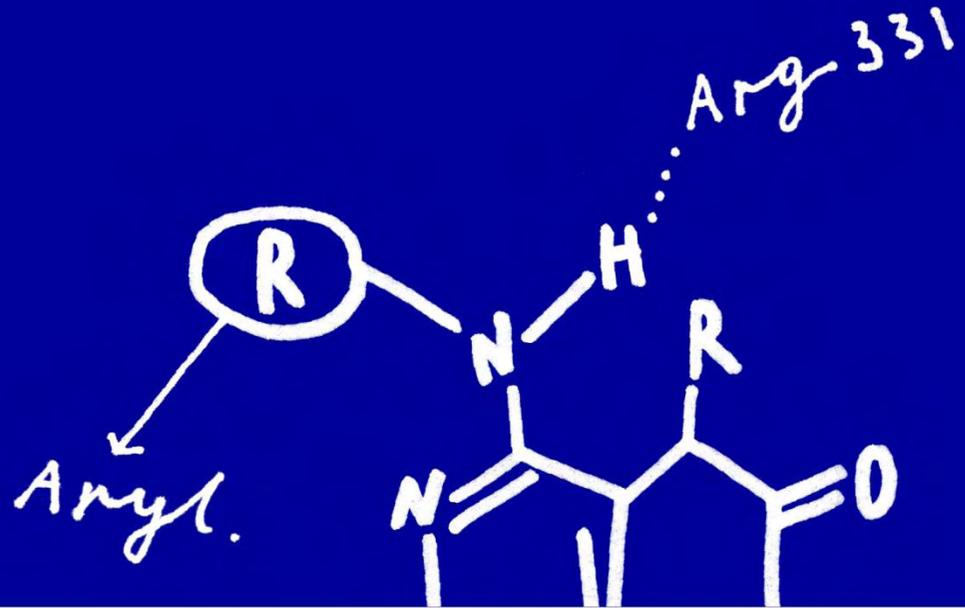


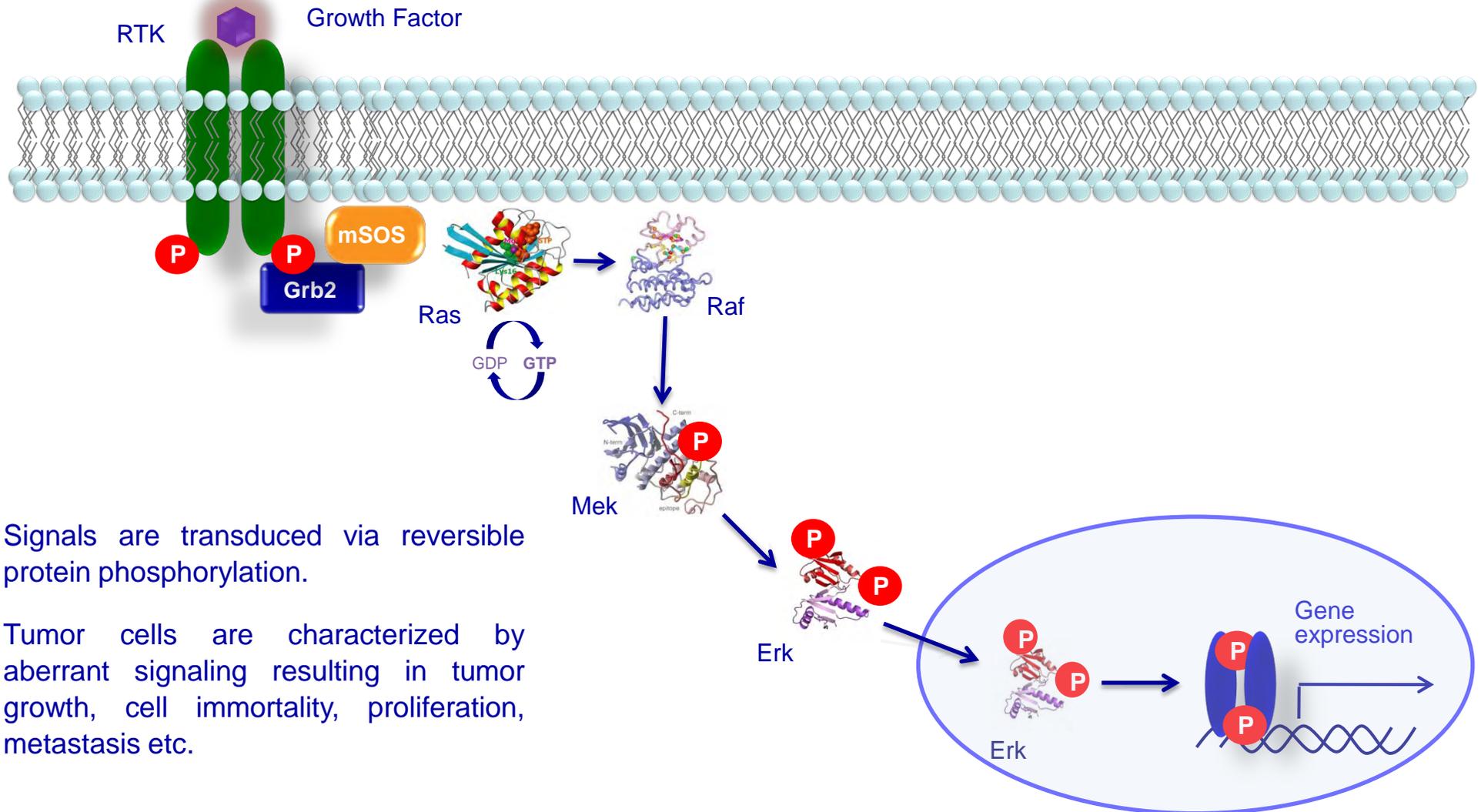
# EVOTEC MUNICH

**Chemical proteomics and quantitative phosphoproteomics to discover novel mechanisms of action of the approved targeted drug Sorafenib**



# Evotec Munich

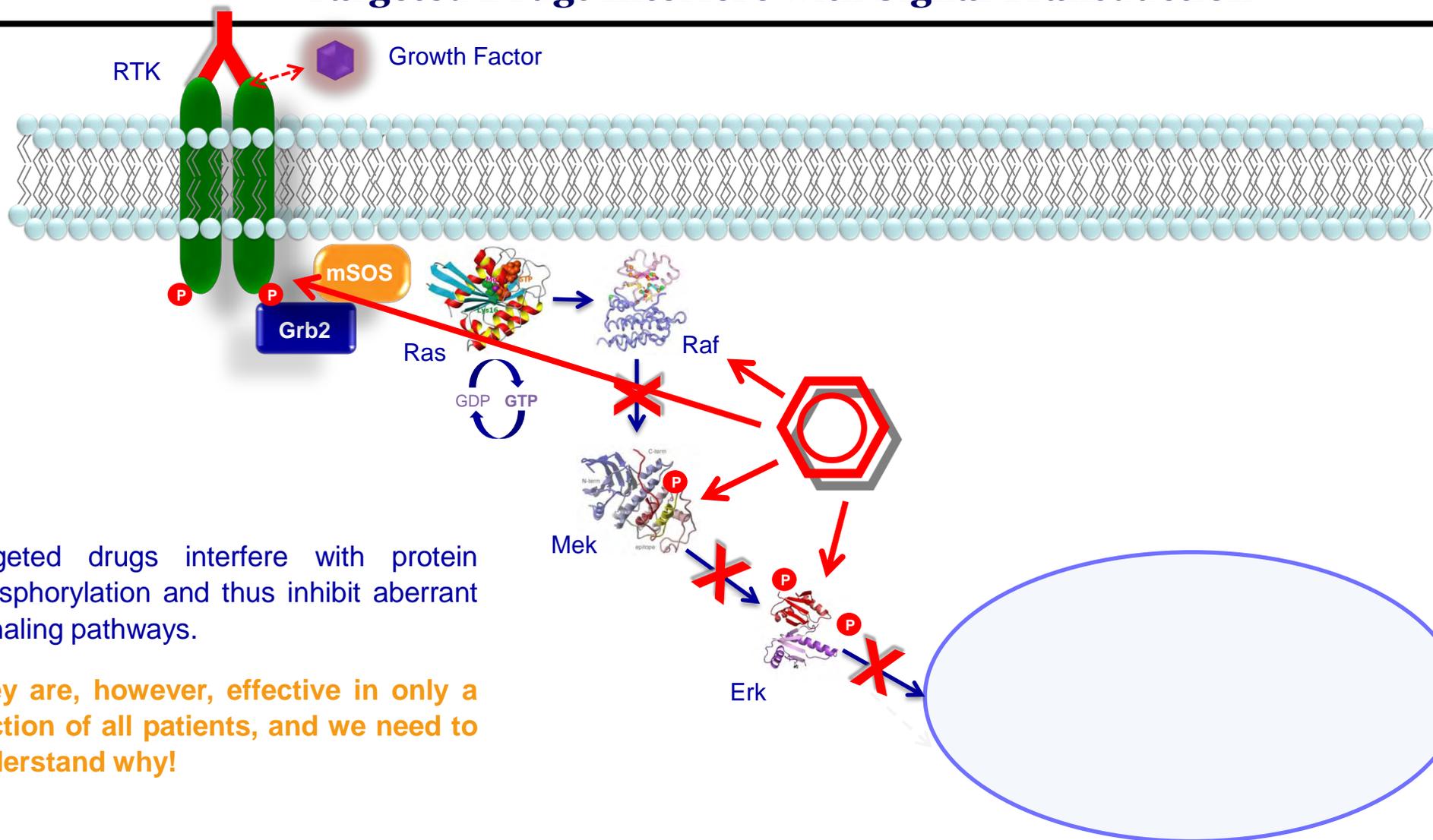
## Targeted Drugs Interfere with Signal Transduction



Signals are transduced via reversible protein phosphorylation.

