The timsTOF Ultra 2 enables systematic understanding of ubiquitination and degradation kinetics of degrader drug targets using slice-PASEF acquisition schemes

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Introduction

Targeted protein degradation is an emerging therapeutic modality in which the degradation of disease-causing proteins by endogenous E3 ubiquitin ligases is mediated by small molecules such as molecular glues or PROTACs. Among the biggest challenges in degrader drug development is the unbiased identification of degradation targets and non-desirable off-targets. Mass spectrometry-based deep proteomic profiling is a powerful approach to detect compound-induced modulation of protein abundances, but it cannot discriminate between on- and off-targets. Global ubiquitinomics based on K-GG remnant profiling addresses this challenge by rapid detection of the compound-induced ubiquitination sites. We combined both, global proteomics and ubiquitinomics in a time-course experiment which allows a detailed understanding of the ubiquitination and degradation kinetics and the hierarchical relationships between various degradation targets.

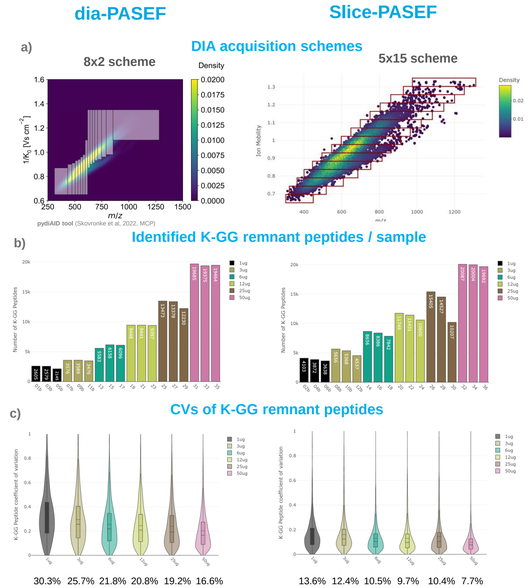


Figure 1

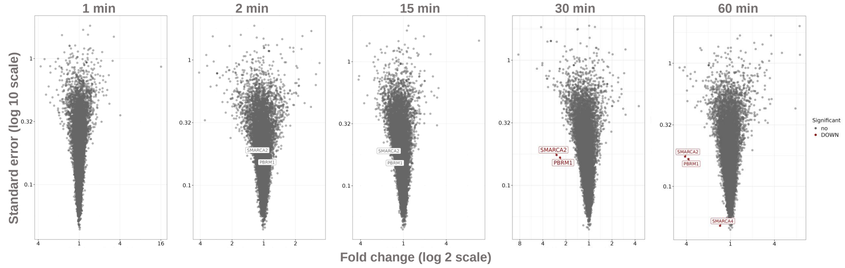
dia-PASEF versus slice-PASEF of a dilution series of K-GG remnant peptides starting from 50 µg input: **a)** illustration of acquisition schemes; **b)** number of identified K-GG remnant peptides; **c)** median coefficients of variation of quantified K-GG remnant peptides. The CVs obtained by the slice-PASEF acquisition method were significantly lower compared to dia-PASEF.

Methods

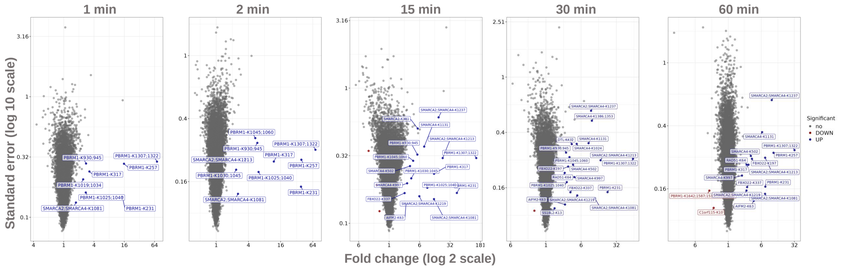
HEK293 cells and human PBMCs were cultivated in 96-well or 48-well plate format and treated in quadruplicates with the VHL-recruiting SMARCA2 PROTAC degrader ACBI2 (provided by opn.me) or the molecular glue degrader Pomalidomide in a time course experiment (one minute to 60 minutes). Cells were lysed and lysates were digested with trypsin in an automated manner. Desalted peptides were analyzed by data-independent (DIA) based acquisition using an adjusted dia-PASEF acquisition scheme on a timsTOF HT system. K-GG remnant peptides were enriched[1] and analyzed on a timsTOF Ultra 2 system using an optimized slice-PASEF[2] acquisition scheme which allows maximum quantification precision. Raw data were analyzed using the DIA-NN software (version 2.1 enterprise) and the results were further analyzed using an in-house data analysis pipeline.

