

# Optimized Ubiquitin Remnant DIA Proteomics Enables High-Throughput Screening of PROTACs

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## INTRODUCTION

In targeted protein degradation drug discovery, mass spectrometry-based proteomics spectrometry is a standard approach to measure the specificity and extent of degradation by monitoring total protein abundance. Adding ubiquitin site measurements can validate that protein reduction is E3-ligase and proteasome-dependent. To better evaluate candidate degraders, we optimized a high-throughput ubiquitin-remnant profiling workflow to complement global proteome analysis. We recently developed an improved antibody-magnetic bead kit that enriches the diGly remnant of ubiquitin left behind by tryptic digestion. The new PTMScan HS Ubiquitin reagent is more than twice as sensitive as previous antibody-based kits and requires as little as 1mg of input protein. The magnetic bead formulation also makes it amenable to automation for high-throughput experimentation. Moreover, the new Orbitrap Astral mass spectrometer offers increased speed and sensitivity, enabling comprehensive and high-throughput analysis of both global proteome and ubiquitin remnant samples.

## METHODS

To demonstrate the utility of the combined KGG-global proteome approach for TPD studies, human prostate cancer cell line LNCaP FGC was treated with androgen receptor (AR) degrader ARCC4 (1) or the standard-of-care inhibitor enzalutamide (Enza) for 4h. Lysates were then subjected to whole-proteome KGG profiling and global proteomics by LCMS. The KGG enrichment was performed on 2.0 mg of input peptide using the Cell Signaling Technology PTMScan HS kit #59322. Enrichment reproducibility was monitored by spiking in three heavy-labeled control peptides at the beginning of the enrichment (cat. #75964).

All LCMS analysis was carried out on a Thermo Orbitrap Astral with data independent acquisition (DIA). The unenriched global proteome was assayed over a 60 min gradient using a 60cm x75um column. KGG samples were analyzed with a 30 min analytical gradient on a 25cm x75um column. Library-free searches and quantitation were performed using Spectronaut software.

## CONCLUSIONS

The Astral DIA method enabled deep coverage of both the global and ubiquitinated proteome. The assay has the sensitivity to detect AR even after significant degradation, including 19 ubiquitinated lysines. The method also identified non-target proteins with increased ubiquitination, a critical observation for drug development. Notably, LYPLA2 exhibited a stronger response to ARCC4 treatment compared to AR.