Tumor cells are characterized by aberrant signaling resulting in tumor growth, cell immortality, proliferation, metastasis etc.

## Targeted Drugs Interfere with Signal Transduction

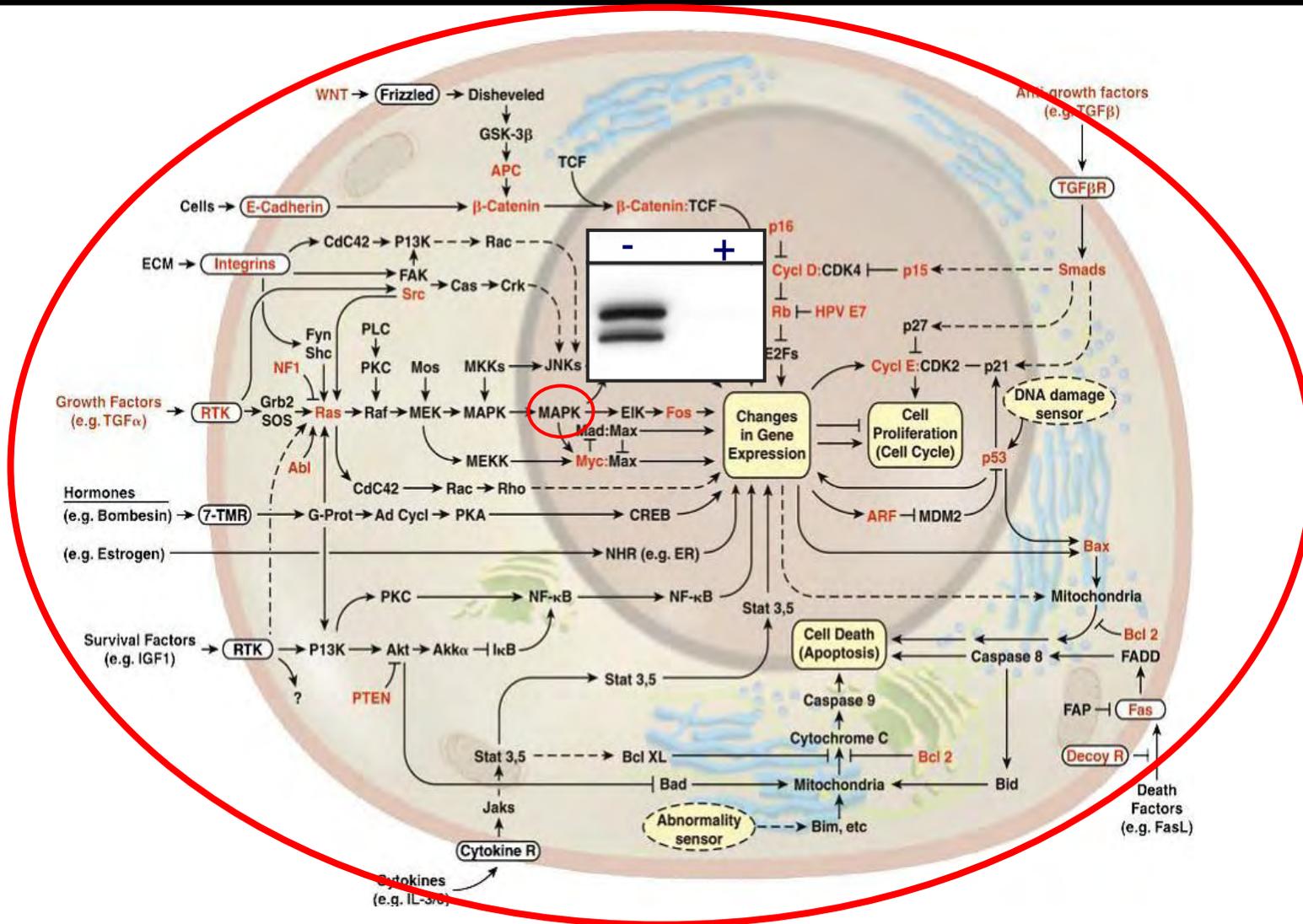


Targeted drugs interfere with protein phosphorylation and thus inhibit aberrant signaling pathways.

**They are, however, effective in only a fraction of all patients, and we need to understand why!**

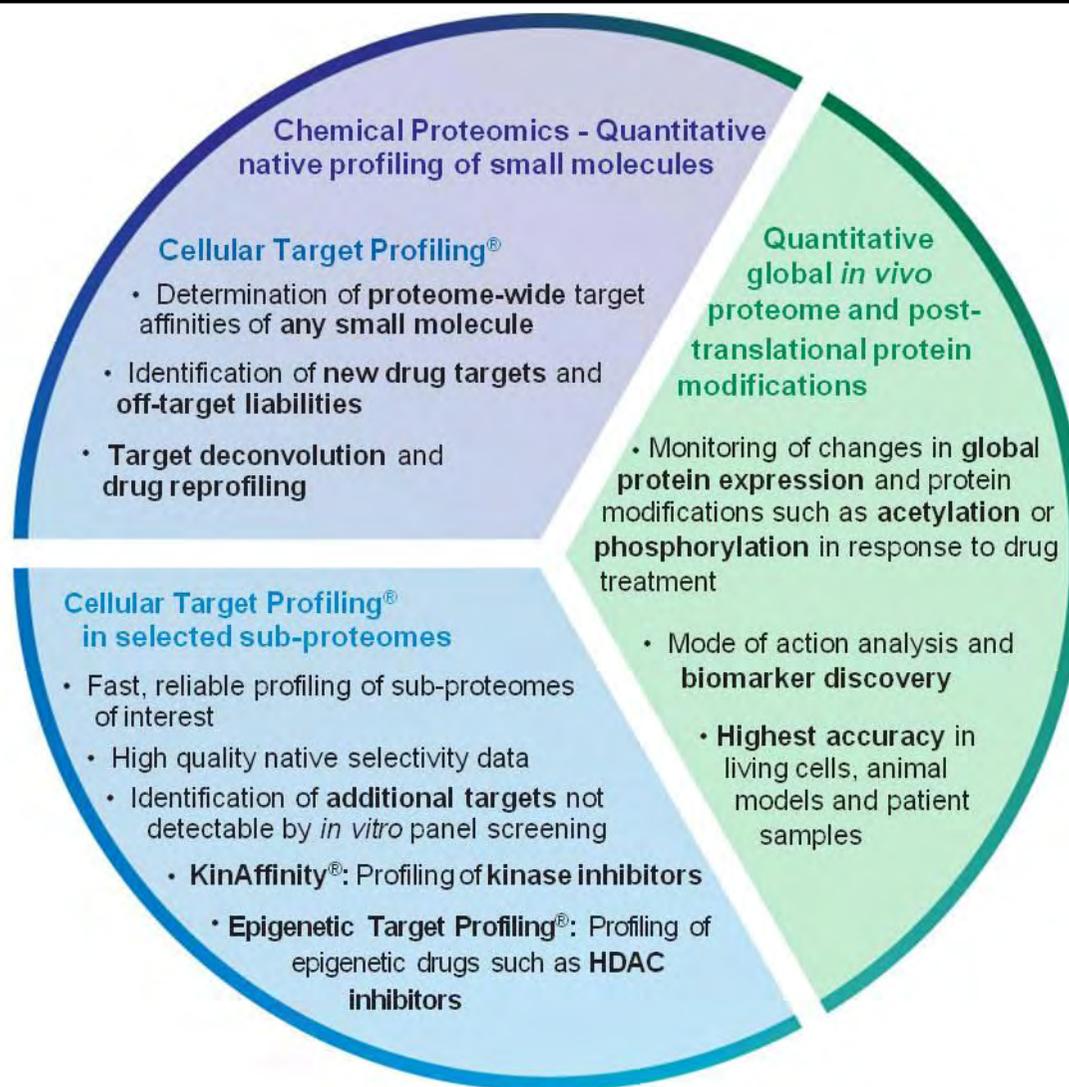
# A Global Understanding of Biological Systems

... by applying mass spectrometry based proteomics



# Evotec Munich

## Technology Offering



## About Evotec Munich

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A leader in chemical proteomics and phosphoproteomics

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### Evotec Munich

- **Evotec's Center of Excellence for Proteomics and Oncology**
- Emerged from Kinaxo Biotechnologies, a Max Planck spin-off founded by the renowned cancer researcher Prof. Axel Ullrich
- Combines highest service quality standards with powerful technological innovation
- Collaborates with leading academic research laboratories including the Matthias Mann lab at the Max Planck Institute
- Has worked with numerous global pharma and biotechnology companies such as



Prof. Dr. Axel Ullrich, Max-Planck Director



## Overview

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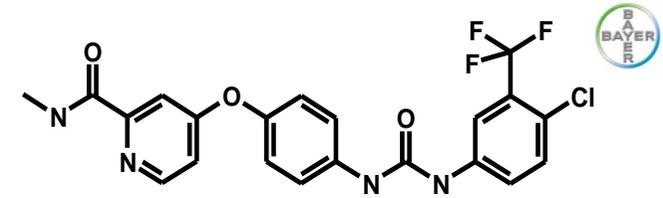
- Identification of the Target Profile of Sorafenib using Cellular Target Profiling
- Phosphoproteomics applied to cultured cell lines (Sorafenib Case Study)
- Outlook

