Unbiased validation of degrader drug neosubstrates by high-sensitivity slice-PASEF mediated global ubiquitinomics



Martin Steger^{1,2}, Vadim Demichev³, Uli Ohmayer¹, Bjoern Schwalb¹, Jutta Fritz¹ and Henrik Daub¹

¹ NEOsphere Biotechnologies GmbH, Martinsried, Germany ² Max-Planck-Institute of Biochemistry, Martinsried, Germany ³Institute of Biochemistry, Charite, Berlin

Targeted protein degradation (TPD) has emerged as a transformative approach to eliminate disease-causing proteins. Mass spectrometry (MS)-based proteomics is particularly attractive for the unbiased discovery of novel molecular glues (and glue targets), since rational design towards a determined target of these drugs is challenging and their target spectrum cannot be predicted *in silico*. Recent advances in both MS hard- and software, and the development of new scan modes such as dia-PASEF and slice-PASEF, have made MS- based proteomics screening technological improvements for screenings of large libraries of degrader drugs against a large fraction of the expressed cellular proteome. Deep proteomic screening involves semi-automated cell treatments and MS sample preparation, single-shot MS runs and data acquisition by dia-PASEF, raw data processing by DIA-NN and further statistical analyses using an in-house programmed biostatistical data analysis pipeline. Using this workflow, we quantify 10-11,000 proteins in human cell lines treated with degrader drugs and we classify neosubstrate candidates according to their likelihood of being primary drug targets. Such candidates are then followed up by a series of unbiased, MS-based validation approaches, such as affinity-enrichment (AE)-MS, proximity-labeling MS and global ubiquitinomics. (K- GG remnant peptide profiling). To demonstrate target ubiquitination by degrader drugs, we developed a ubiquitinatinomics workflow, which we coupled with slice-PASEF MS to maximize quantitative precision and coverage especially for low input samples.

NEOsphere's pipeline for screening of degrader drugs

Cell treatment

• Cell treatments in 96-well plates • Semi-automated sample preparation Scalable to efficiently screen entire degrader libraries of 10,000s of compounds at unparalleled turnaround times

