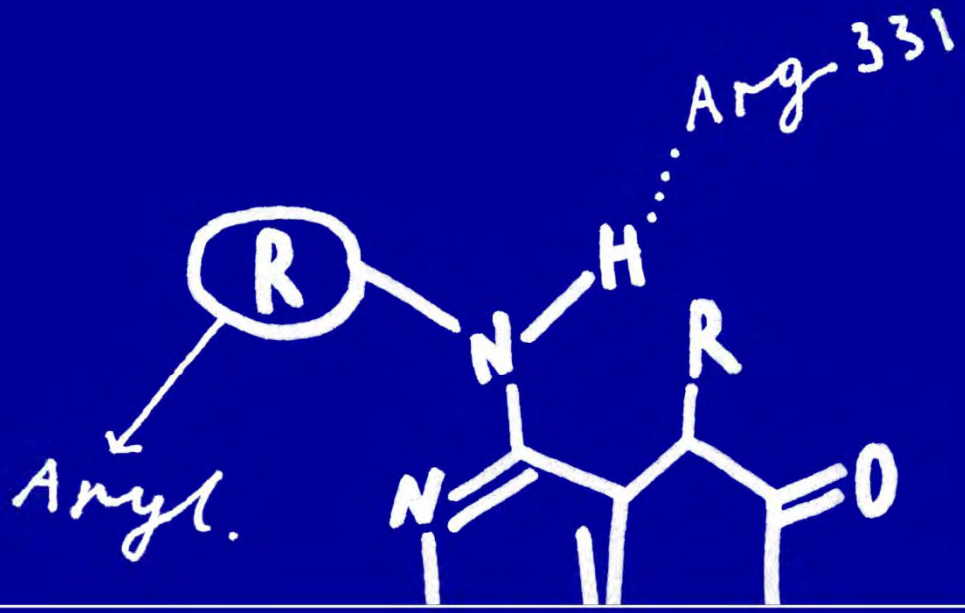
evotec

'RESEARCH NEVER STOPS'

Building innovative drug discovery alliances

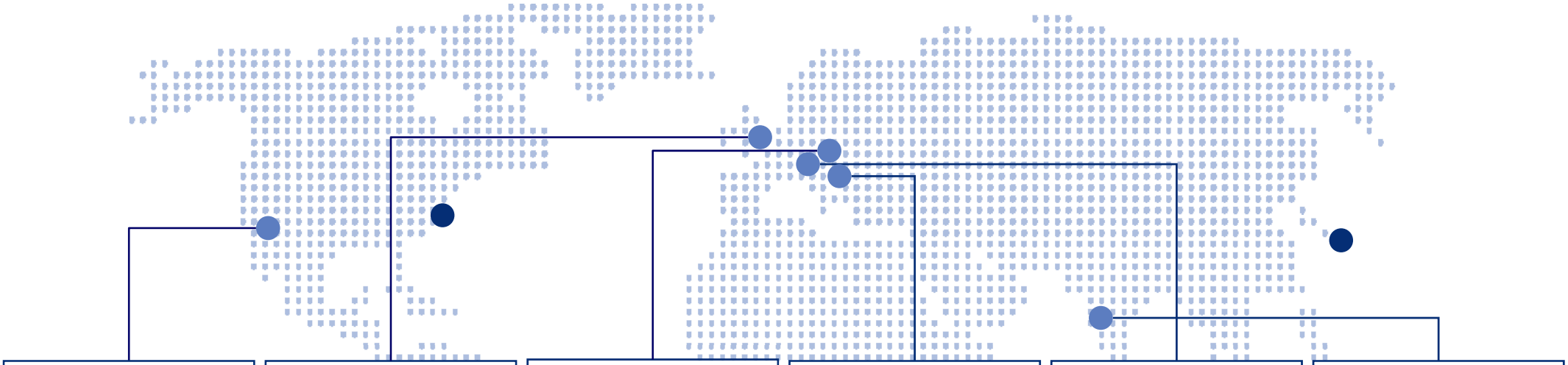
Mode of action analysis and biomarker discovery by phospho-proteomics



Evotec AG, May 15th 2013

Global reach for global projects – more than 600 employees

Evotec worldwide



San Francisco, US
~30 employees
- Compound Procurement
- Compound QC and storage

Abingdon, UK
~215 employees
- Medicinal chemistry
- ADMET
- Structural biology

Hamburg, Germany
~200 employees
- Screening
- HTS,NMR
- *in vitro & in vivo* biology

Munich, Germany
~25 employees
- Phospho-proteomics
- Chemical proteomics

Göttingen, Germany
~40 employees
- Metabolics
- Regenerative Medicine

Thane, India
~130 employees
- Library synthesis & mgmt.
- Development chemistry

● Sales representation (Boston, Tokyo)
● Operations & sales representation

About Evotec Munich

A leader in chemical proteomics and phosphoproteomics

Evotec Munich

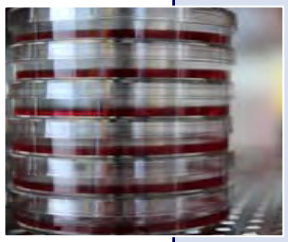
Prof. Dr. Axel Ullrich, Max-Planck Director

- **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 developed by leading proteomics scientists such as Dr. Henrik Daub, Evotec Munich's SVP Technology & Science
- 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

AstraZeneca, Daiichi-Sankyo, Roche, Bristol-Myers Squibb, UCB, Johnson & Johnson, Eisai, Takeda Pharma, Boehringer Ingelheim, Bayer HealthCare

Technology Overview

Cellular Target Profiling® and Mode-of-Action Studies



Cellular Target Profiling®

- Peer-reviewed chemical proteomics technology to both identify and quantify interactions with cellular compound targets

1. **Cellular compound selectivity analysis in a native context**
2. **Target deconvolution of hit compounds from phenotypic screens**

Mode-of-Action Studies

- Quantitative and unbiased analysis of protein modification and expression on a proteome-wide scale
- High-end quantitative mass spectrometry to monitor 10,000+ phosphorylation sites, 1,000+ acetylation sites or 6,000+ proteins, e. g. upon drug treatment

3. ***In vivo* mode-of-action analysis in cells, tissues or patient samples**
4. **Discovery of biomarkers candidates**

Evotec Munich infrastructure

High-end mass spectrometry equipment and proprietary software tools

2 Agilent 1200

5 Easy nLCs

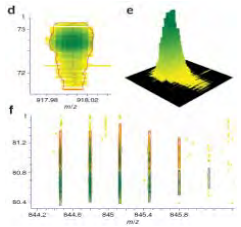
High resolution columns

Column ovens

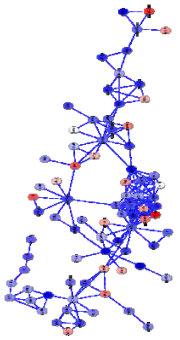
1 LTQ-Orbitrap Discovery
4 LTQ-Orbitrap Velos

1 TSQ Vantage

1 Q Exactive



Max Quant software



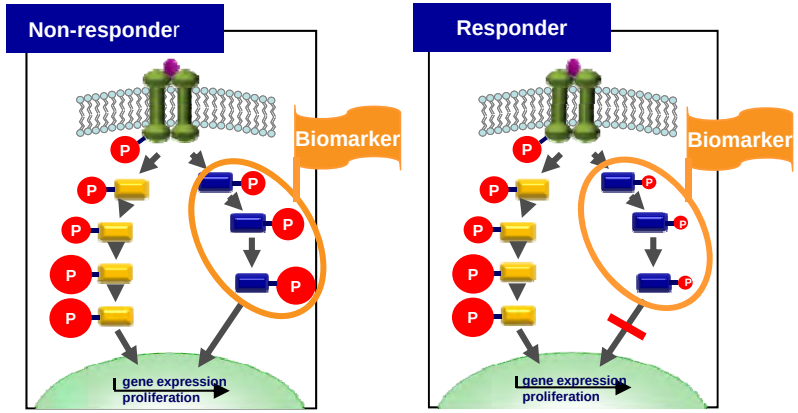
Proprietary data mining tools

Chromatography | Interfaces | Mass Spectrometry | Data Analysis and Interpretation