# Case Study Sorafenib

## Rationale

### Sorafenib (Nexavar®)

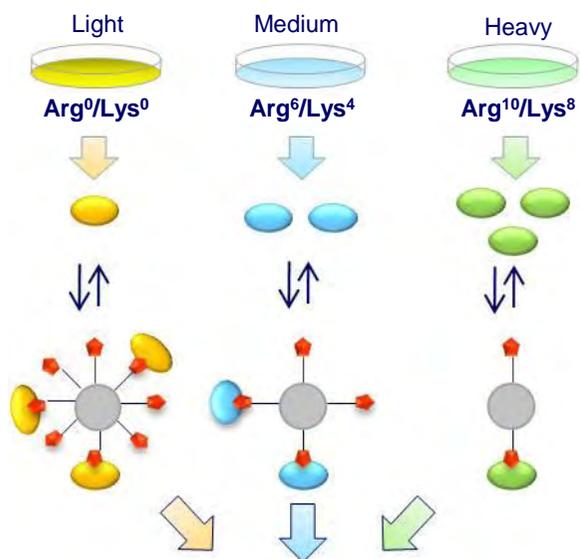
- Multi-kinase inhibitor (known targets are bRAF, VEGFR, RET, FLT3)
- Strong anti-proliferative and anti-angiogenic effects
- Approved for treatment of renal-cell carcinoma and hepatocellular carcinoma; shows promising activity in several different cancer types
- Human prostate cancer cells (PC3) are sensitive to sorafenib treatment, even though this effect cannot be explained by inhibition of the reported main targets



**Sorafenib's mode-of-action  
remains unclear in PC-3 cells**

# Cellular Target Profiling<sup>®</sup>

## Workflow

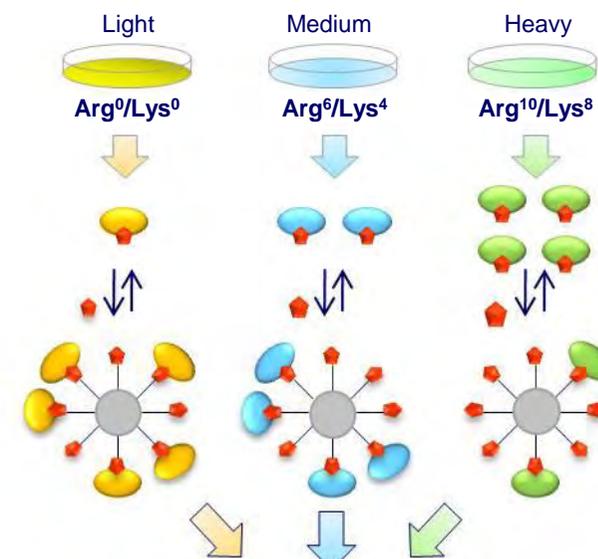


### Proteome labeling

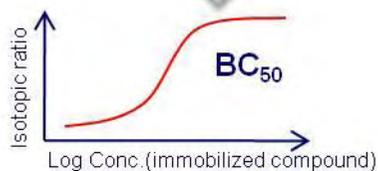
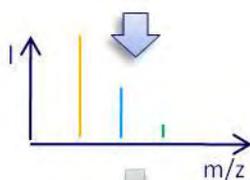
Metabolic  
Chemical

### Compound / target binding

← Labeled lysates are incubated with immobilized compound at different densities of coupled compound.  
Different concentrations of soluble compound are added to the mixture of labeled lysate at a fixed density of coupled compound. →



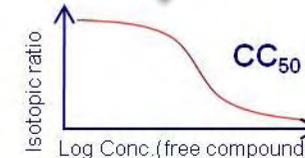
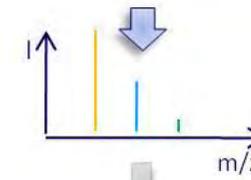
### LC-MS/MS (1)



### Identification and quantification of proteins

Identification and determination of the relative amounts of the captured proteins by liquid chromatography and quantitative mass spectrometry

### LC-MS/MS (2)

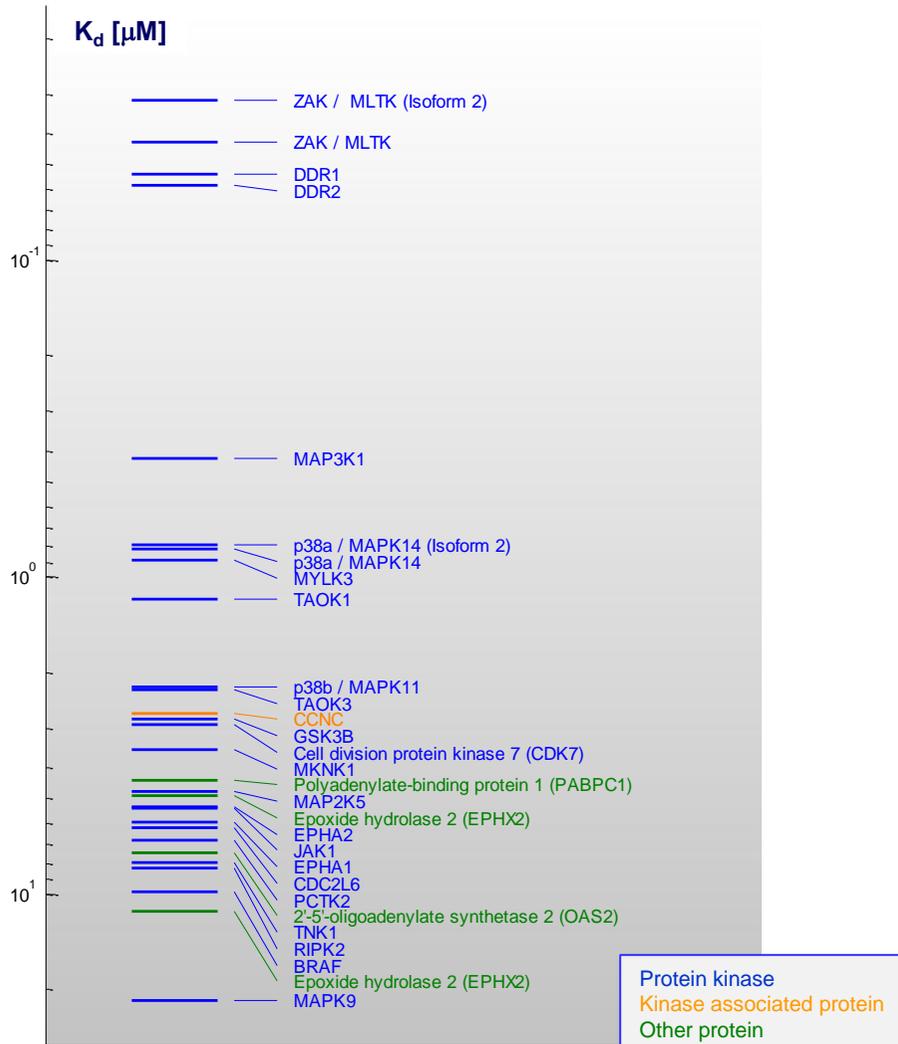


### Determination of $K_{d,free}$ values

Generation of compound/target curves for immobilized and free compound. Application of **Cheng-Prusoff** equation to determine the  $K_{d,free}$  values of all target proteins

# Cellular Target Profiling<sup>®</sup>

## Target Profile of Sorafenib in PC3 cells



- ZAK, DDR1, DDR2, MAP3K1, MAPK14/p38 $\alpha$  and MYLK3 bind Sorafenib with affinities better than 1 $\mu\text{M}$  in PC3 cells
- These kinases modulate a wide range of cellular responses such as **apoptosis**, **cell migration** or **cell proliferation** and might therefore contribute to the drug's effects in PC3 cells

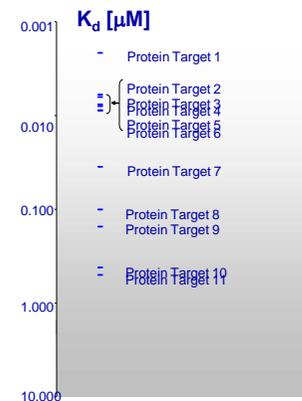
# Cellular Target Profiling - Phosphoscout

From Target Profile to the Mode-of-Action

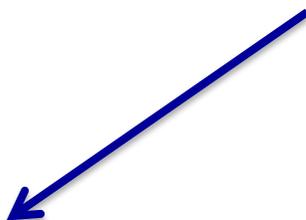
• Cellular Target Profiling



Target Profile



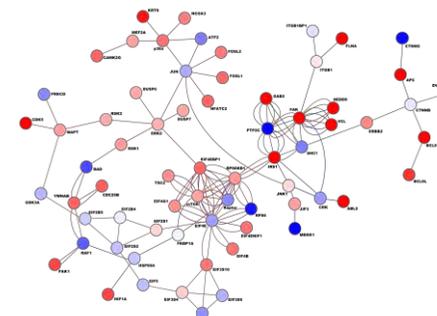
Impact on Signaling Pathways?



