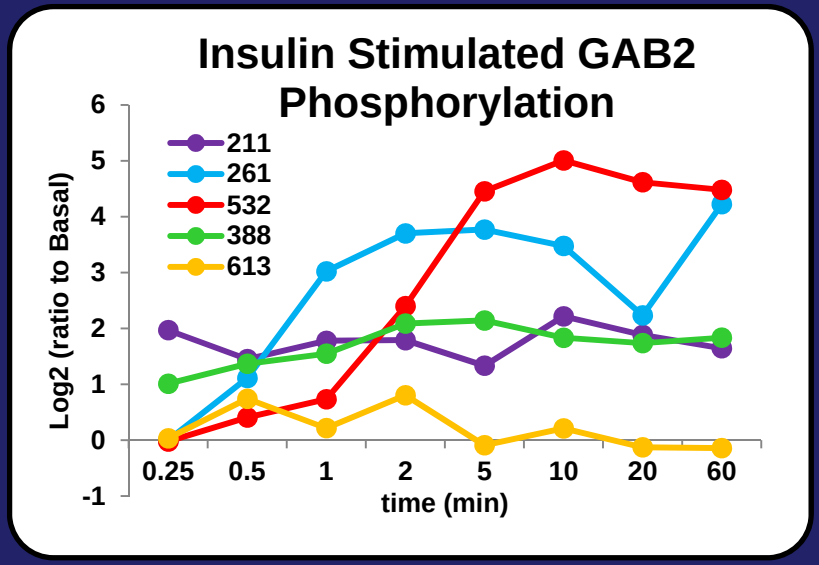
- 45 out of 98 phospho
DIA criteria.
- 42 out of 45 phospho
with <20% CV



Targeted quantification of predicted AKT and mTOR substrate phosphorylation using label-free DIA