Quantitative proteomics and *in vivo* PTM analysis

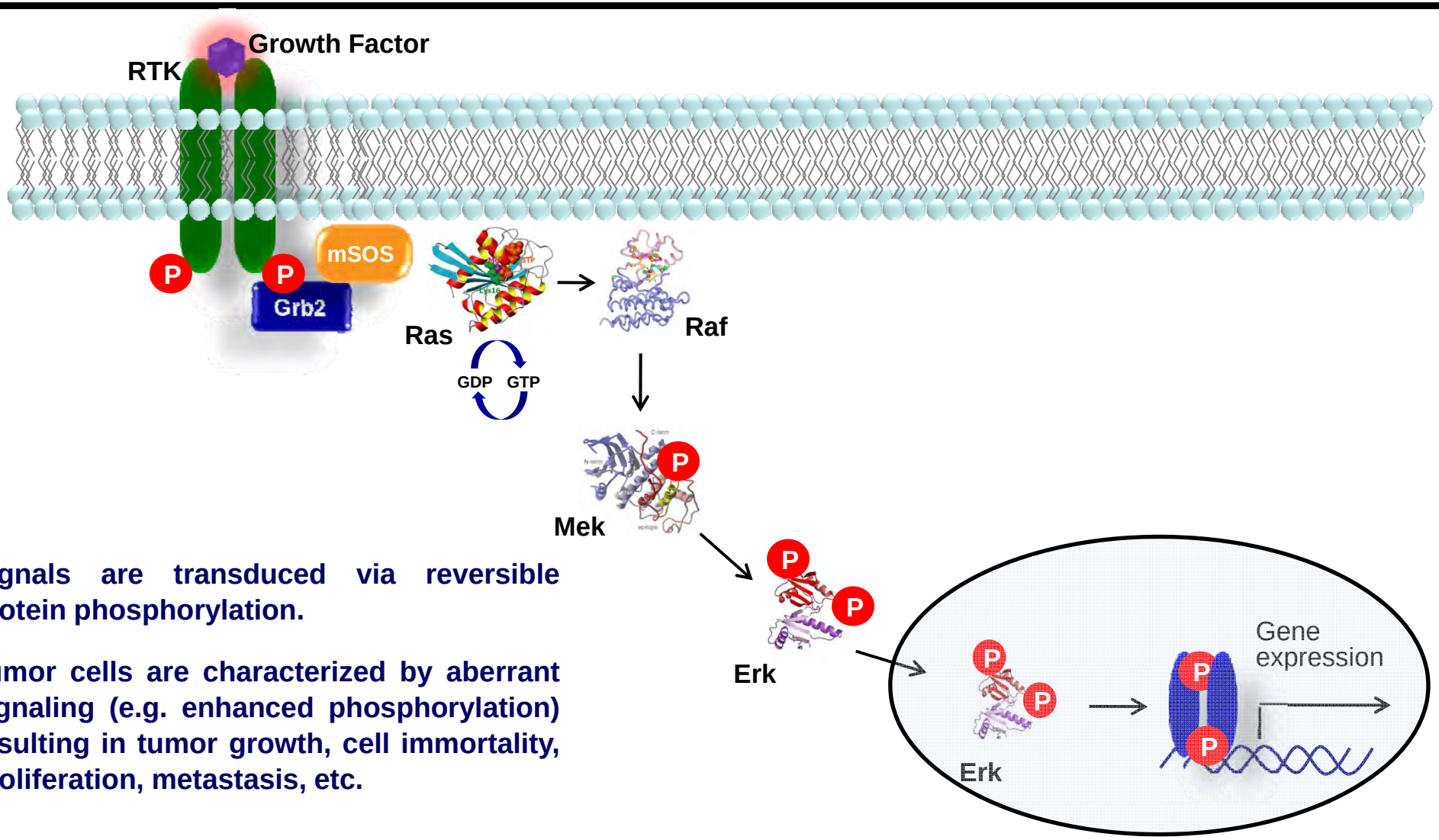
Mode of Action analysis of targeted drugs & biomarker identification

- High-end mass spectrometry and software applications enable comprehensive quantitative analyses of the **proteome** or protein modifications such as **phosphorylation** or **acetylation** in living cells, tissues, or patient samples
- Monitoring of global protein expression changes and signaling pathway regulation to determine the influence of drug treatment, disease state or genetic interference on biological systems
- Applications include **mode of action analysis of targeted drugs** and **discovery of biomarkers (protein expression, phosphorylation, acetylation) for patient stratification**



Cellular Signal Transduction

Protein phosphorylation is the key event

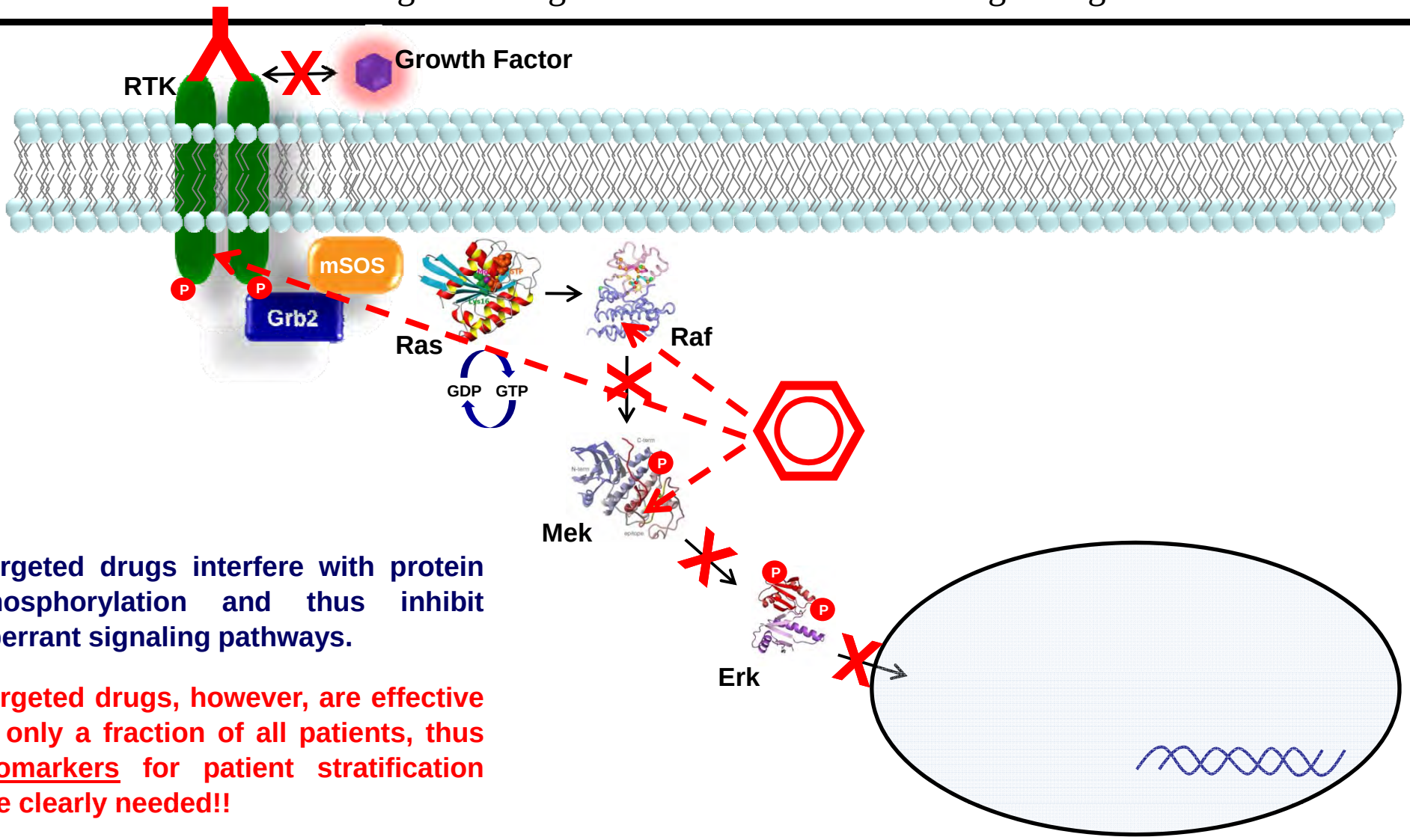


Signals are transduced via reversible protein phosphorylation.

Tumor cells are characterized by aberrant signaling (e.g. enhanced phosphorylation) resulting in tumor growth, cell immortality, proliferation, metastasis, etc.

Cellular Signal Transduction

Targeted drugs interfere with aberrant signaling



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

Targeted drugs, however, are effective in only a fraction of all patients, thus biomarkers for patient stratification are clearly needed!!

Biomarker Study for Dasatinib (Sprycel®)

Identification of a response prediction marker in NSCLC cell lines

Rationale

- 342,000 deaths from lung cancer (20% of all cancer deaths) in Europe (2008), with 85% of all lung cancer incidences are non-small cell lung cancer (**NSCLC**).
- Recent clinical studies showed clinical activity of **dasatinib** (targeting BCR/ABL, Src kinases, ephrin receptors and PDGFRβ) in NSCLC patients.
- Neither Src activation nor EGFR or K-Ras mutation could predict response to dasatinib treatment.
- Can we identify a phosphorylation signature that predicts response to dasatinib treatment in NSCLC?

Quantitative & global unbiased analysis of basal cellular protein phosphorylation of a NSCLC cell line panel