• PhosphoScout



Mode-of-Action analysis



# PhosphoScout®

## Global quantitative phosphoproteomics

- **Global, unbiased** and **quantitative** method to monitor dynamic phosphorylation events for systematic understanding of cellular behavior
- Reproducible quantification of >15,000 phosphorylation events in a single experiment
- Identification and quantification of phosphorylation sites in living cells, animal models and patient samples
- **Mode of action analysis** of targeted cancer drugs and **biomarker discovery**



The collage features several scientific articles and a MaxQuant advertisement:

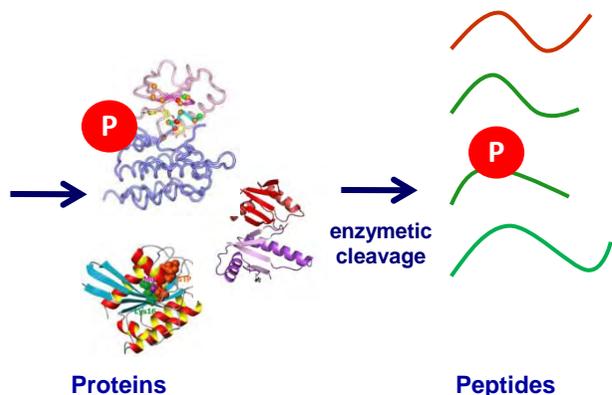
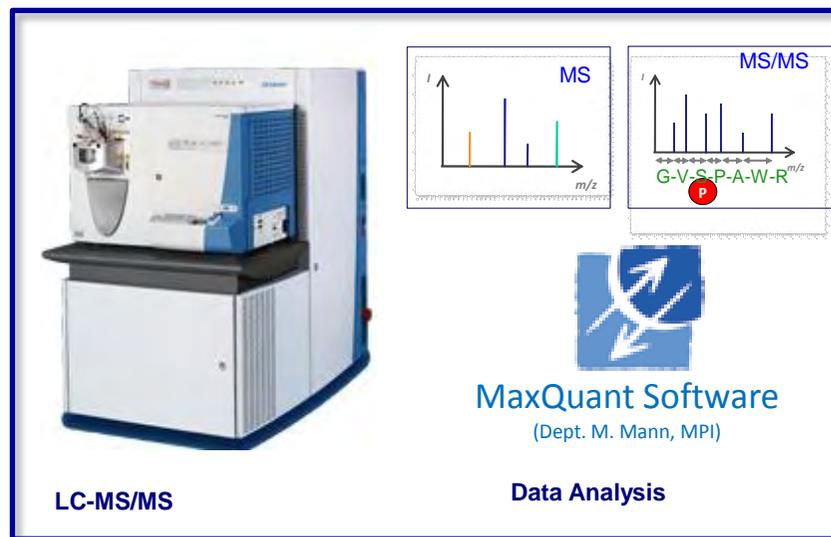
- Resource (Cell):** "Global, In Vivo, and Site-Specific Phosphorylation Dynamics in Signaling Networks" by Jäger et al. (2010). Abstract: Cell signaling mechanisms often depend on phosphorylation, but knowledge of the sites in which transition mechanisms has been generatively accumulated, mostly through the study of individual molecules in specific pathways. More recently, the emergence of high-resolution mass spectrometry has enabled a global and unbiased view of phosphorylation dynamics in cells and organisms. The PhosphoScout method is a high-resolution, high-coverage, and high-precision method for the identification and quantification of phosphorylation sites in cells and organisms. The PhosphoScout method is a high-resolution, high-coverage, and high-precision method for the identification and quantification of phosphorylation sites in cells and organisms.
- Resource (Cell):** "SILAC Mouse for Quantitative Proteomics Uncovers Kiflin-3 as an Essential Factor for Red Blood Cell Function" by Marra et al. (2010). Abstract: Stable isotope labeling by amino acids in cell culture (SILAC) has become a standard tool for quantitative proteomics. However, the use of SILAC in mammalian cells is limited by the low incorporation efficiency of heavy amino acids. We have developed a novel method for the generation of heavy amino acid-labeled mice, which allows for the identification and quantification of phosphorylation sites in cells and organisms.
- Learning Edge Essay (Cell):** "Is Proteomics the New Genomics?" by Vogelstein and Kinzler (2002). Abstract: The past decade has seen a revolution in the way we study the genome. The discovery of the human genome sequence and the subsequent development of high-throughput sequencing technologies have opened up new frontiers in the study of the genome. Proteomics, the study of the structure and function of proteins, is emerging as a complementary field to genomics.
- Cell:** "MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification" by Jäger et al. (2009). Abstract: Efficient analysis of very large amounts of raw data for peptide identification and protein quantification is a principal challenge in mass spectrometry (MS)-based proteomics. Here we describe MaxQuant, an integrated suite of algorithms specifically developed for high-resolution, quantitative MS data. Using correlation analysis and graph theory, MaxQuant detects peaks, filters false and stable noise and assigns identified (SILAC) peptide pairs in three-dimensional objects in *m/z*, retention time and signal intensity space. By integrating multiple mass measurement and correcting for mass measurement effects, we achieve mass accuracy in the pp.b. range, a novel measure of dataset homogeneity, and increase the proportion of identified phosphorylation sites by 75% for SILAC peptide pairs via unambiguous assignment of unique and internal-charge state and individual mass peaks. MaxQuant automatically quantifies control labeled (heavy) peptides for SILAC proteomics.

# PhosphoScout®

## Global Quantitative Phosphoproteomics Workflow



Isobaric labeling (SILAC)

LC-MS/MS

MS

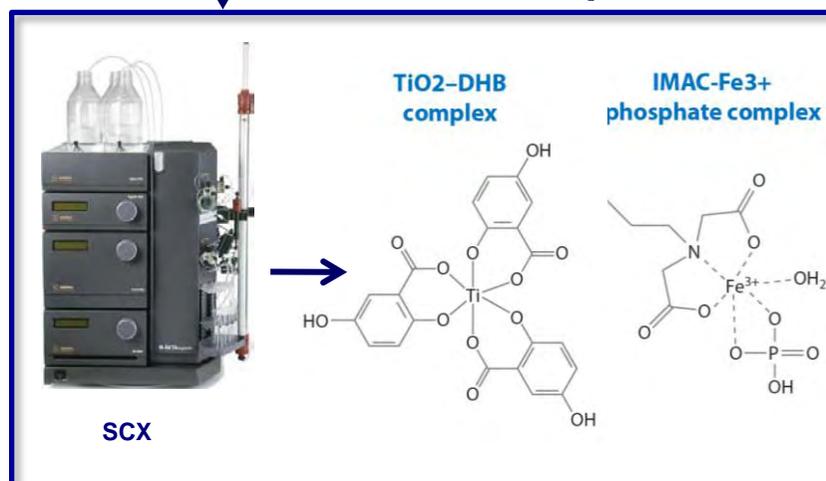
MS/MS

G-V-S-P-A-W-R

MaxQuant Software  
(Dept. M. Mann, MPI)

Data Analysis

Global phosphoproteome enrichment



SCX

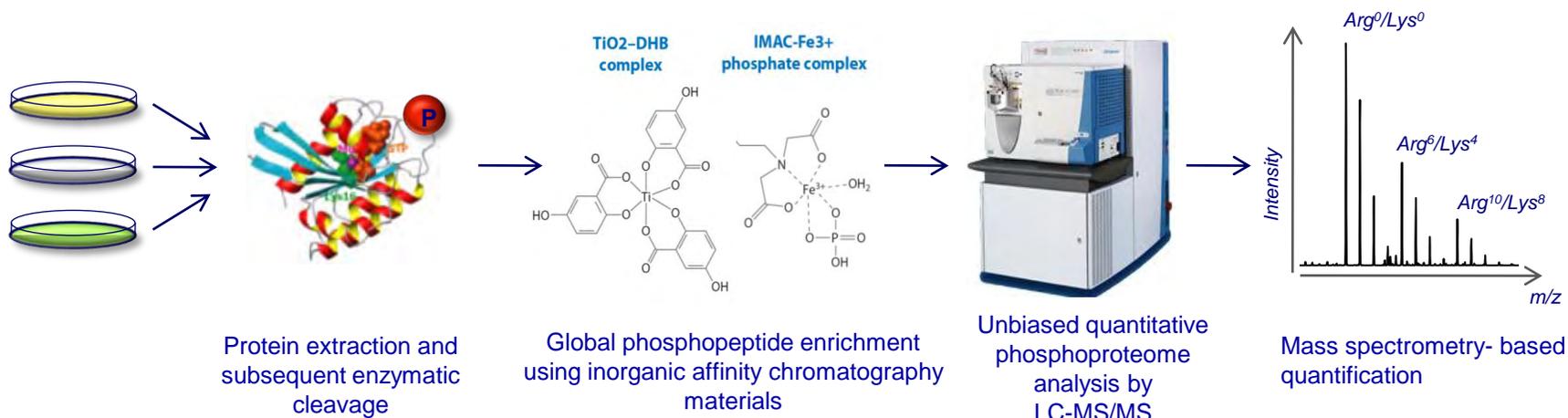
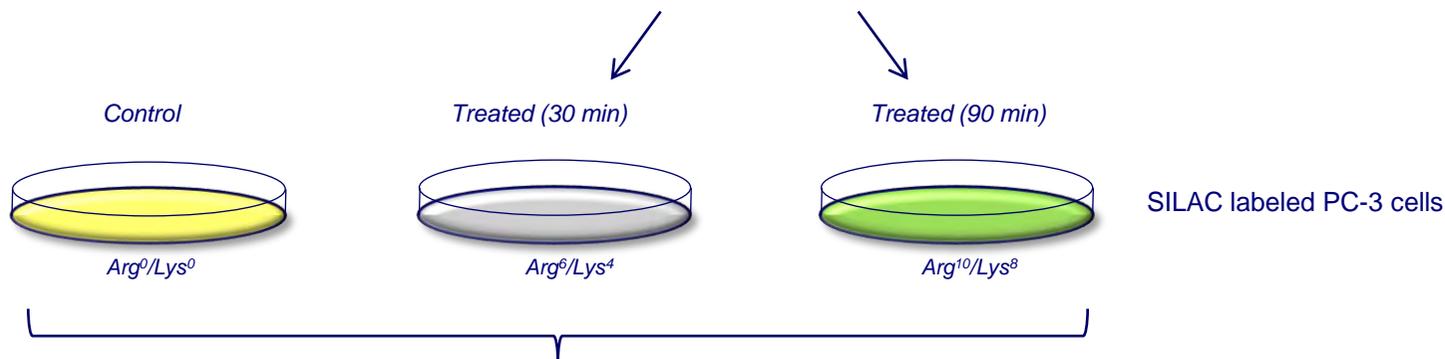
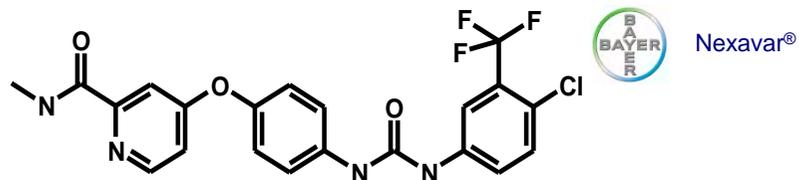
TiO<sub>2</sub>-DHB complex