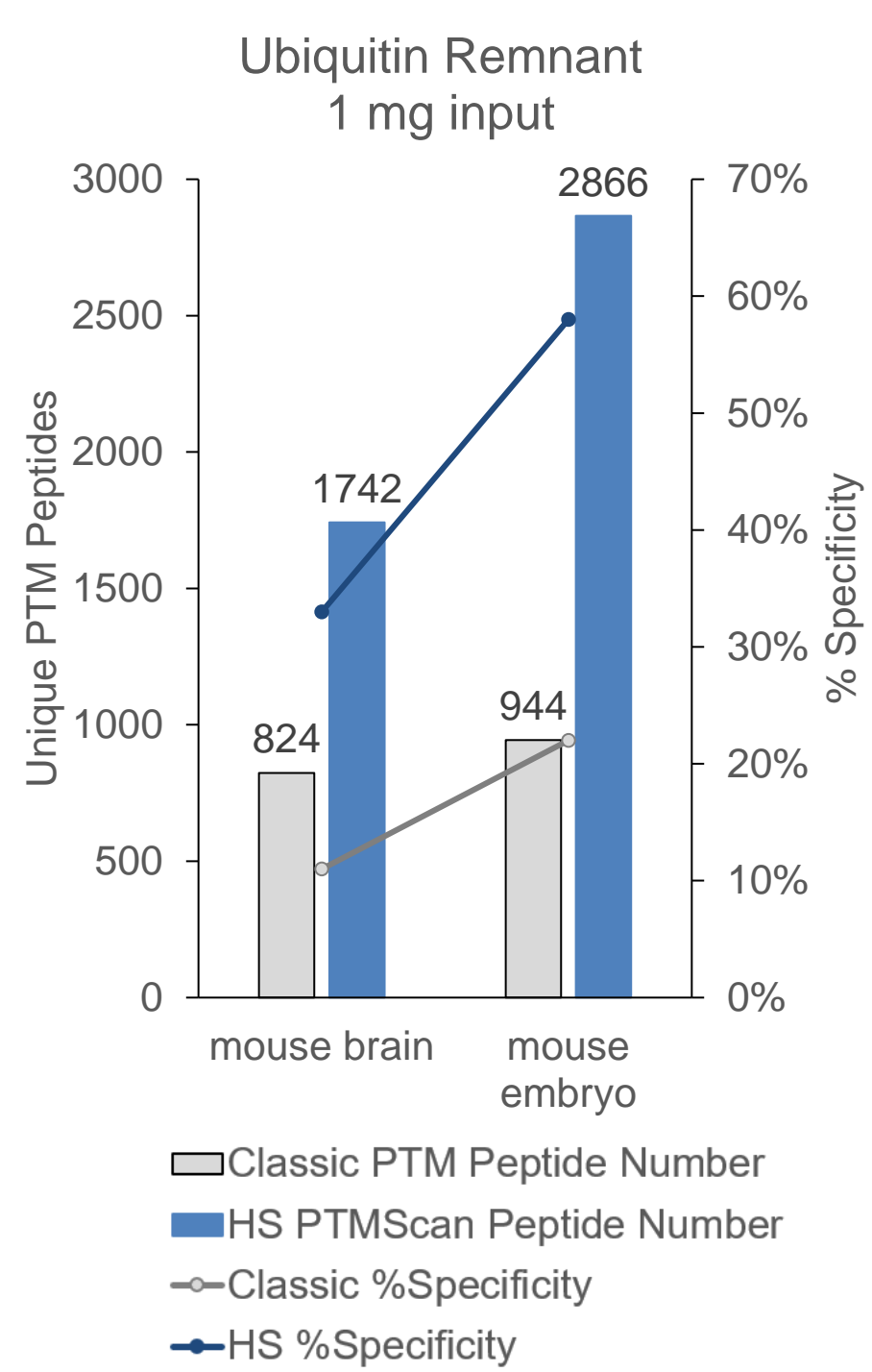
Correlating changes in ubiquitination sites with protein expression levels enabled differentiation between drug-induced off-target effects and putative substrate proteins. Correlating changes in ubiquitination sites with protein expression levels enabled differentiation between drug-induced off-target effects and putative substrate proteins. The presence of elevated ubiquitination sites supports a VHL-mediated degradation pathway for specific substrates. Furthermore, mapping ubiquitination sites onto the three-dimensional structure of substrate proteins may elucidate potential E3 ligase binding regions.

Incorporating PTMScan HS ubiquitin profiling with total proteomics provided insights into AR's vulnerability to VHL-mediated degradation. This research underscores the value of integrating ubiquitin and protein profiling to evaluate drug candidates and guide further medicinal chemistry optimization.