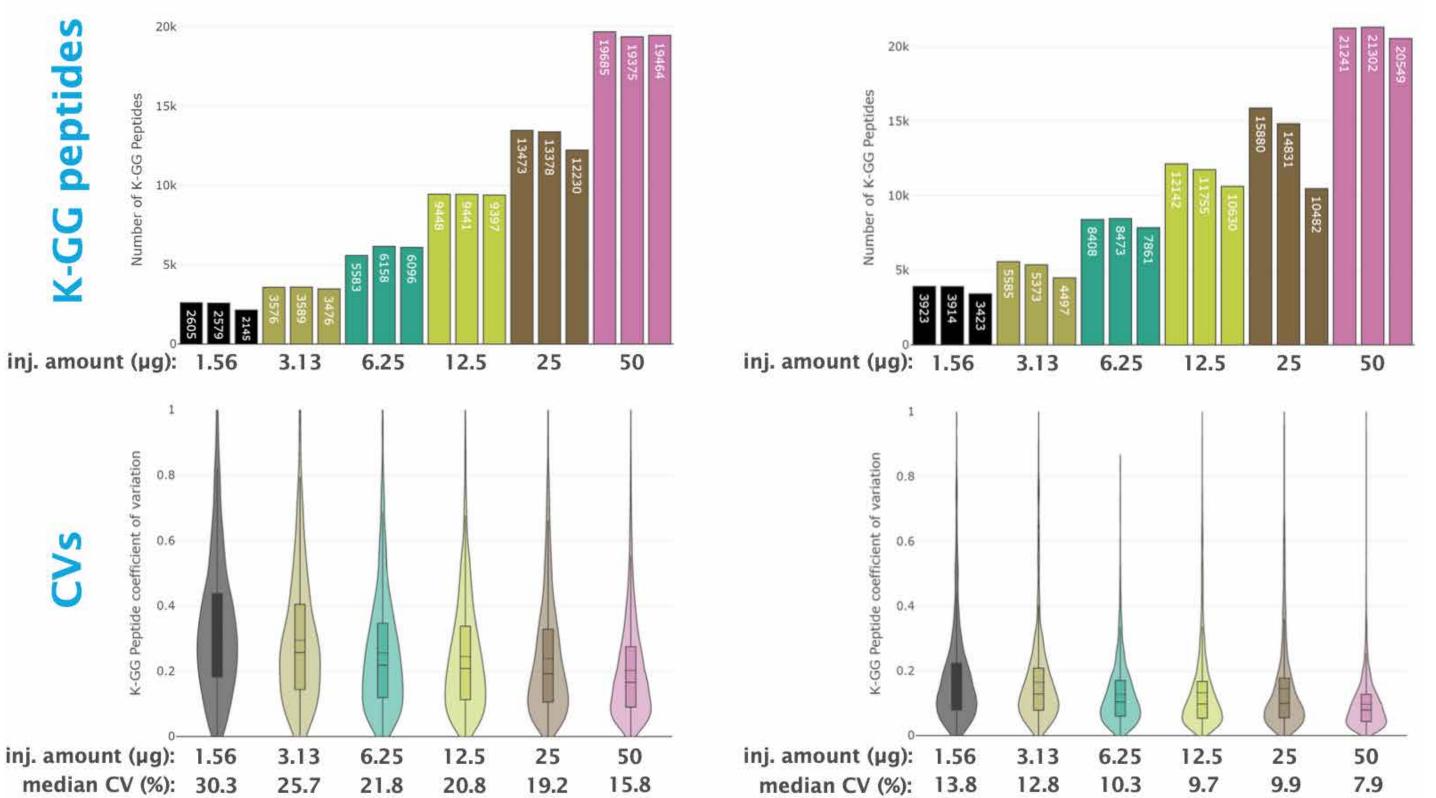
Ultra-sensitive detection and precise quantification of K-GG remnant peptides using slice-PASEF

dia-PASEF



and automated sample preparation

- Highly optimized dia-PASEF methods and in-house produced LC columns • Screening-compatible. Quantification of up to 11,000 protein groups (~10,500 genes) per sample (18 SPD)
- Data completeness of >99% at protein group level
- Highest precision. Coefficients of variation (CVs) of ~5% in screening mode
- Data processing using DIA-NN for highest coverage, precision and data completeness
- Custom-made statistical analysis pipeline to **maximize the yield of putative neosubstrates**, through optimized filtering, normalization and batch correction modules
 - Reviewing of data and scoring of proteins to identify putative direct degrader targets
- Pharmacological and genetic validation of E3 ligase and ubiquitin-proteasome system dependency
- Detection of degrader-induced E3 ligase binding by interactomics (affinity-enrichment (AE)-MS and cellular proximity labeling)
- slice-PASEF powered single-shot ubiquitinomics to demonstrate degrader-induced target ubiquitination under physiological conditions (i.e., without proteasome inhibitor)



Establishing K-GG remnant profiling for low protein inputs. K-GG peptides were enriched from HEK293 cells and the resulting eluate was diluted for simulation of low protein input samples. For each condition samples were acquired each with dia-PASEF and slice-PASEF scan modes and the raw data processed with DIA-NN.