IMAC-Fe<sup>3+</sup> phosphate complex

Unbiased, global quantitative phosphoproteome analysis

# Case Study Sorafenib

## Quantitative Phosphoproteomics Workflow



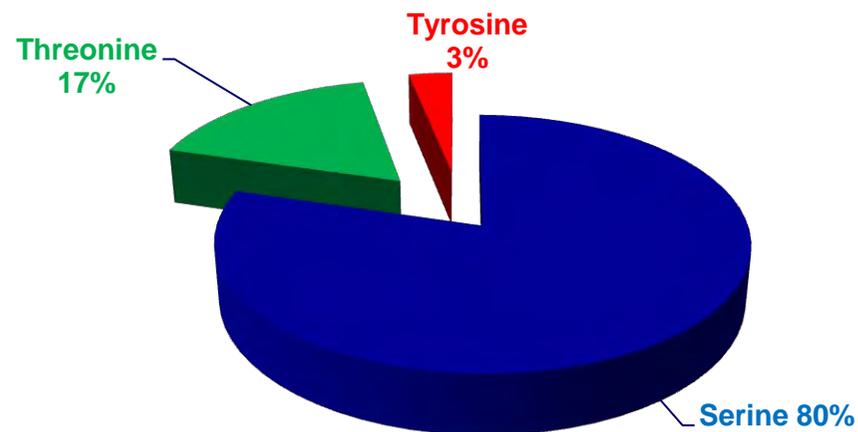
# Case Study Sorafenib

## Identification of Regulated Phosphorylation Sites

	All P-sites	Kinases
No. of detected phosphorylation sites	15,825	961
No. of detected proteins with phosphorylation sites	3,931	228
No. of regulated sites	1,012	68
No. of proteins with regulated phosphorylation sites	605	40

- Only phosphorylation sites that could be localized within the peptide sequence with high confidence are considered in our analysis
- Identification of differentially regulated phosphorylation sites at a false discovery rate of 5% based on a global rank test

Zhou *et al.*, *Bioinformatics* 23 (2007) 2073



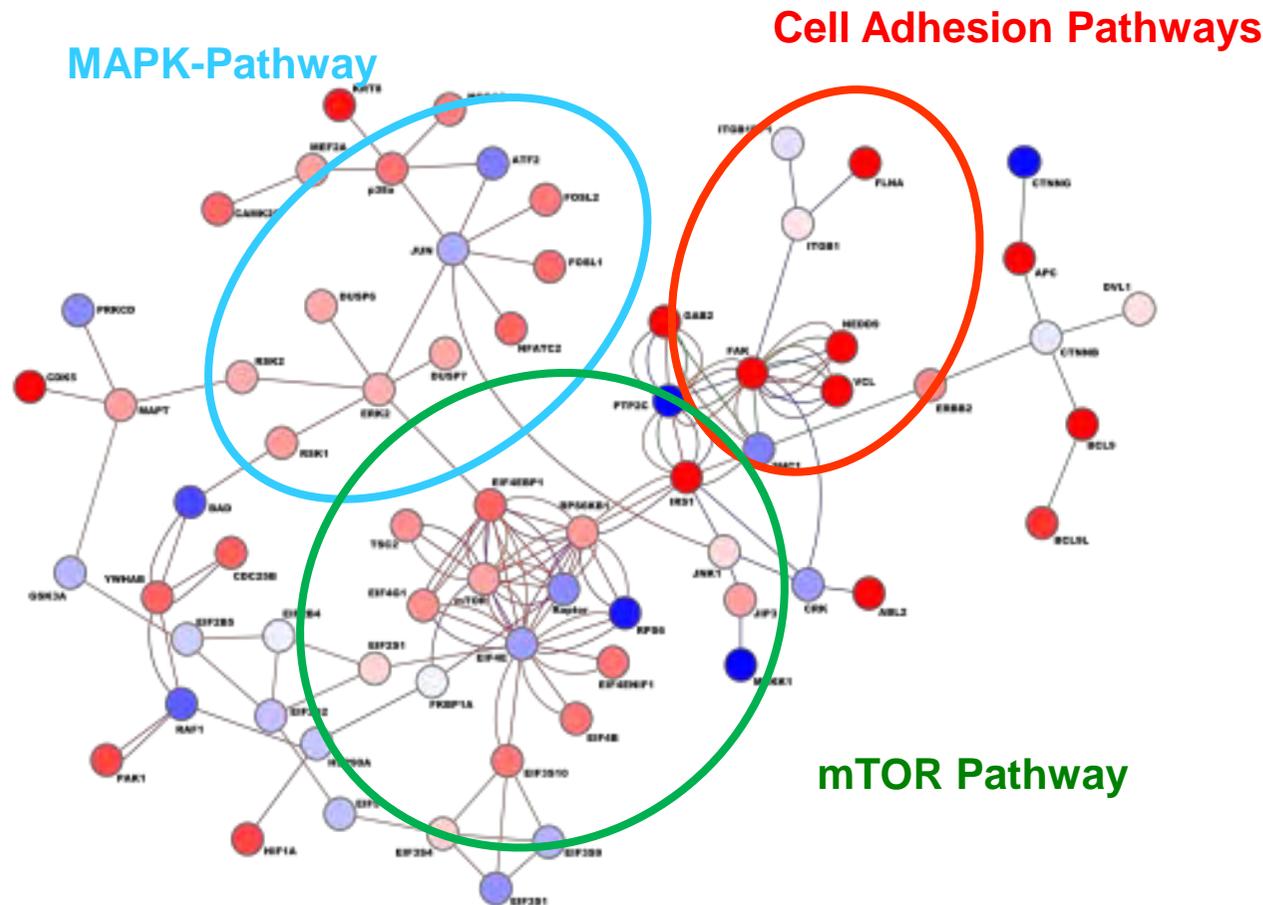
# Case Study Sorafenib

## Affected Signaling Pathways

KEGG Pathway	Proteins with detected P-sites	Proteins with regulated P-sites
Insulin signaling pathway	52	19
MAPK signaling pathway	74	21
mTOR signaling pathway	22	9
ErbB signaling pathway	40	13
Axon guidance	34	10
Prostate cancer	21	7
Non-small cell lung cancer	17	6

# Case Study Sorafenib

## Integrating data with protein-protein networks

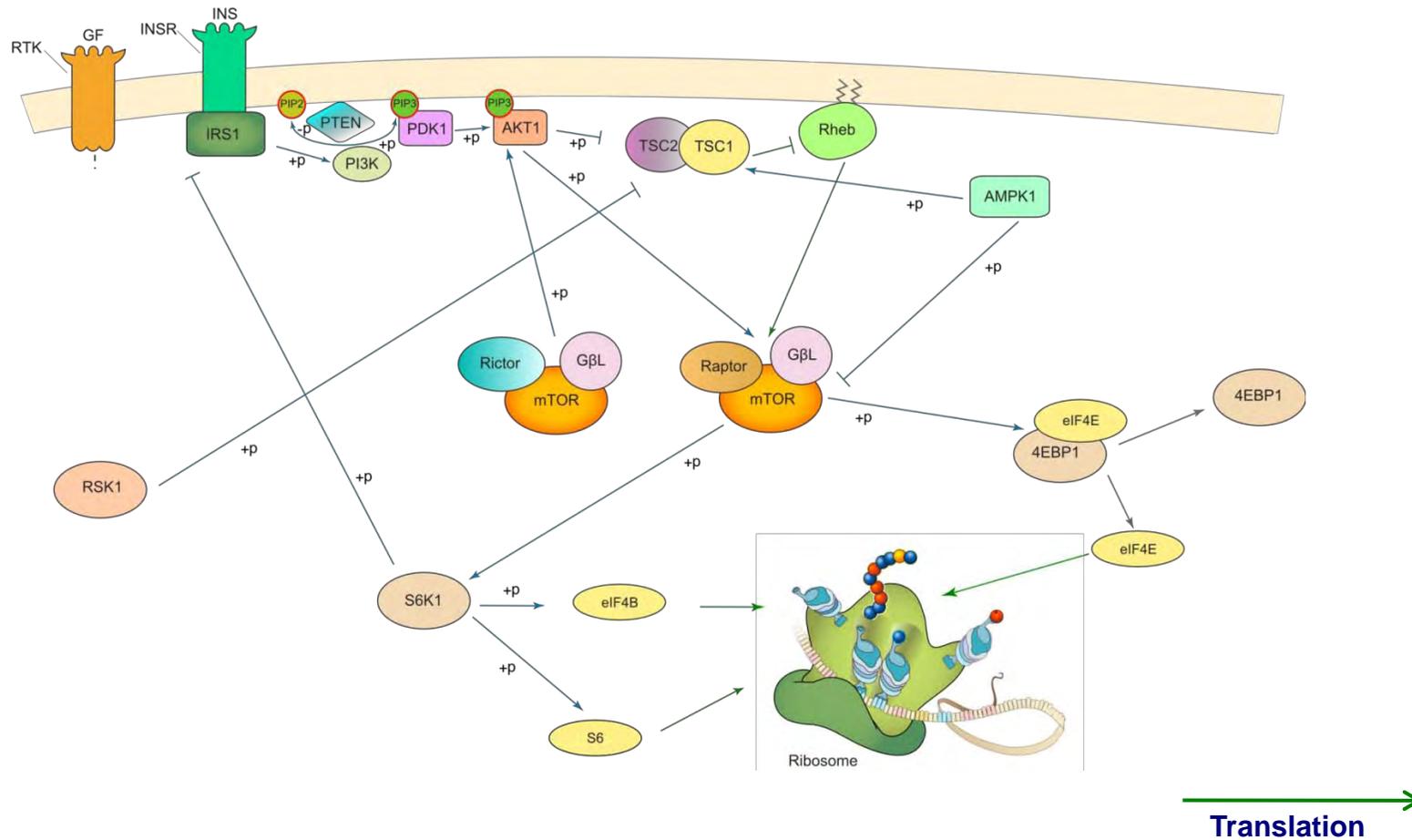


- The „SubExtractor“ algorithm combines phosphoproteomic data with information from STRING
- Identification of differentially regulated subnetworks and individual proteins in a biological context