The Cell Line Panel

Response prediction in 19 NSCLC cell culture models

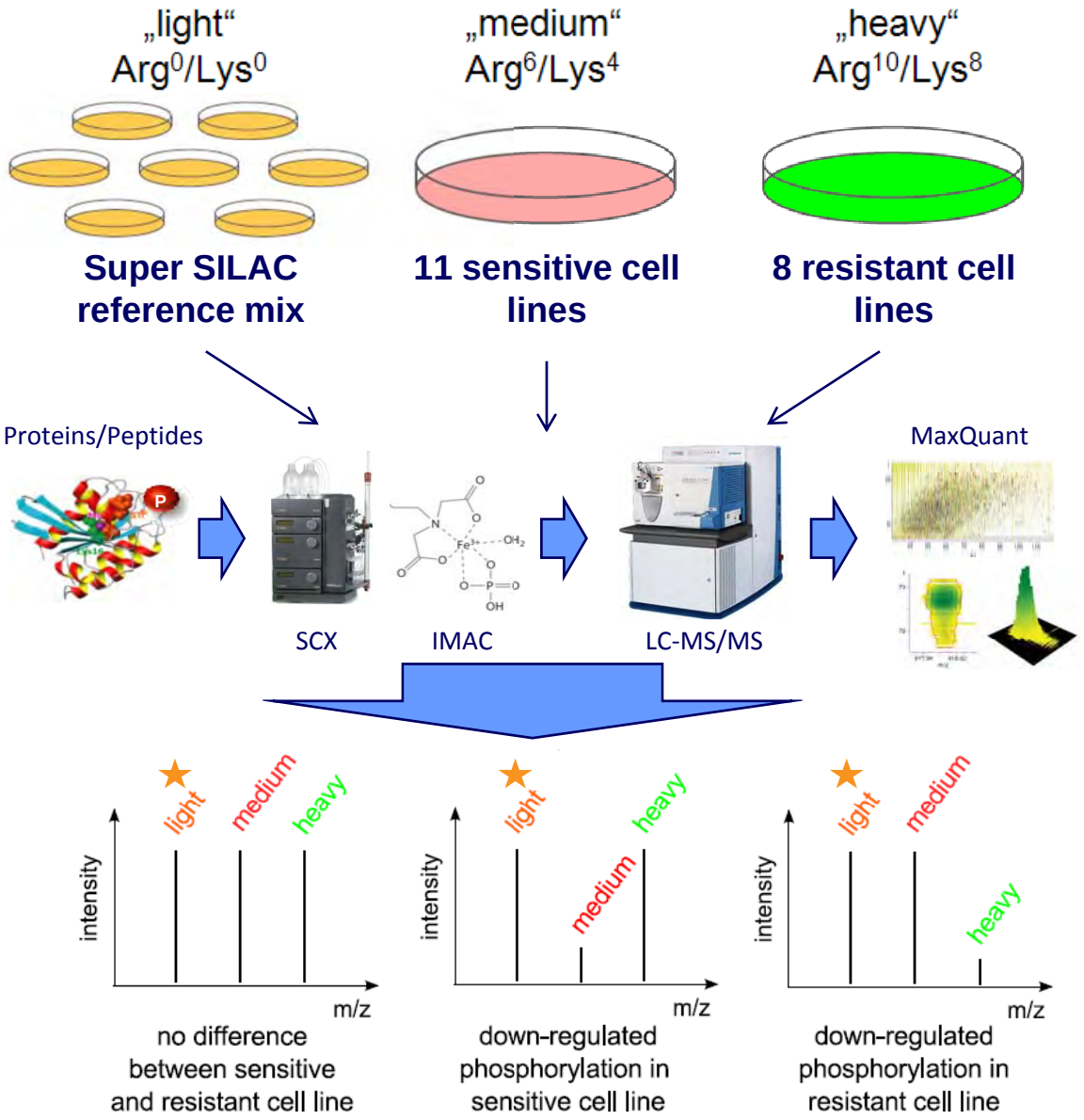
#	Cell Line	Indication	Origin	Supplier No	TP53 mutation description	IC50 (µM) Sos et al.	IC50 (µM) this study	Group
1	HCC366	NSCLC	DSMZ	ACC 492	-	0.482	0.017	+
2	PC9	NSCLC	MPI for Neurological Research		c.743G>A; Arg->Gln; p.R248Q	0.4603	0.02	+
3	H2030	NSCLC	ATCC	CRL-5914	c.785G>T; Gly->Val; p.G262V	0.1183	0.022	+
4	HCC827	NSCLC	ATCC	CRL-2868	-	0.1456	0.033	+
5	HCC2279	NSCLC	MPI for Neurological Research		c.701A>G; Tyr->Cys; p.Y234C	0.139	0.045	+
6	LouNH91	NSCLC	DSMZ	ACC 393	-	0.113	0.068	+
7	H1666	NSCLC	ATCC	CRL-5885	WT	0.175	0.076	+
8	H1648	NSCLC	ATCC	CRL-5882	c.102_103ins1; Leu->?; p.?	0.0593	0.079	+
9	H2009	NSCLC	ATCC	CRL-5911	c.818G>T; Arg->Leu; p.R273L	0.7465	0.085	+
10	H322M	NSCLC	MPI for Neurological Research		c.743G>T; Arg->Leu; p.R248L	0.0819	0.311	+
11	HCC4006	NSCLC	ATCC	CRL-2871	-	0.8376	0.95	+
12	H520	NSCLC	ATCC	HTB-182	c.438G>A; Trp->STOP; p.W146X	11.56	1.43	-
13	H157	NSCLC	MPI for Neurological Research		c.892G>T; Glu->STOP; p.E298X	10.54	2.63	-
14	Calu6	NSCLC	ATCC	HTB-56	c.586C>T; Arg->STOP; p.R196X	22.54	2.8	-
15	H460	NSCLC	ATCC	HTB-177	WT	24.16	3.9	-
16	H1395	NSCLC	ATCC	CRL-5868	WT	31.12	4.7	-
17	H2077	NSCLC	MPI for Neurological Research		-	10.07	4.75	-
18	H2172	NSCLC	ATCC	CRL-5930	-	16.71	5.85	-
19	HCC78	NSCLC	DSMZ	ACC 563	-	13.9	17.05	-