Detecting degrader-induced neosubstrate ubiquitination from low protein input on a large scale

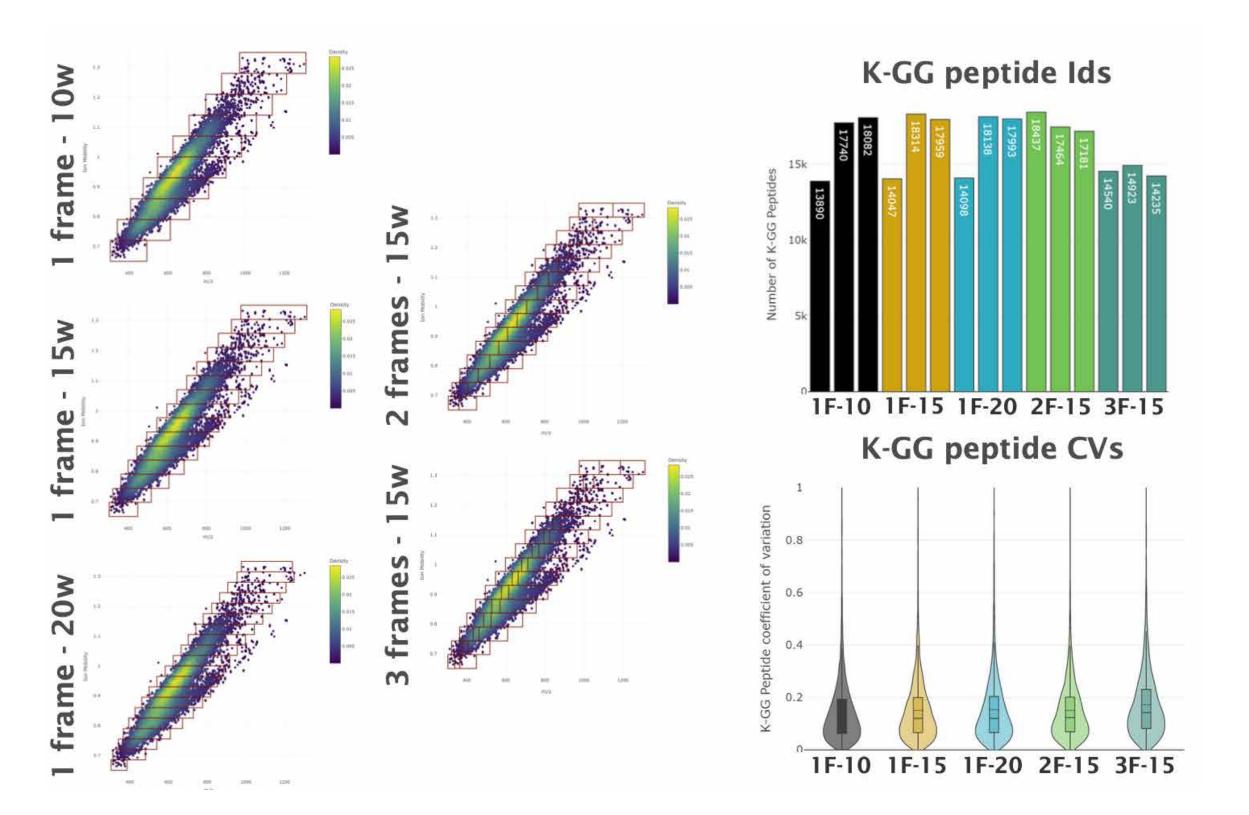
Data acquisition **Biostatistics and data** Ubiquitin remnant Raw data Drug treatment