# Case Study Sorafenib

## Detailed visualization of regulated phosphosites

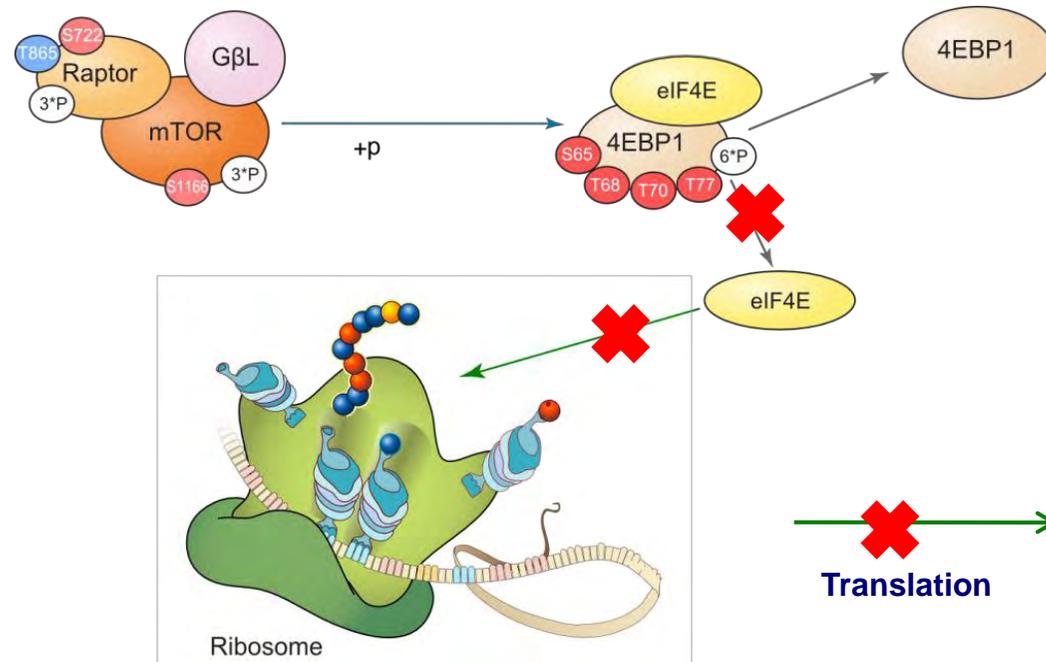
PC3 cells with PTEN mutation → activated PI3K/Akt/mTOR-pathway





# Case Study Sorafenib

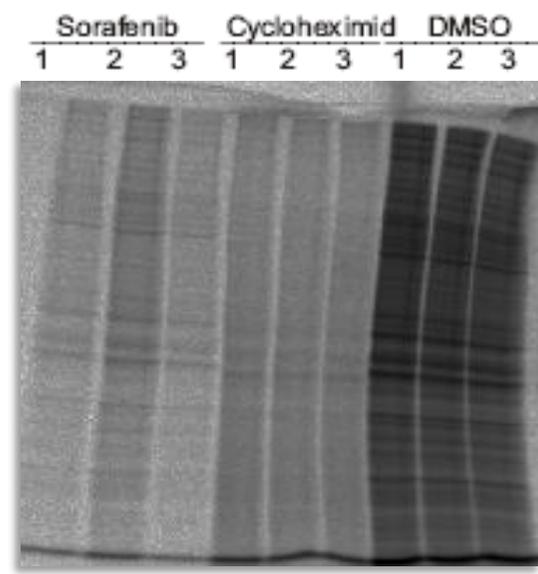
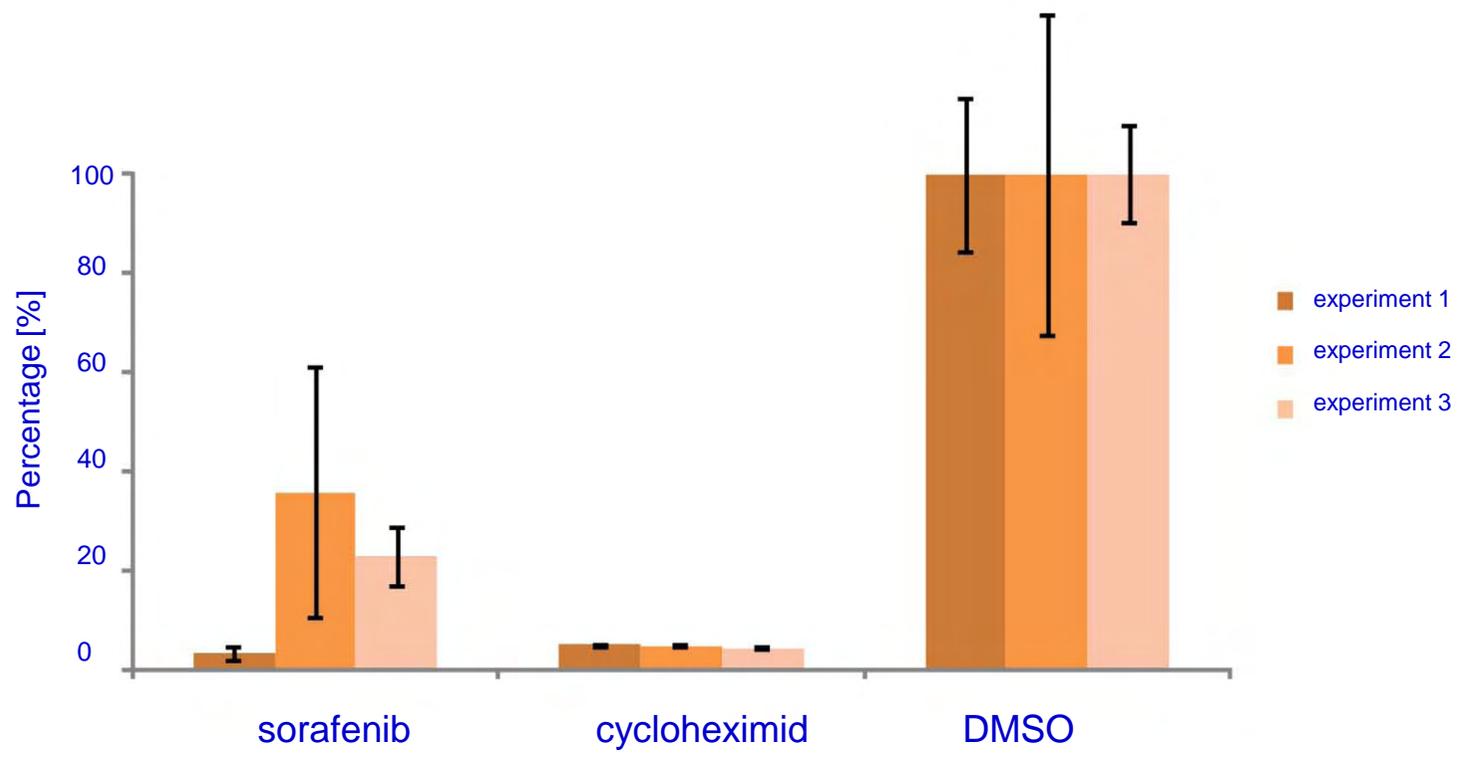
## Detailed visualization of regulated phosphosites



# Case Study Sorafenib

## Validation of sorafenib's effect on translation

PC3 - 30min S<sup>35</sup> pulse after treatment with sorafenib, cycloheximid or DMSO for 90min



# Case Study Sorafenib

## Summary

### Phosphoproteomics analysis of Sorafenib

- Statistically validated and reproducible information about the drug's mode of action
- Revealed previously unknown inhibition of the mTOR pathway
- Might lead to additional therapeutic applications with a better understanding drug efficacy, resistance mechanisms etc.

#### Biomarker discovery for sorafenib



**KINAXO**  
Biotechnologies GmbH

#### **KINAXO Biotechnologies and Bayer Vital GmbH Collaborate in Phosphoproteomics Biomarker Identification**

*Martinsried, Leverkusen, Germany, October 15, 2009.* KINAXO Biotechnologies GmbH and Bayer Vital GmbH announced today that they will enter into collaboration. KINAXO will apply its quantitative phosphoproteomics technology PhosphoScout® for the identification of novel biomarkers in a clinical trial conducted by Bayer Vital.

KINAXO's phosphoproteomics platform allows annotation and quantification of regulated phosphorylation sites. Since the majority of targeted compounds used as anti-cancer drugs influence cellular signal transduction pathways, analysis of phosphorylation patterns in relation to drug administration reveals a compound's molecular mode of action. Characteristic phosphorylation sites predicting response to treatment, resistance mechanism or synergistic effects can hereby be identified as biomarkers which allow for personalized treatment plans.