responsive

non-responsive

The General Workflow

... applying the Super-SILAC strategy

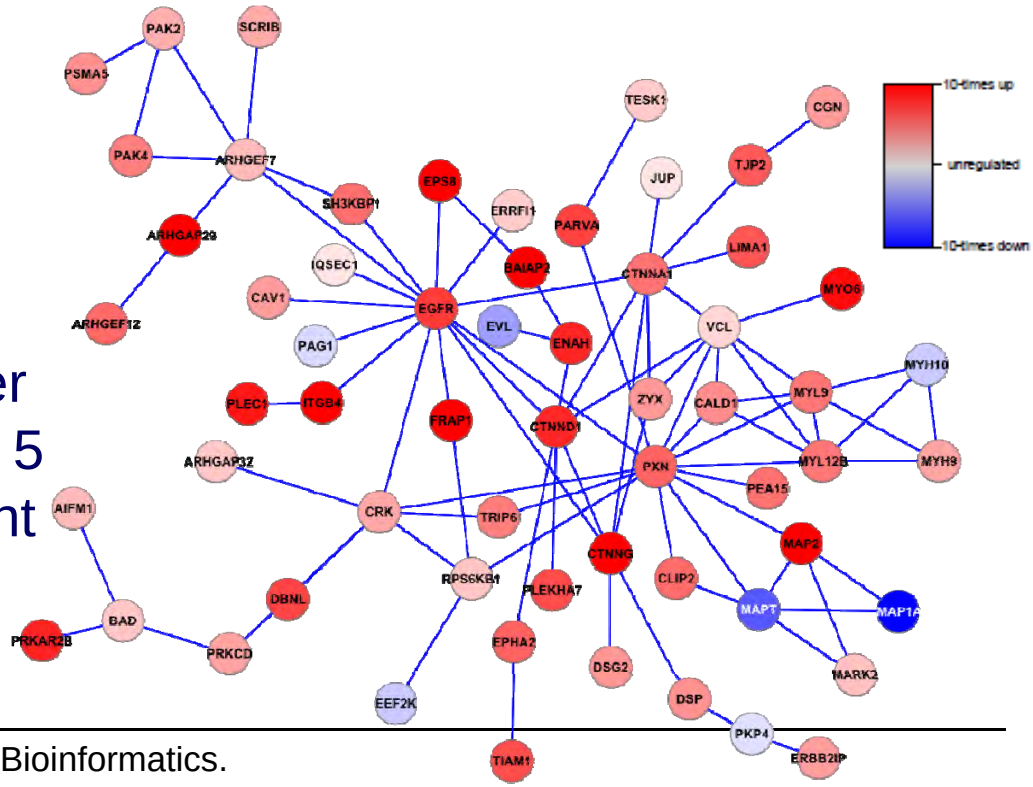


Geiger *et al.*, Nat Methods. 2010;7(5):383-5

General Proteomics Results

... identified phosphorylation sites

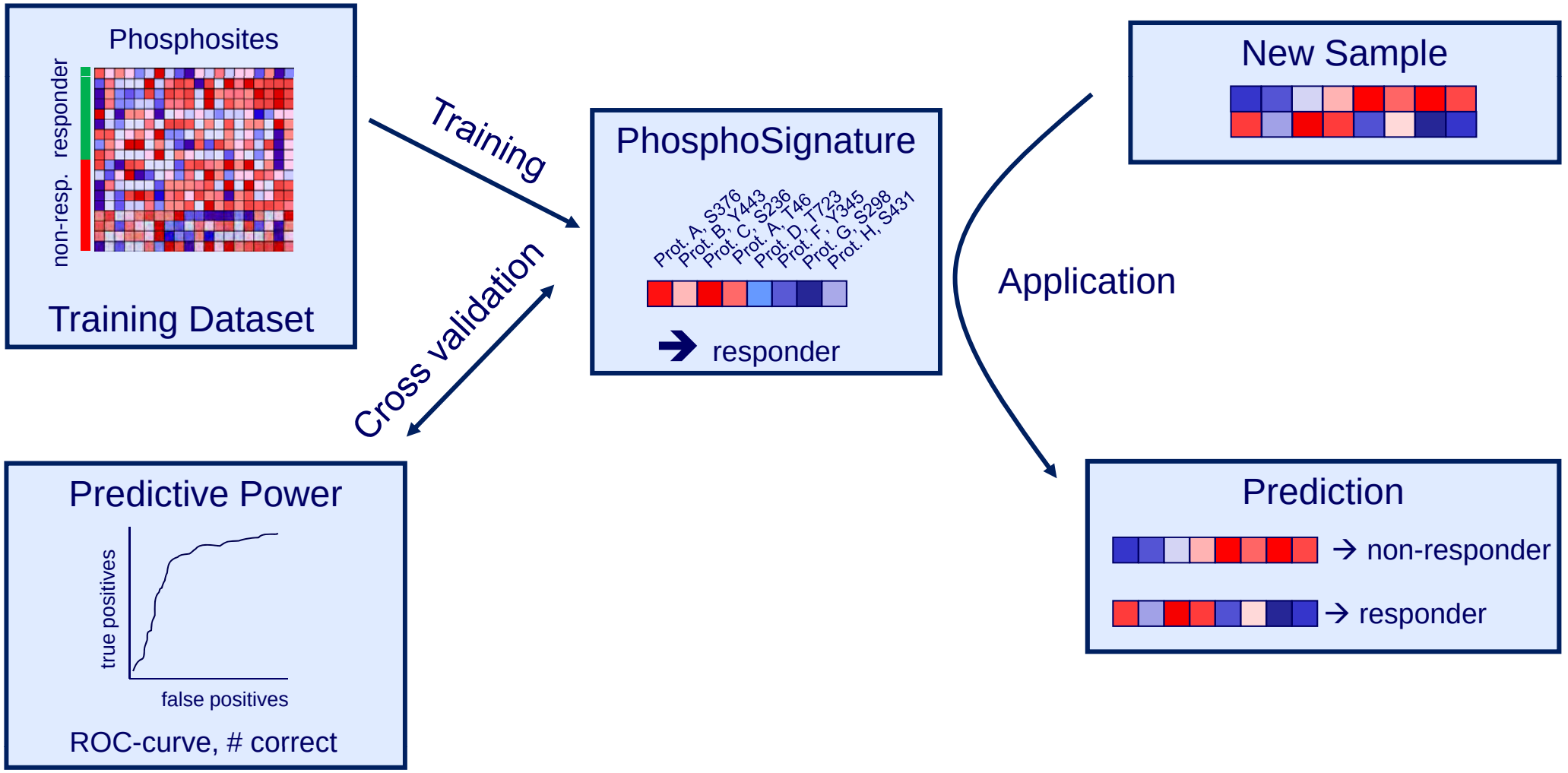
- 34,747 P-sites identified, 88% having a cell line to Super-SILAC ratio < 4
- 83.2% serine, 15.3% threonine and 1.5% tyrosine phosphorylations
- 25,020 P-sites were rated to be class-I (localization probability > 75%)
- 58 P-sites were found to be differentially regulated between sensitive and resistant cell lines
- 53 P-sites (91%) were found to be stronger phosphorylated in sensitive cell lines, only 5 P-sites stronger phosphorylated in resistant cell lines



Klammer *et al.*, BMC Bioinformatics. 2010; 28;(11):351

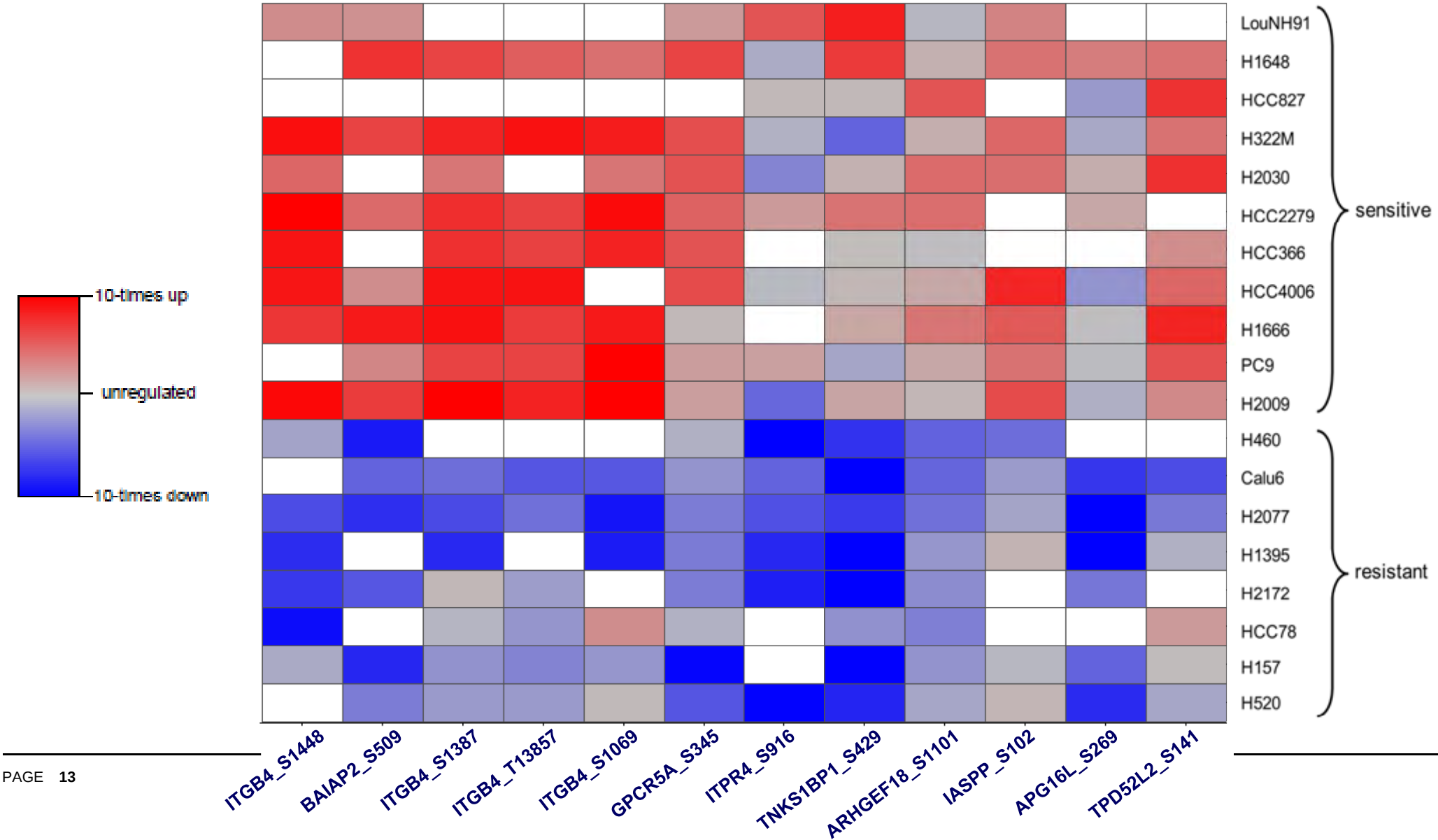
Phosphosignatures as Biomarkers

Statistical identification and validation of the phosphosignature



The Phosphosignature

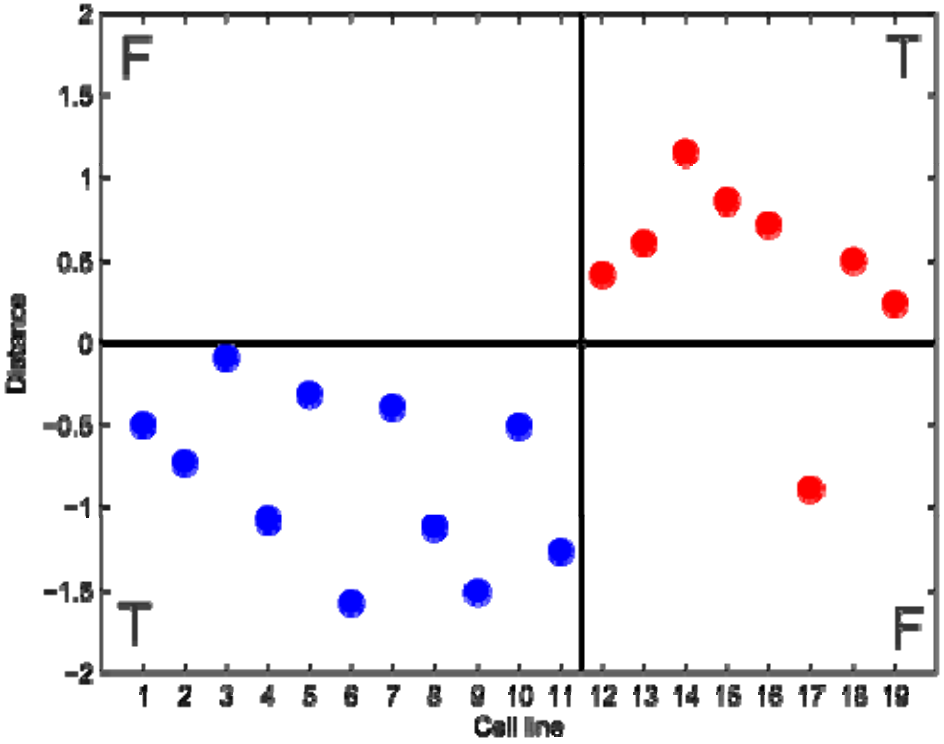
Heat map of the 12 phosphosites across the cell line panel