Single-shot LC-MS

Data analysis & identification of novel degrader targets

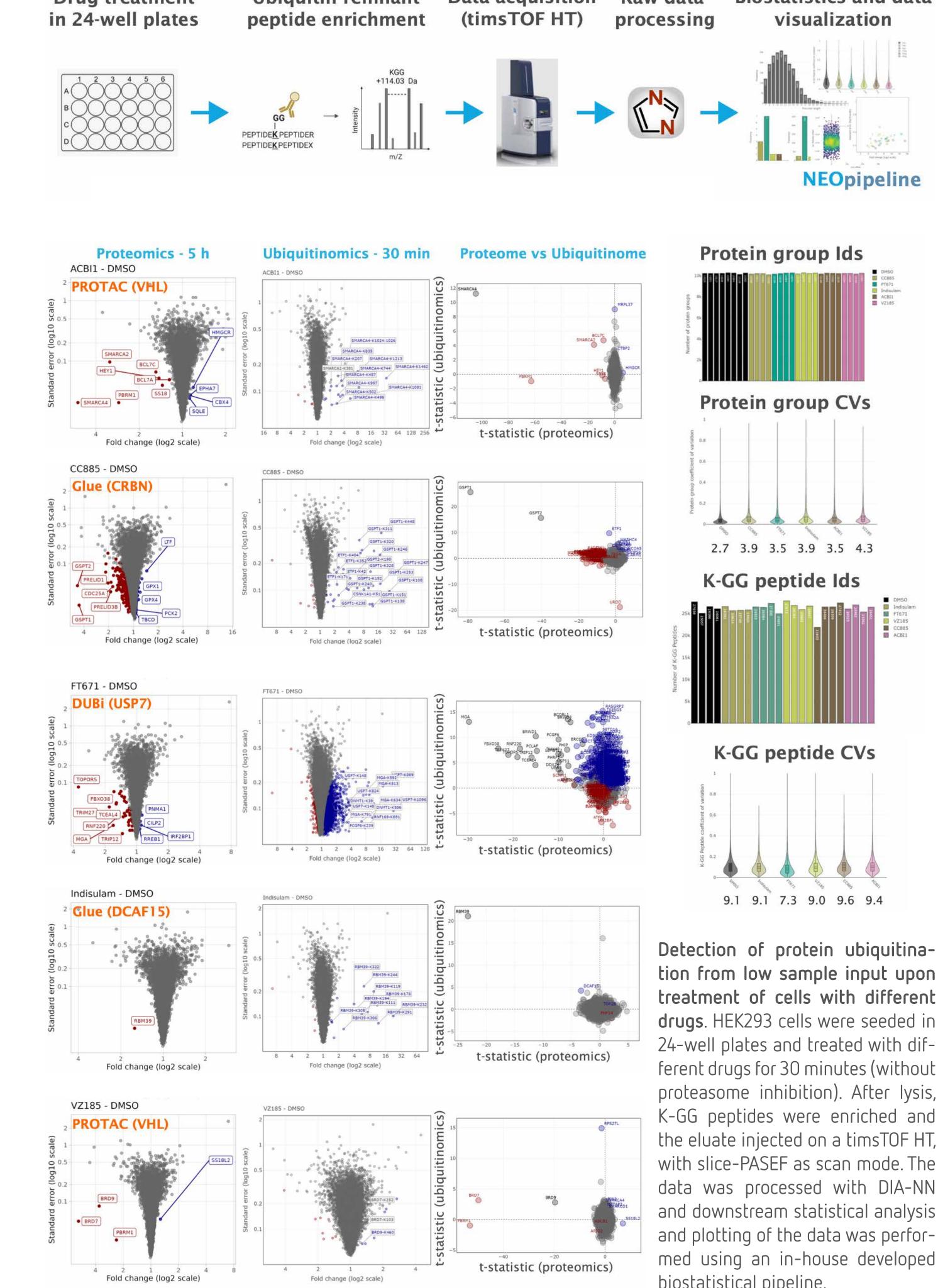
Mechanistic target validation

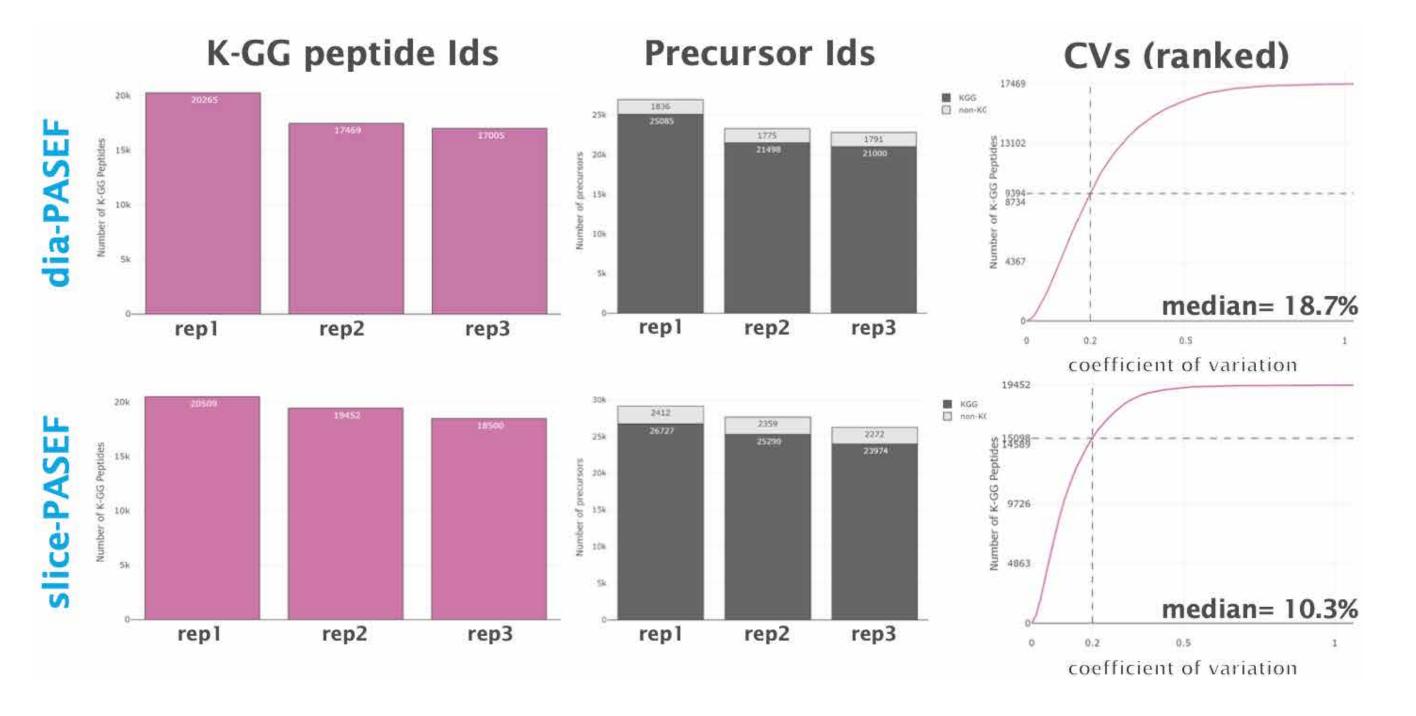
slice-PASEF methods for ubiquitinomics



Testing of five slice-PASEF methods for ubiquitin remnant profiling. K-GG peptides were enriched from HEK293 cells and for each run, an amount equivalent to 30 µg of total protein was separated using a 45 min LC gradient before injection into a timsTOF HT. The data was acquired using different slice-PASEF methods and processed with DIA-NN. K-GG peptide lds and their coefficients of variation (CVs) were scored.

Direct comparison of dia-PASEF and slice-PASEF scan modes for K-GG remnant profiling





slice-PASEF outperforms dia-PASEF in terms of quantification precision. K-GG remnant peptides were enriched from 200 µg of HEK293 cell lysate. The eluate was injected six times (3 x dia-PASEF and 3 x slice-PASEF, equivalent to ~30 µg of protein input) on a timsTOF HT and the raw data processing with DIA-NN.

tion from low sample input upon treatment of cells with different drugs. HEK293 cells were seeded in 24-well plates and treated with different drugs for 30 minutes (without proteasome inhibition). After lysis, K-GG peptides were enriched and the eluate injected on a timsTOF HT, with slice-PASEF as scan mode. The data was processed with DIA-NN and downstream statistical analysis and plotting of the data was performed using an in-house developed biostatistical pipeline.