## Biomarkers

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### Definition Biomarker

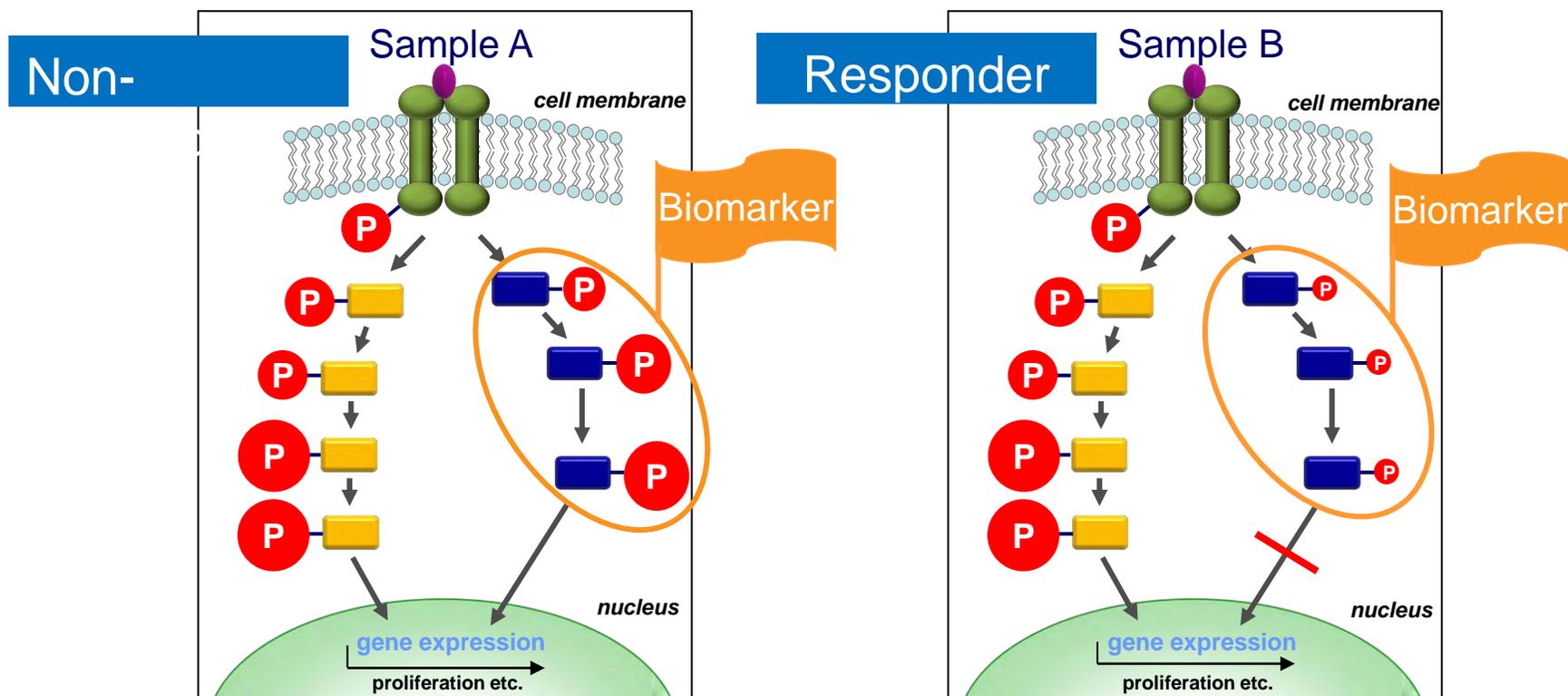
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“A biomarker is a characteristic that is objectively measured and evaluated as an indicator of **normal biologic processes, pathogenic processes, or pharmacologic responses** to a therapeutic intervention.” (NIH)

- **Diagnostic** marker (e.g. PSA level for the diagnosis of prostata carcinoma)
- **Prognostic** marker (e.g. mRNA levels predicting the risk of forming metastasis for breast cancer patients)
- **Drug response** marker (e.g. FDG-PET for monitoring tumor size)
- **Stratification marker** (e.g. HER2 overexpression to stratify patients that are likely to benefit from Herceptin therapy)

# Biomarkers for Sorafenib

## Rationale for Biomarker Discovery

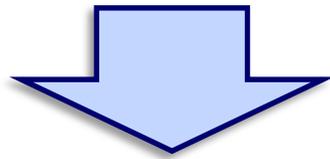


Hypothesis: Patient samples A and B are distinguishable due to their individual phosphorylation patterns that correlate with the drug's effects. These 'phosphosignatures' represent biomarkers for patient stratification.

# In-vivo Phosphoproteomics

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Cell culture on plastic dishes might not always recapitulate all aspects of *in vivo* tumor physiology in particular cancer cell growth in a three-dimensional environment in contact to other cells of non-tumor origin.



Extending global phosphoproteomics on animal models such as mouse xenograft models.



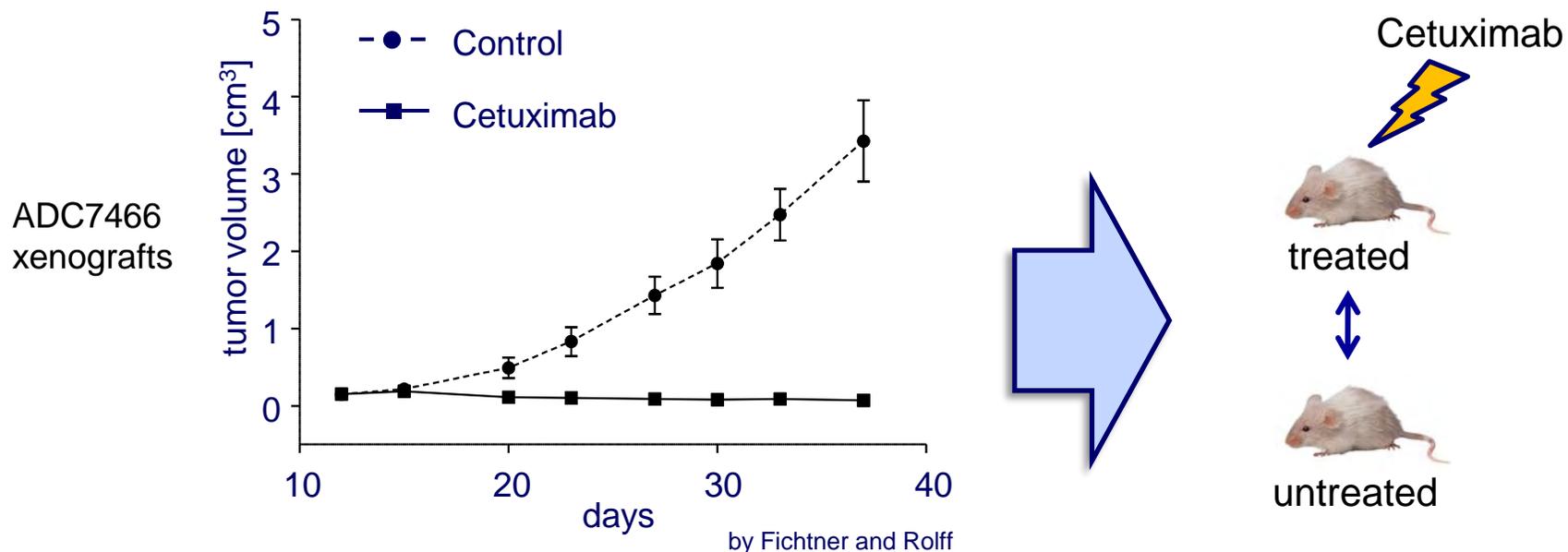
# Mode of Action Analysis in Xenograft Models

... responsive to cetuximab

**Cetuximab:** monoclonal antibody directed against EGFR given for the treatment of metastatic colorectal and head/neck cancer

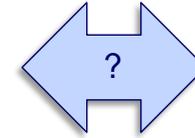
ID	Age	Sex	Smoker	Tumor-stage	Prior treatment	EGFR	K-ras	p53	Cetuximab response
ADC7466 (NSCLC)	57	M	s	pT2 pN1 cM0 G3 R0	No	wt	wt	R196P	++++

adapted from Fichtner et al., *Clin Cancer Res* 2008;14(20)



A (untreated)

B (treated)



# Case Study Cetuximab

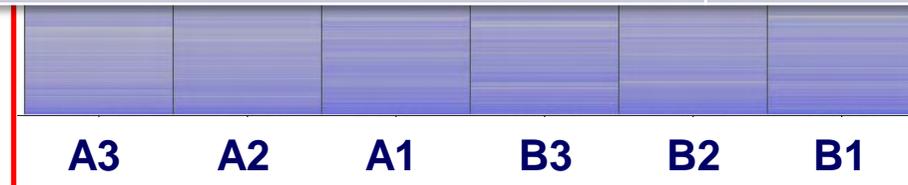
Treated vs. untreated

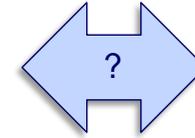
A (-)

B (+)



Found at least in 2 rep. from A & B	All P-sites (shared)	Unique Human
No. of class I sites with at least 2 ratios (A&B)	8.911	5.529
No. of regulated sites	1.072	755
No. of proteins with regulated class I sites	674	484