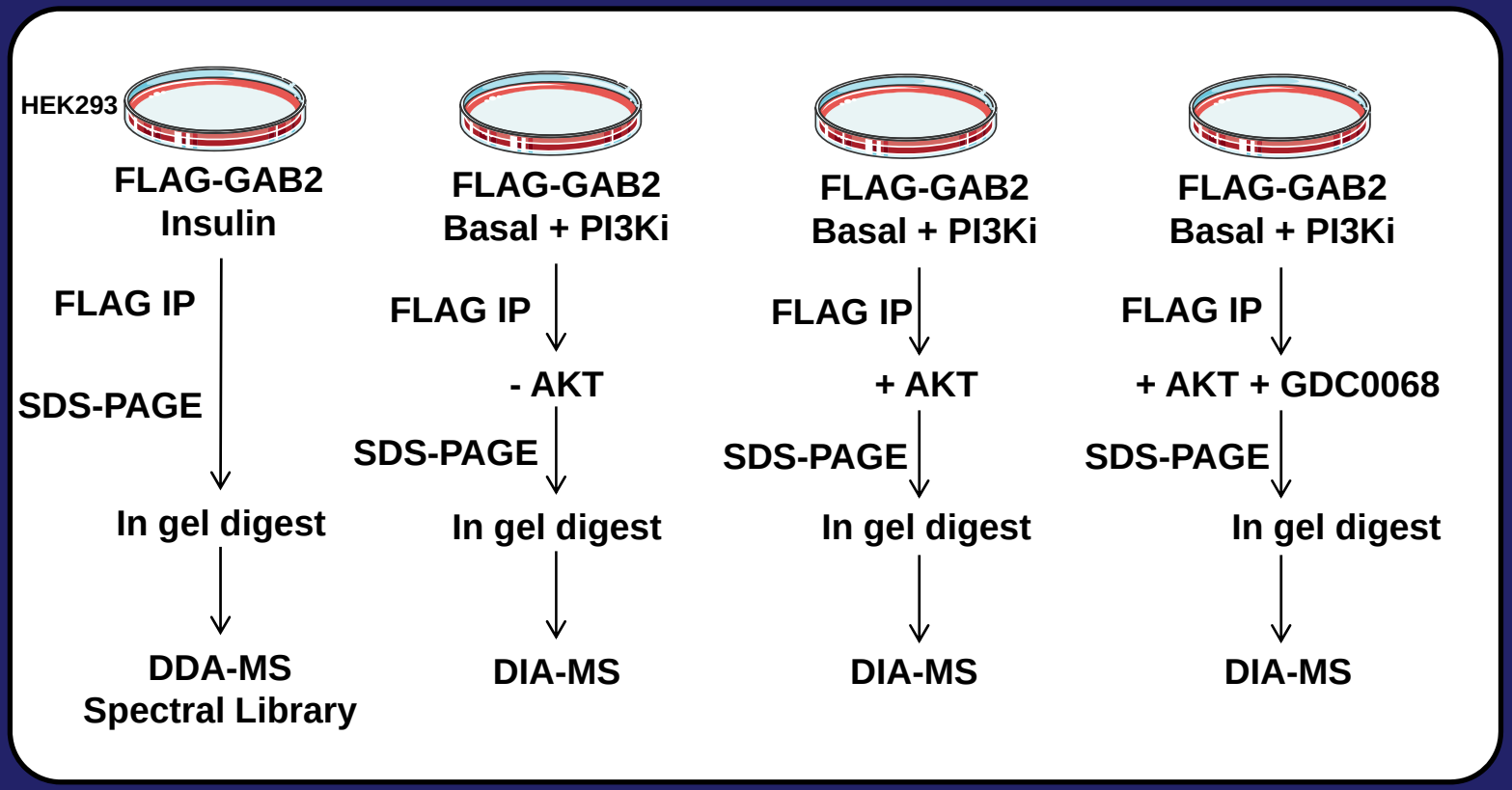


GAB2 (GRB2-associated-binding protein 2) – docking protein with PH domain, PxxP motifs for SH2/SH3 adaptor activity and binds to numerous signalling molecules in PI3K/AKT pathway.

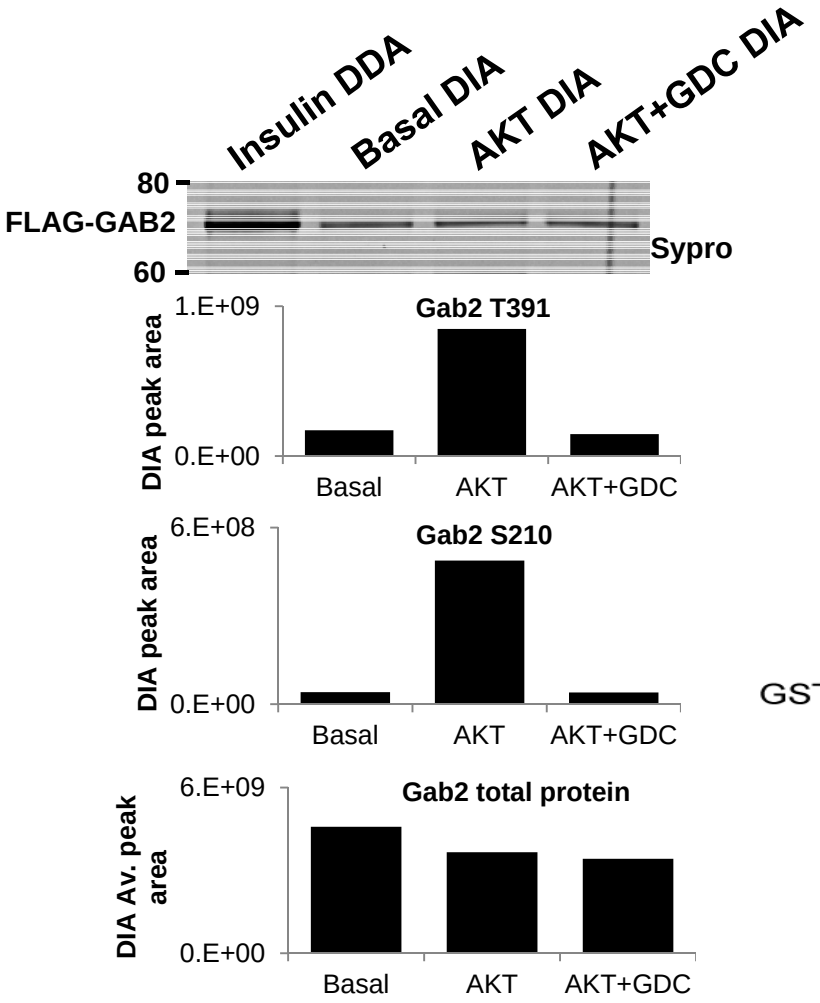


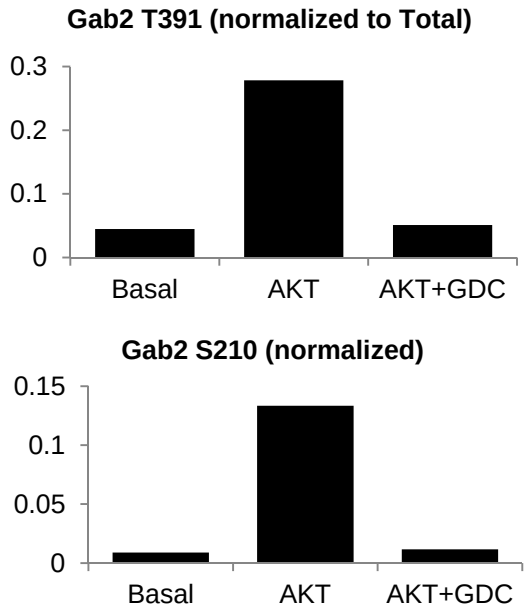
Humphrey, *et. al., Cell Metab.* (2013)