The Phosphosignature

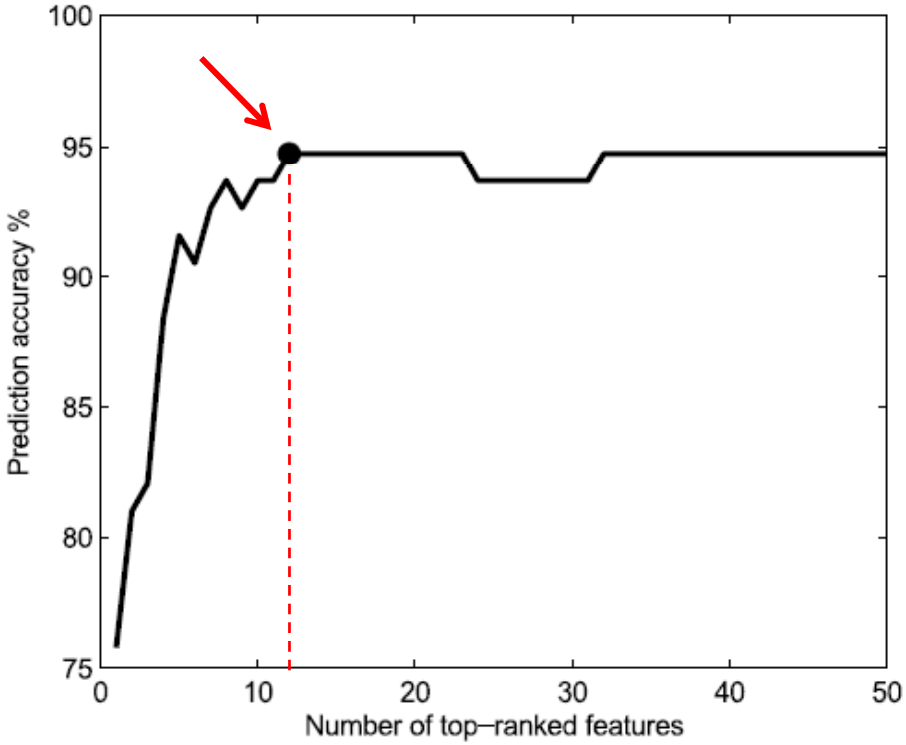
Classification results

Classification results represented by distance to the respective SVM's separating hyperplane.



Phosphosignature separates sensitive and resistant cells with a prediction accuracy of **94%**.

Prediction accuracy depending on the number of features in the phosphosignature.

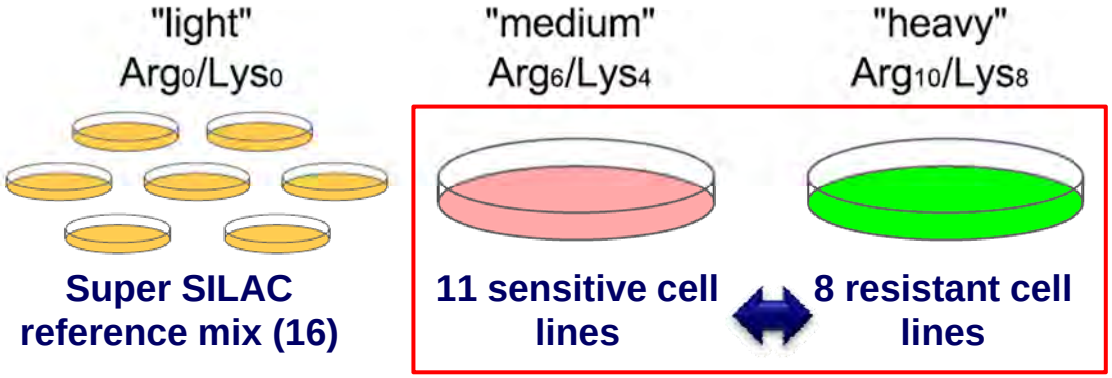


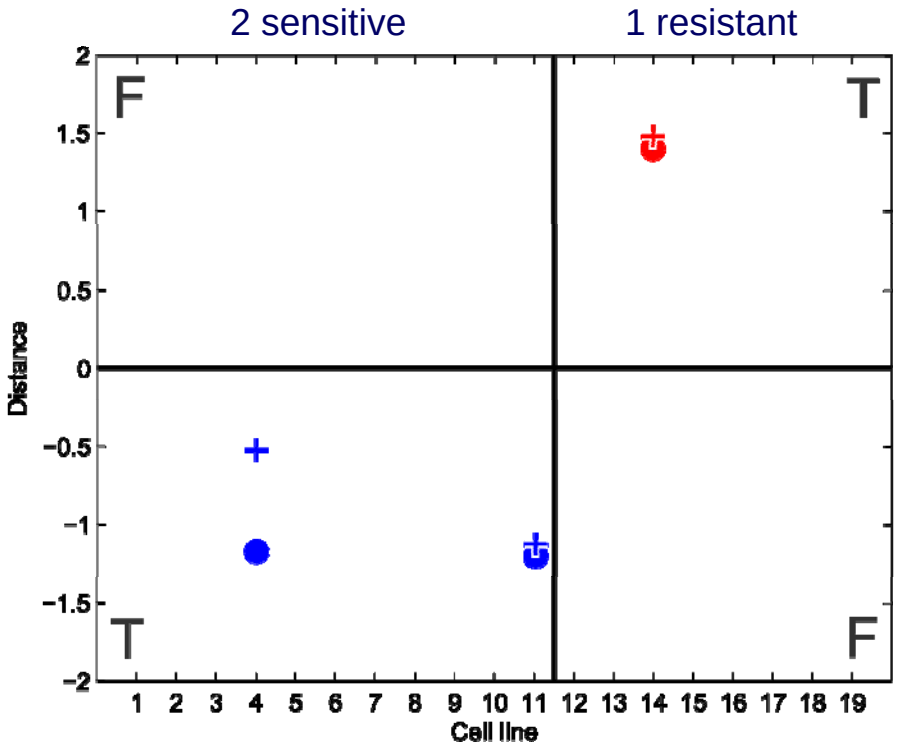
12 features are enough for maximal response prediction.

Phosphosignature Validation

Is the identified signature robust?

Test for SILAC labeling Effects: Label Switch experiment





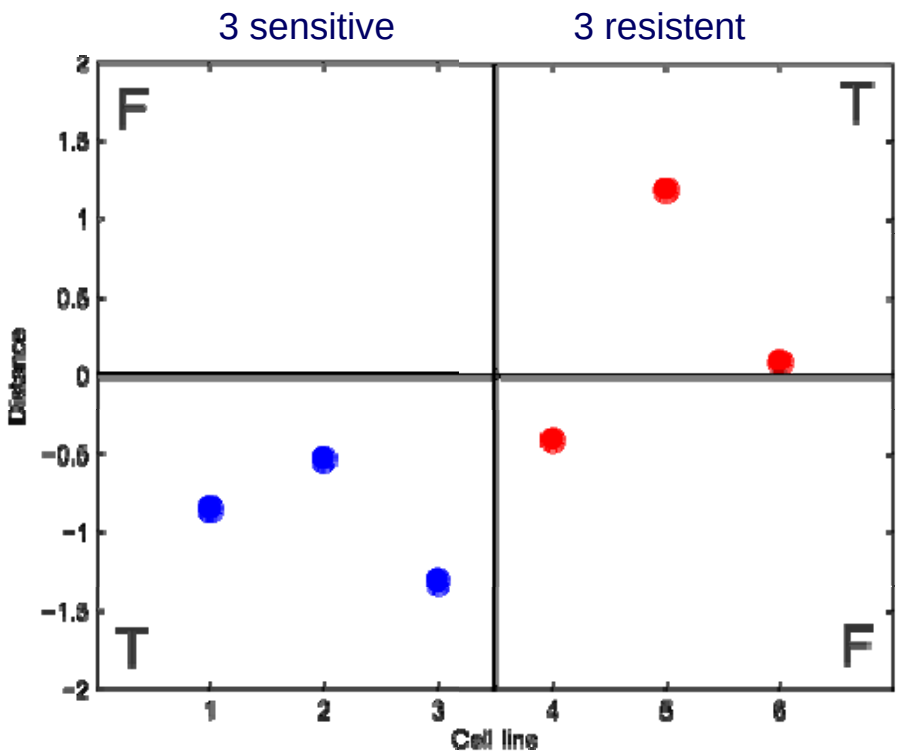
For two of the three label switches, the prediction is identical to the original data. In one case, the difference is somewhat larger, but still correctly classified.

Phosphosignature Validation

Classification results for signature validation

Application of the phosphosignature to a panel of breast cancer cell lines

#	Cell Line	Indicati	(µM) g et al.	IC50 (µM) this paper	Group
1	MDA-MB-231	Breast can	0095	0.036	+
2	HCC1937	Breast can	070	0.082	+
3	BT-20	Breast can	1652	0.497	+
4	BT-549	Breast can	057	1.71	-
5	MDA-MB-468	Breast can	125	2.8	-
6	MCF7	Breast can	.524	3.27	-



5 of the 6 breast cancer cell lines were classified correctly (prediction accuracy of **83%** only one resistant sample was wrongly predicted, indicating the applicability of phosphosignature in other cancer types.