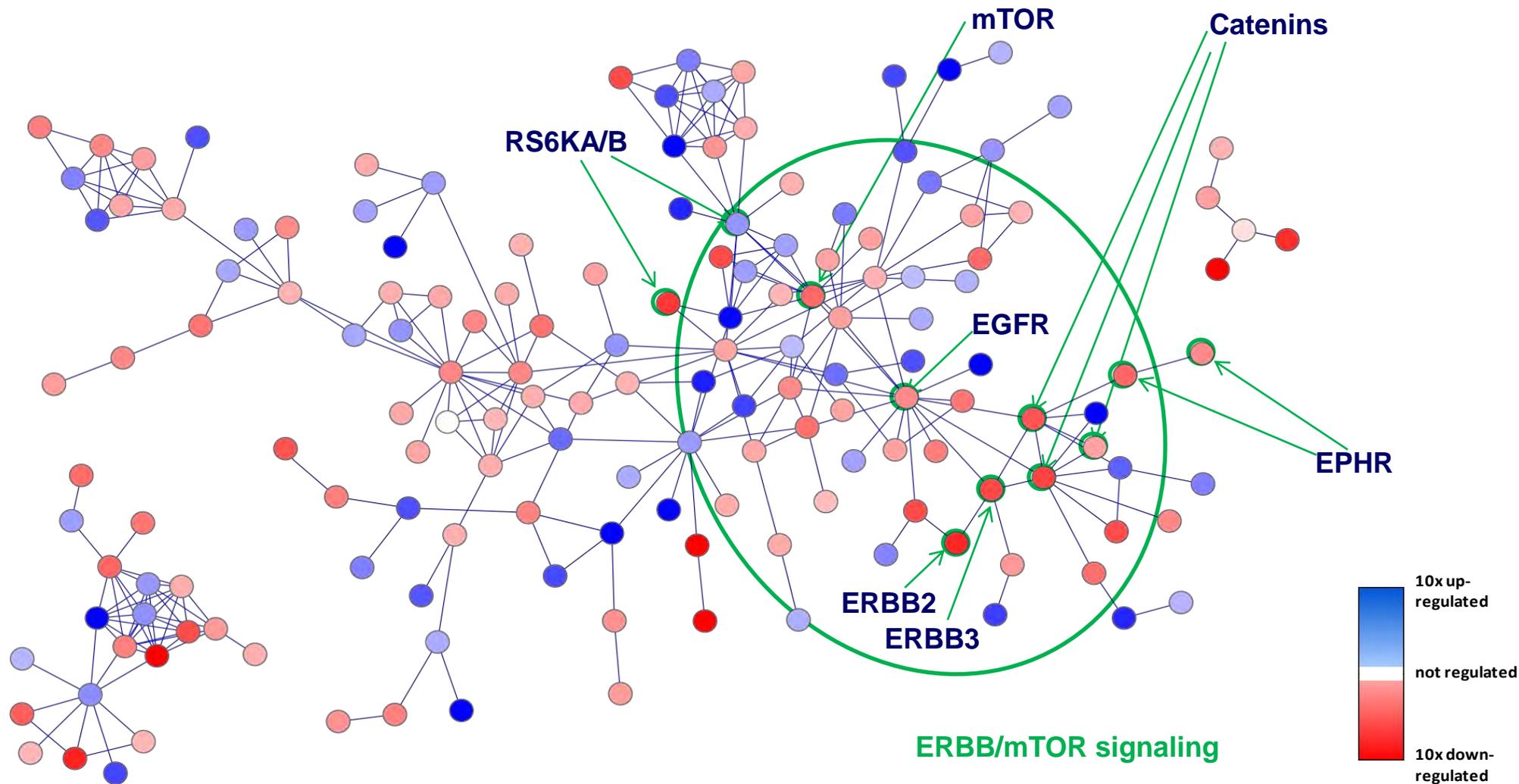
# Case Study Cetuximab

Overall results

KEGG Pathway	Proteins with detected p-sites	Proteins with regulated p-sites
Cell junctions	23	11
Focal adhesion	82	17
ErbB signaling pathway	51	12
Regulation of actin cytoskeleton	82	22
GO term	Proteins with detected p-sites	Proteins with regulated p-sites
cytoskeleton organization	351	62
GTPase regulator activity	456	65
regulation of kinase activity	219	35
regulation of cell cycle	310	41
regulation of signal transduction	465	62

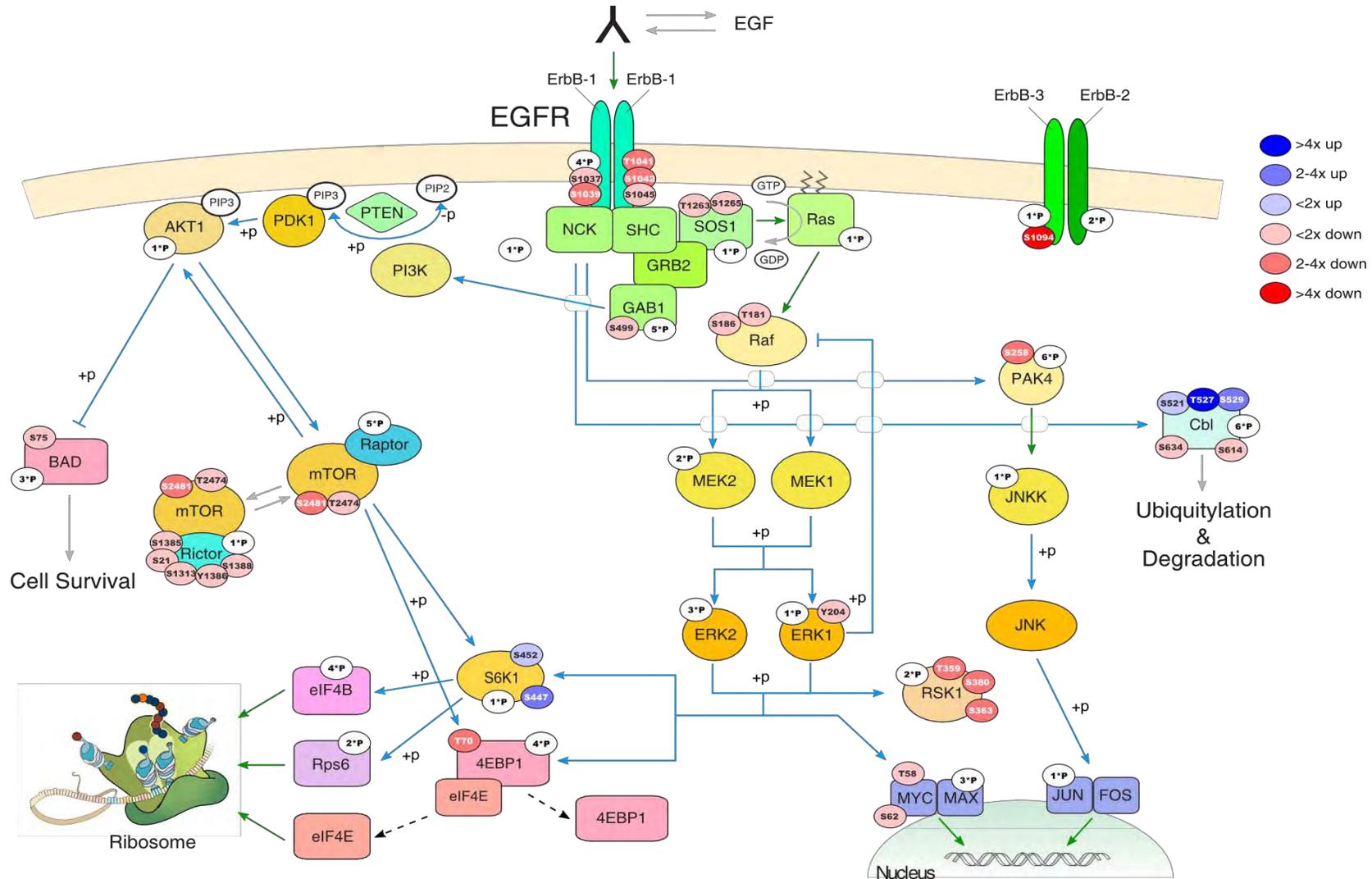
# Case Study Cetuxumab

Integration with protein-protein interaction networks



# Case Study Cetuximab

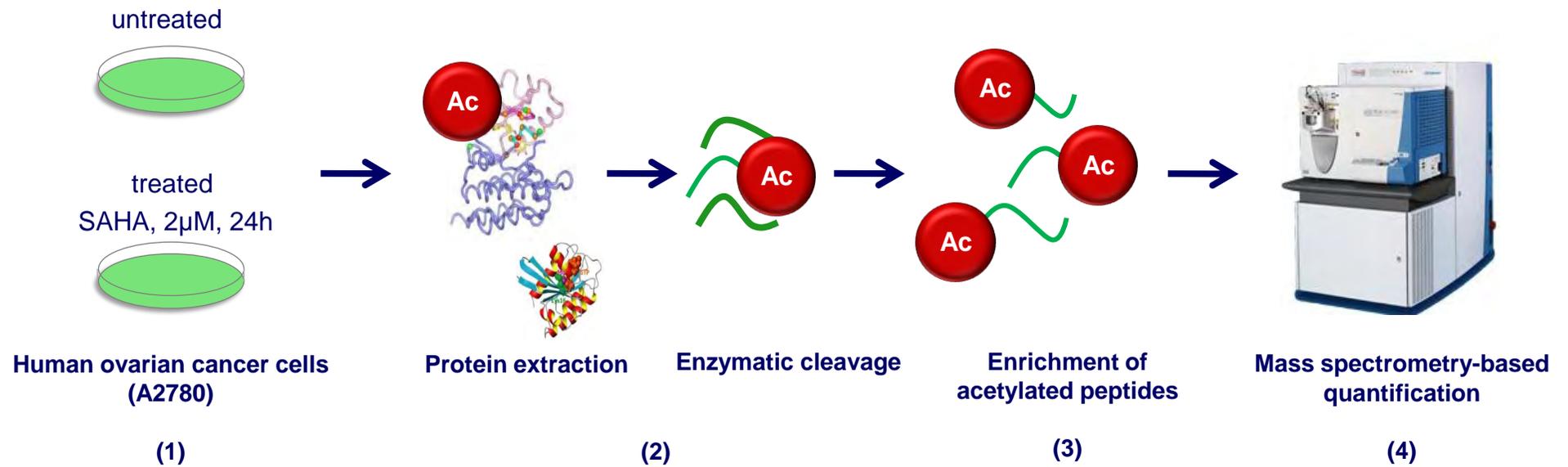
## Mapping of phosphorylation sites



# Outlook

Extending global analysis on other PTM's

## Acetylomics:



# Acknowledgements

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- Klaus Godl
- Stefan Müller
- Jutta Fritz
- Andreas Tebbe
- Henrik Daub
- Martin Klammer



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**Thank you for your attention!**

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