Results

a) Proteomics (HEK293 cells treated with ACBI2)



b) Global Ubiquitinomics (no proteasome inhibition)



c) Kinetics of Proteomics and Ubiquitinomics

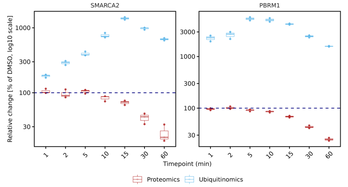


Figure 2

Data of proteomics and ubiquitinomics time course experiment. HEK293 cells were treated with ACBI2 for 1 – 60 minutes **a)** Proteomics data of indicated time points. Significantly downregulated proteins are highlighted in red. **b)** Ubiquitinomics data of the indicated time points. Significantly upregulated K-GG remnant peptides are highlighted in blue. **c)** Ubiquitination and degradation kinetics of SMARCA2 and its complex partner PBRM1.

Reliably distinguish off-target effects from downstream (secondary) regulatory events

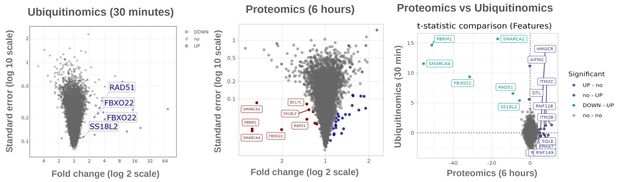
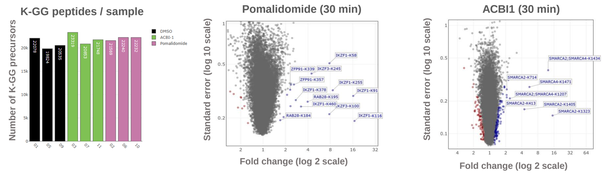


Figure 3

Besides the known degradation targets SMARA2/4 and PBRM1 we identified significantly upregulated K-GG remnant peptides of RAD51, FBXO22, and SS18L2 after 30 minutes. All three proteins were significantly downregulated after 6 hours validating all of them as off-targets of ACBI2 with slower ubiquitination and degradation kinetics in HEK293 cells.

Global Ubiquitinomics of human PBMCs treated with degrader drugs

a) Proof-of-concept (pilot study with ACBI1 and Pomalidomide)



b) Time course experiment of human PBMCs treated with Pomalidomide

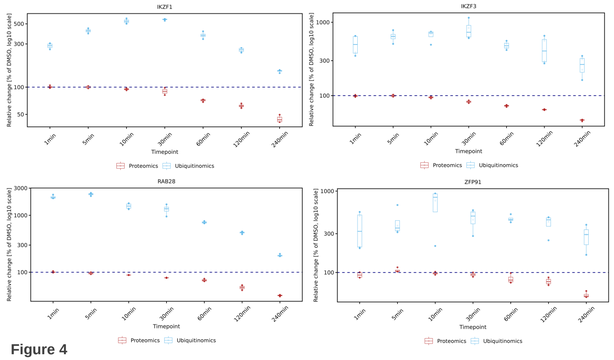


Figure 4

a) PBMCs were treated in triplicates with Pomalidomide or ACBI1 (opn.me). Number of quantified K-GG remnant peptides and the volcano plots are depicted. **b)** time course experiment: ~ 5 million PBMCs were treated in quadruplicates for various time points with 10 µM Pomalidomide followed by proteomics and ubiquitinomics profiling. Depicted are the ubiquitination and degradation kinetics profiles of the known degradation targets IKZF1, IKZF3, RAB28, and ZFP91.

Summary

The very high sensitivity of the timsTOF Ultra 2 combined with the highly-precise quantification enabled by the slice-PASEF acquisition scheme allowed the detection of significantly upregulated K-GG remnant peptides from both, human cell lines as well as isolated human PBMCs. The data from time-course experiments demonstrate instant ubiquitination of the primary degradation targets already 1 minute after treatment while off-targets exhibited slower kinetics. The applicability of the technology with low or very low input samples enables increased throughput for Ubiquitinomics in 48-well or 96-well plate format applying NEOsphere's fully automated compound treatment, sample preparation, and data analysis pipeline.

One very relevant aspect of analyzing rapid compound-induced ubiquitination in human PBMCs is its potential for the analysis of therapeutically relevant degradation targets and the applicability alongside clinical trials.

Conflict of Interest

M.S., U.O., B.S., and B.S. are employees and shareholders of NEOsphere Biotechnologies GmbH. P.S., L.A., and D.H. are employees and shareholders of Bruker Daltonics.

- The high sensitivity of the timsTOF Ultra 2 combined with slice-PASEF acquisition schemes enables highly sensitive detection and very precise quantification of K-GG remnant peptides from low-input samples
- NEOsphere's fully automated Proteomics and Ubiquitinomics platform supports discovery, validation, and optimization of degrader drugs for high-value targets

Technology

1 Steger et al 2024 (doi: https://doi.org/10.1101/2024.10.18.618633)

2 Szyrwiel et al, 2022 doi: https://doi.org/10.1101/2022.10.31.514544