Proteins spanning the Phosphosignature

12 phosphorylation sites on 9 proteins

- Integrin beta-4 (**ITGB4**): cell-cell / cell matrix interaction
- Brain-specific angiogenesis inhibitor 1-associated protein 2 (**BAIAP2**): regulation of actin cytosceleton
- Rho guanine nucleotide exchange factor 18 (**ARHGEF18**): regulation of actin cytosceleton

- RelA-associated inhibitor (**IASPP**): functionally connected to p53
- Retinoic acid-induced protein 3 (**GPRC5A**): functionally connected to p53

- Inositol 1,4,5-trisphosphate receptor type 3 (**ITPR3**): receptor for inositol 1,4,5-trisphosphate, mediating release of intracellular calcium.
- 182 kDa tankyrase-1-binding protein (**TNKS1BP1**): binds to Tankyrase-1/2
- Autophagy-related protein 16-1 (**APG16L**): plays an essential role in autophagy
- Tumor protein D54 (**TPD52L2**): interacts with MAL2

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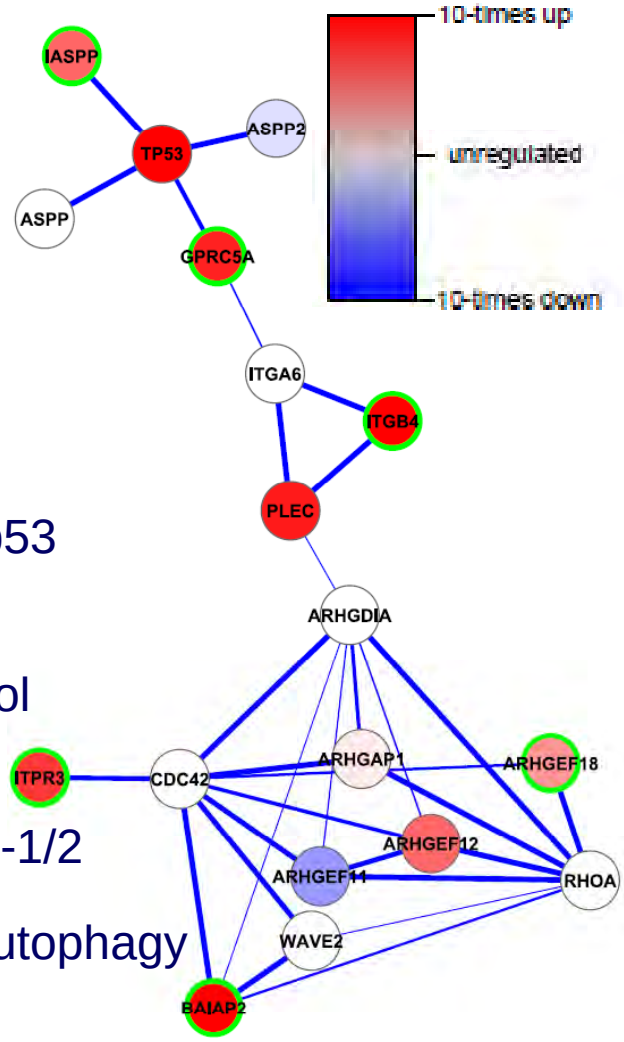
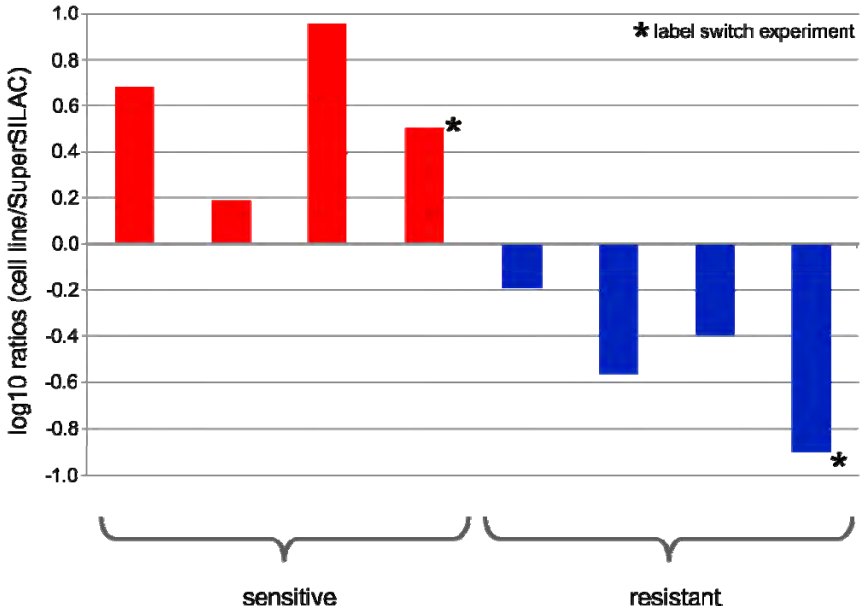


Integrin β-4 (ITGB4)

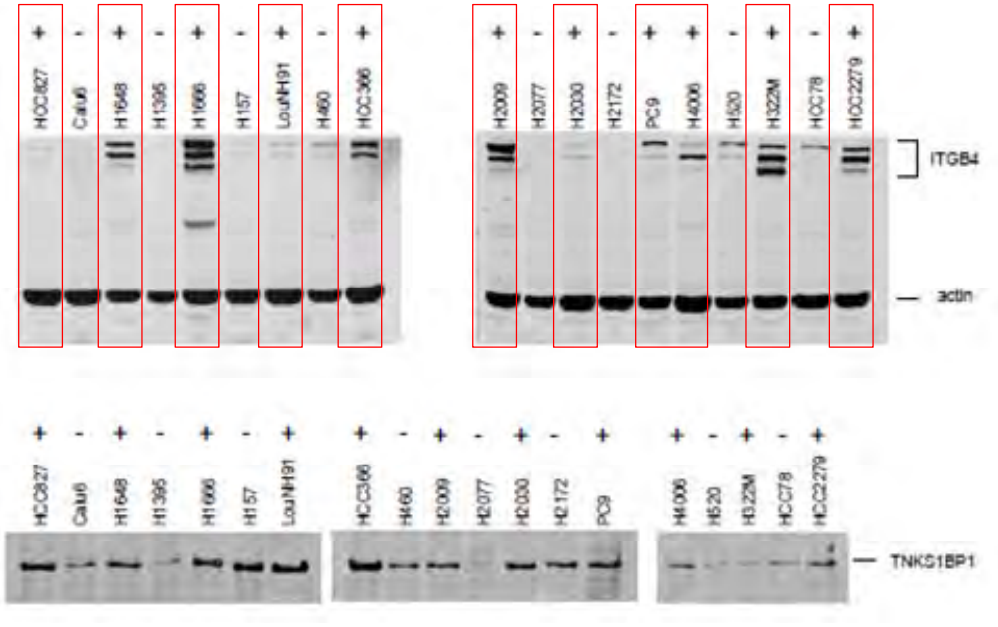
A protein surrogate marker for its phosphorylation

Difference in ITGB4 phosphorylation due to differential protein expression?

MS data (non-phosphopeptides)



Western Blot analysis



- ITGB4 is differentially expressed between responsive and resistant cell lines
- ITGB4 alone classifies 8 of 11 sensitive and all resistant models correctly (84%)

Summary

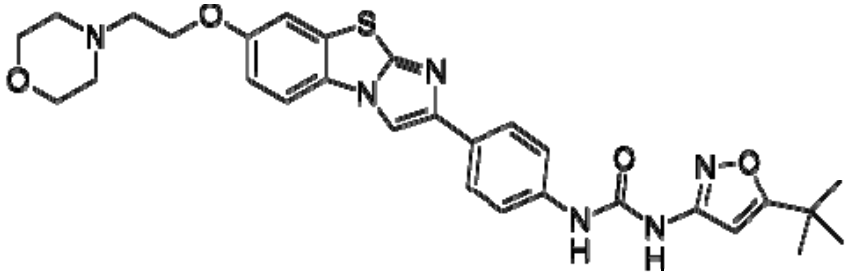
- We successfully identified a response prediction marker from global and unbiased quantitative phosphoproteomics experiments in a preclinical setting.
- The final signature consists of 12 phosphosites located on 9 different proteins.
- The phosphosignature was highly predictive for the sensitivity to treatment with dasatinib in NSCLC as well as breast cancer cell lines.
- These 12 phosphorylations are candidate biomarkers for predicting response in solid tumors to dasatinib.
- Analysis of non-phosphorylated peptides and western blot analysis showed that the protein expression of ITGB4 is likely to be predictive for sensitivity to dasatinib treatment as well.
- Validation of the phosphosignature in the clinic will prove general applicability

Biomarker discovery in Patient Samples

Predictive Phospho-Signatures

Global Analysis of the Phosphoproteome of Human Blasts Reveals Predictive Phosphorylation Markers for the Treatment of Acute Myeloid Leukemia with AC220

- Pathway activation as biomarker for kinase inhibitors
- Successful discovery of phospho-signature for dasatinib in NSCLC cell lines
- Here: application to discovery from clinical samples
- AC220 (Quizartinib): selective FLT3 inhibitor