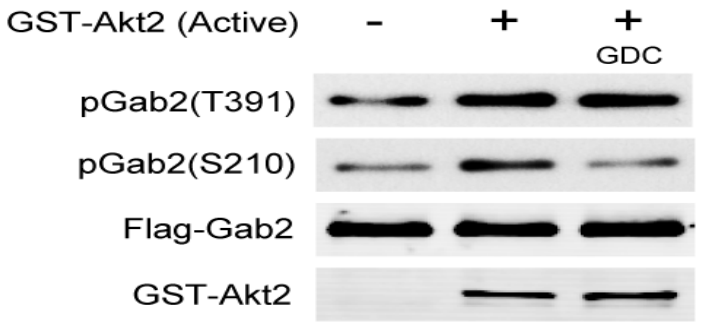
AKT *In vitro* kinase analysis of GAB2 by DIA



In vitro kinase analysis by DIA: AKT phosphorylates GAB2 at S210 and T391

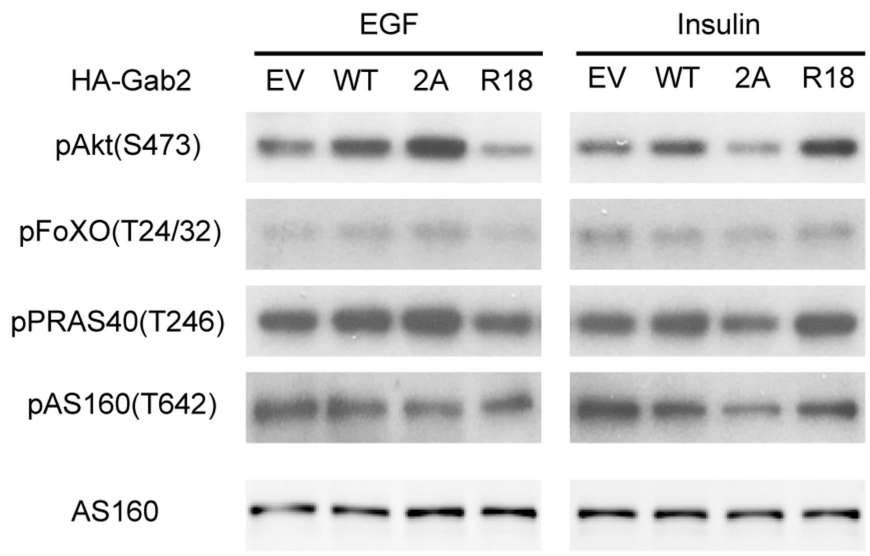




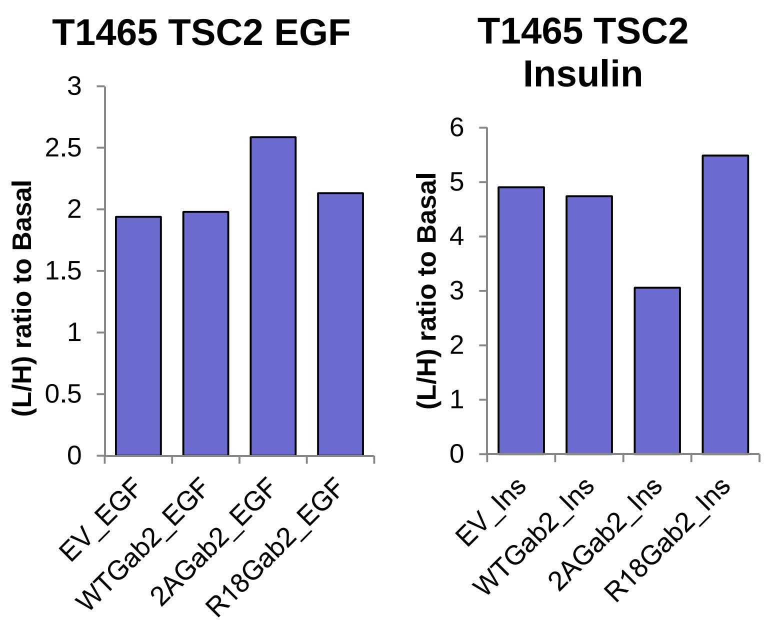


4- 14-3-3 binding of GAB2: insulin versus EGF

Phosphorylation of S210 and T391 recruits 14-3-3 and negatively regulates EGF signalling
(Brummer, *et al., EMBO J* (2008))



SID-DIA-MS



Conclusions and future work

Phosphoproteomic analysis using DIA is a viable strategy for targeted analysis with and without SID normalization.

Future work:

- **further work with false-discovery rates for identification**
- **further work with normalization methods**
- **quantification of total protein abundances**

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