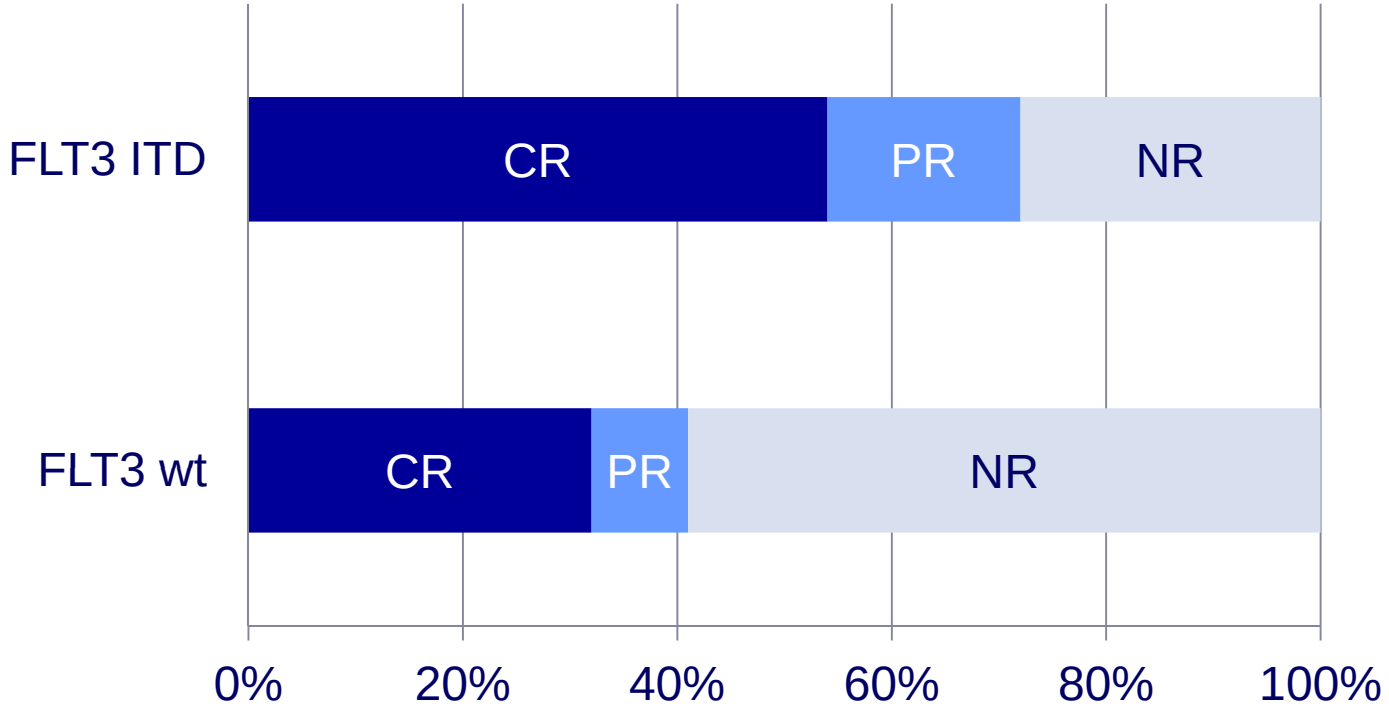


Klammer , M. et al., MCP 11 (2012) 651
Cortes, J., Abstract #41; Levis, M: Abstract #673

Biomarker discovery in Patient Samples

Predictive Phospho-Signatures

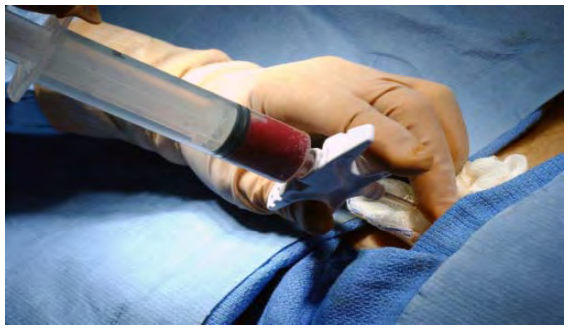
Phase II trial (ACE) for mono therapy in AML just completed:



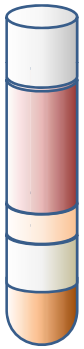
- CR: complete responder
- PR: partial responder
- NR: non responder

Phosphoproteomics Workflow

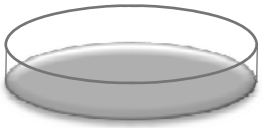
Preparation and MS-Analysis of AML Cells



Ficoll



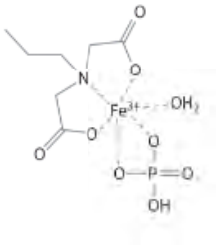
Blasts



Spike-in SILAC reference

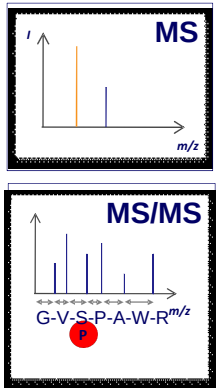
Trypsin digestion

SCX chromatography (12 fractions)



IMAC

LC-MS/MS



MaxQuant

Schaab, Pfeifer et al: „Global Phosphoproteome Analysis of Bone Marrow Samples from Patients with Acute Myeloid Leukemia“, MCP submitted

AML patient sample phosphoproteomics

Phosphoproteome Stability

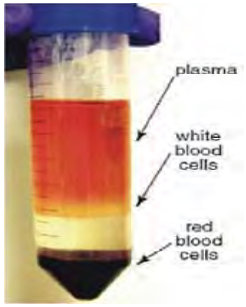
Bone marrow aspirate of AML patient

24h

7h

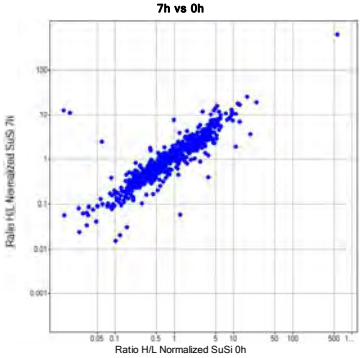
0h

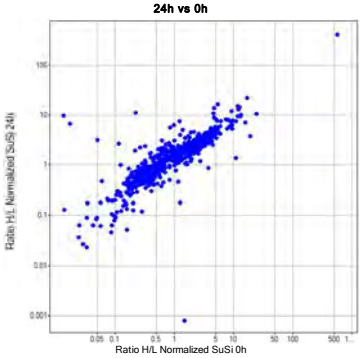
at room temperature



Ficoll

Storage of vital cells in liquid nitrogen (wash in PBS, add DMSO, FCS)





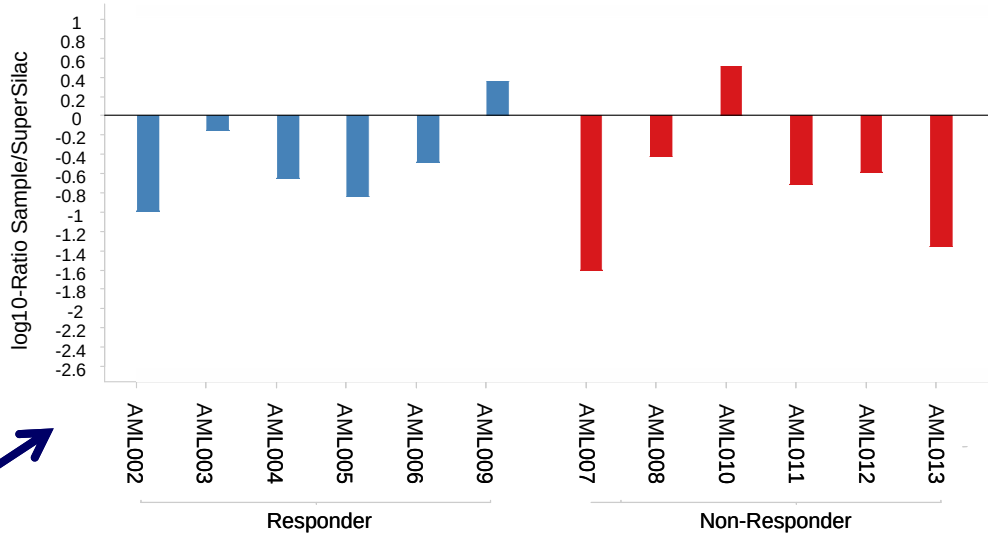
- On average ~4.500 phosphorylation sites from less than 500 µg protein
- Super-SILAC quantification allows for ~99% coverage, with 71% of phosphosite ratios in the range between 0.2 and 5
- Super-SILAC quantification indicates phosphoproteome stability at RT

Sample Collections

Training and Validation Samples

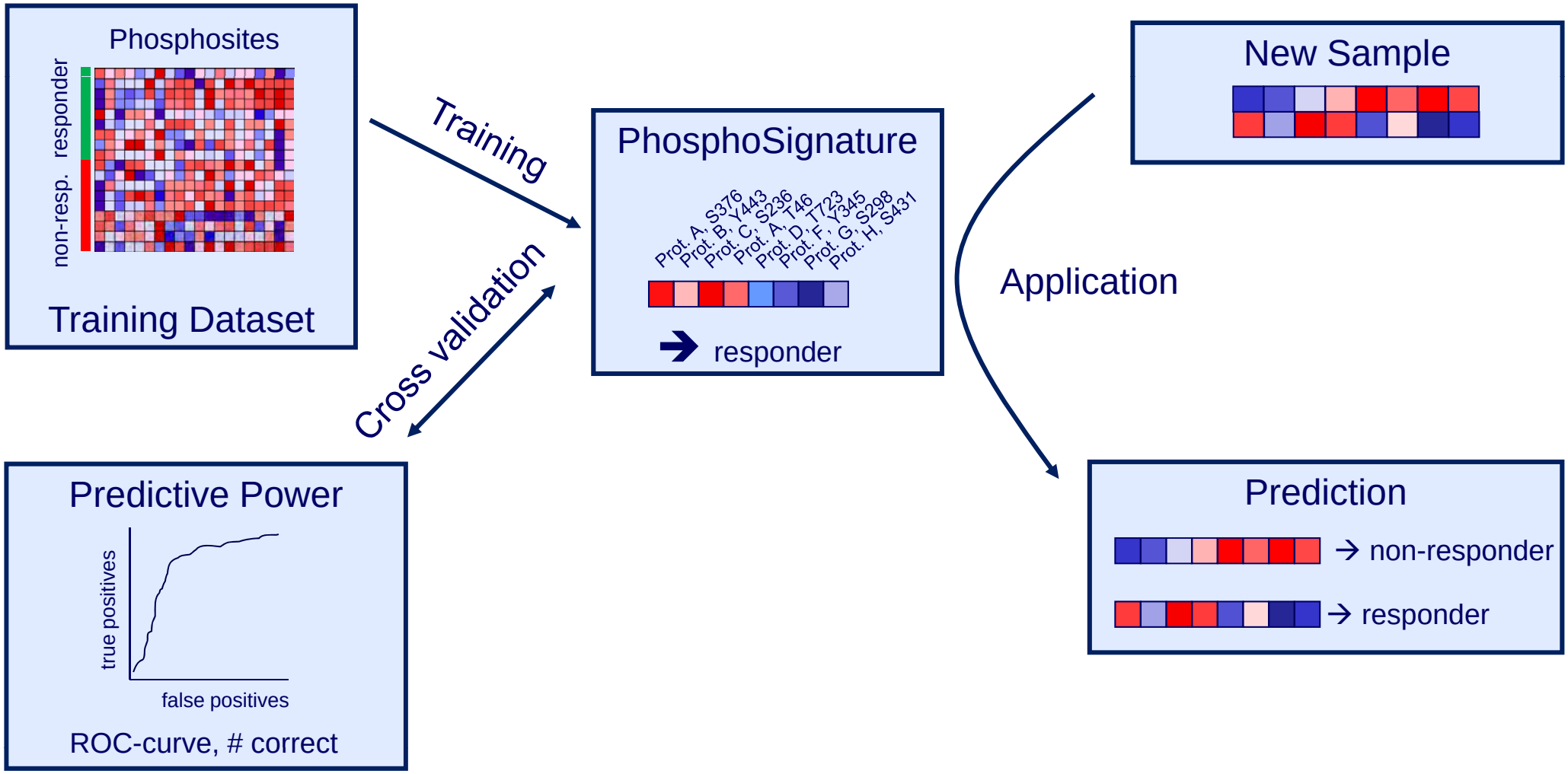
Collection	Responder	Non-Responder
Training	6	6
Validation	6	3

- Pre-treatment samples
- FLT3-ITD positive
- Responder := CR + PR
- ~ 300 µg protein per sample extracted on average
- FLT3 phosphorylation of Y694/ Y699 on STAT5A/ STAT5B is **not predictive** for AC220 response



Biomarker Discovery Workflow

Training and Validation



Phospho-Signature

Identification in Training Data Set

- Detection of 13,236 phosphorylation sites; 7,831 class-I sites
- No predictive markers found on proteins involved in FLT3-pathway

- Selection of 5 features:

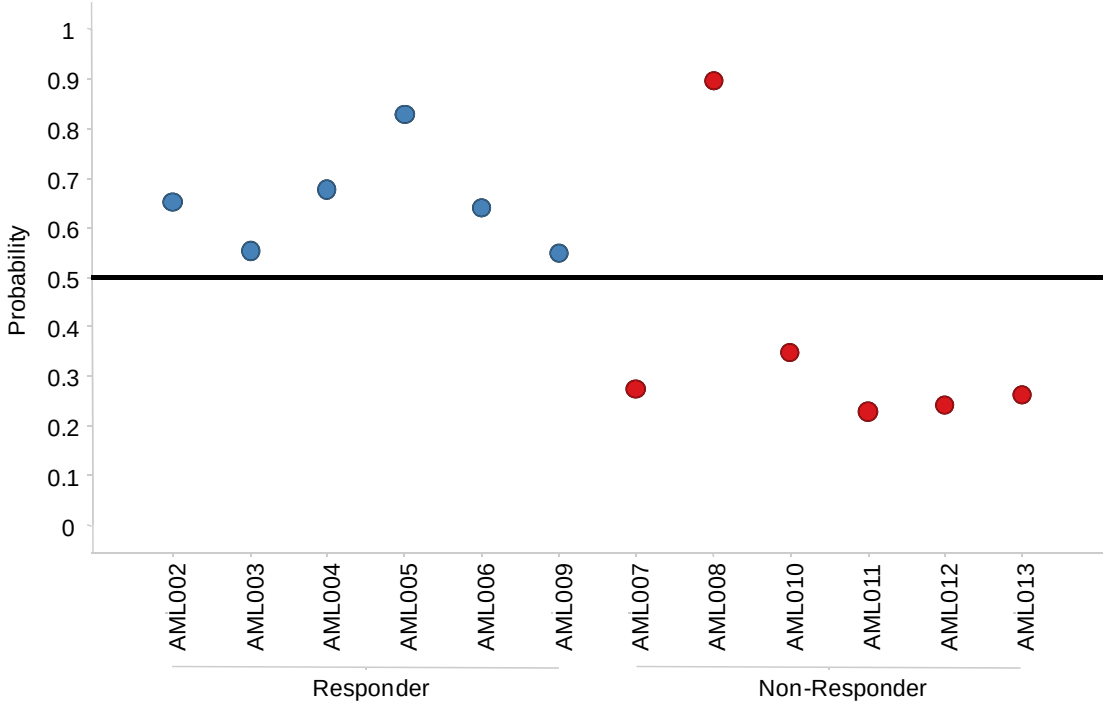
Uniprot id	Gene name	Site	Protein Name	Diff (Log10)	SV weight
Q7L9B9	EEPD1	S160	endonuclease/exonuclease/phosphatase family domain-containing protein 1	-1.05	-0.75
Q9H165	BCL11A	S630	B-cell lymphoma/leukemia 11A	-0.68	-0.54
Q9H6Z4	RANBP3	S333	Ran-binding protein 3	-0.94	-0.31
Q92834	RP3	S961	x-linked retinitis pigmentosa GTPase regulator	0.64	+0.88
P02545	LMN1	S458	Lamins A/C	-0.76	-0.75

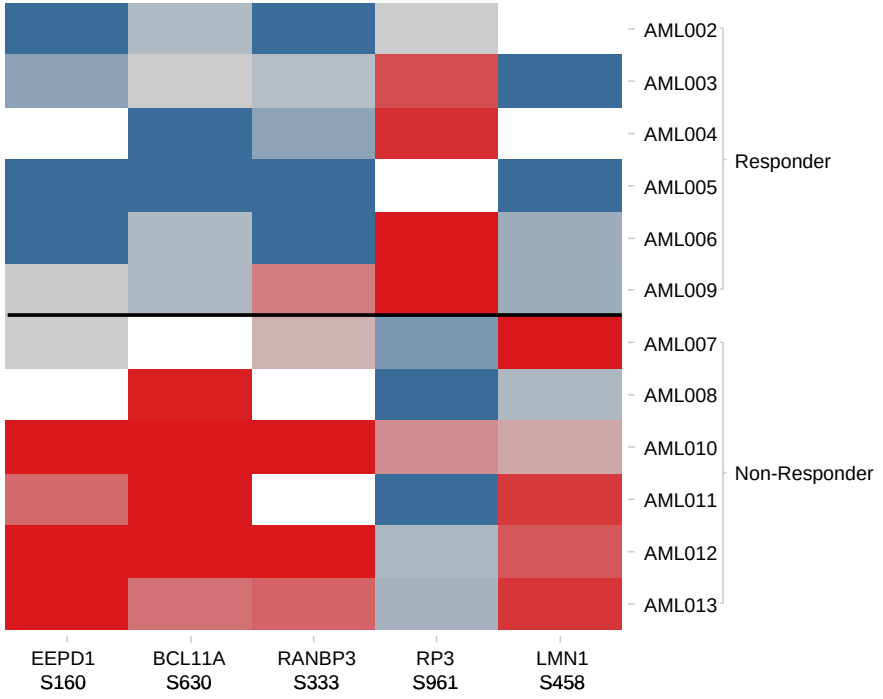
Klammer, M. et al. (2012). "Identification of Significant Features by a Global Mean Rank Test." Bioinformatics, submitted
Abeel, T. et al. (2010). Bioinformatics 26(3): 392-398

Cross-Validation

High accuracy on training samples

- Leave-One-Out Cross-Validation (feature selection and SVM training)
  → Accuracy: 92%
- AML008: marked reduction of marrow blasts (from 95% to 5-10%), but 5-10% circulating blasts → stable disease





Validation

Independent Validation Collection

- Two misclassifications: AML031 and AML033
- AML033: FLT3-ITD positive cells were sensitive, patient progressed with FLT3-wt clone
- Accuracy 78% or 88% (without AML033)

Probe	Quelle	Ansprech-verhalten	Vorhersage
AML014	Baltimore	CRi	responder
AML020	Baltimore	CRi	responder
AML025	Baltimore	NR	non-responder
AML030	Philadelphia	CRp	responder
AML031	Philadelphia	CRi	non-responder
AML032	Philadelphia	CRi	responder
AML033	Philadelphia	SD	responder
AML034	Philadelphia	NR	non-responder
AML035	Philadelphia	CRi	responder

Conclusions

- A global and unbiased quantitative phosphoproteomics approach was successfully performed on human blasts
- ~4,600 phosphorylation sites can be identified from $2x10^7$ cells
- Translation to targeted platform (MRM, immuno-based assay)
- Signature of 5 phosphorylation markers predict response for AC220 (accuracy 80-90%)

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