## REFERENCES

- Salami J, Alabi S, Willard RR, Vitale NJ, Wang J, Dong H, Jin M, McDonnell DP, Crew AP, Neklesa TK, Crews CM. Androgen receptor degradation by the proteolysis-targeting chimera ARCC-4 outperforms enzalutamide in cellular models of prostate cancer drug resistance. *Commun Biol.* 2018 Aug 2;1:100. doi: 10.1038/s42003-018-0105-8. PMID: 30271980; PMCID: PMC6123676.
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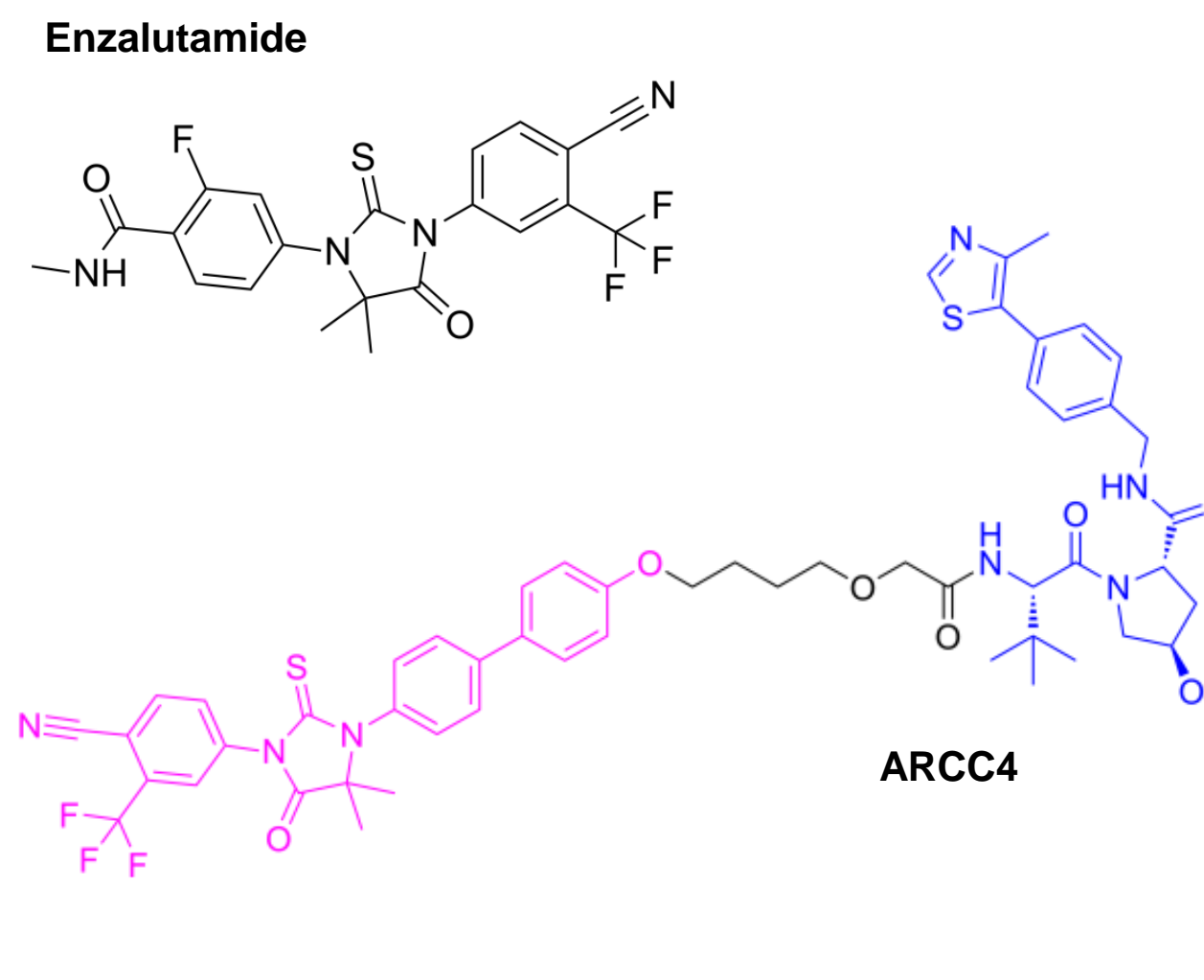
### Robust Ubiquitin Remnant (K-ε-GG) Enrichment



- PTMScan® HS Benefits:**
- Ab crosslinked to beads, does not co-elute with PTM peptides
  - Magnetic beads amenable to automation
  - Optimized buffers improved sensitivity & specificity

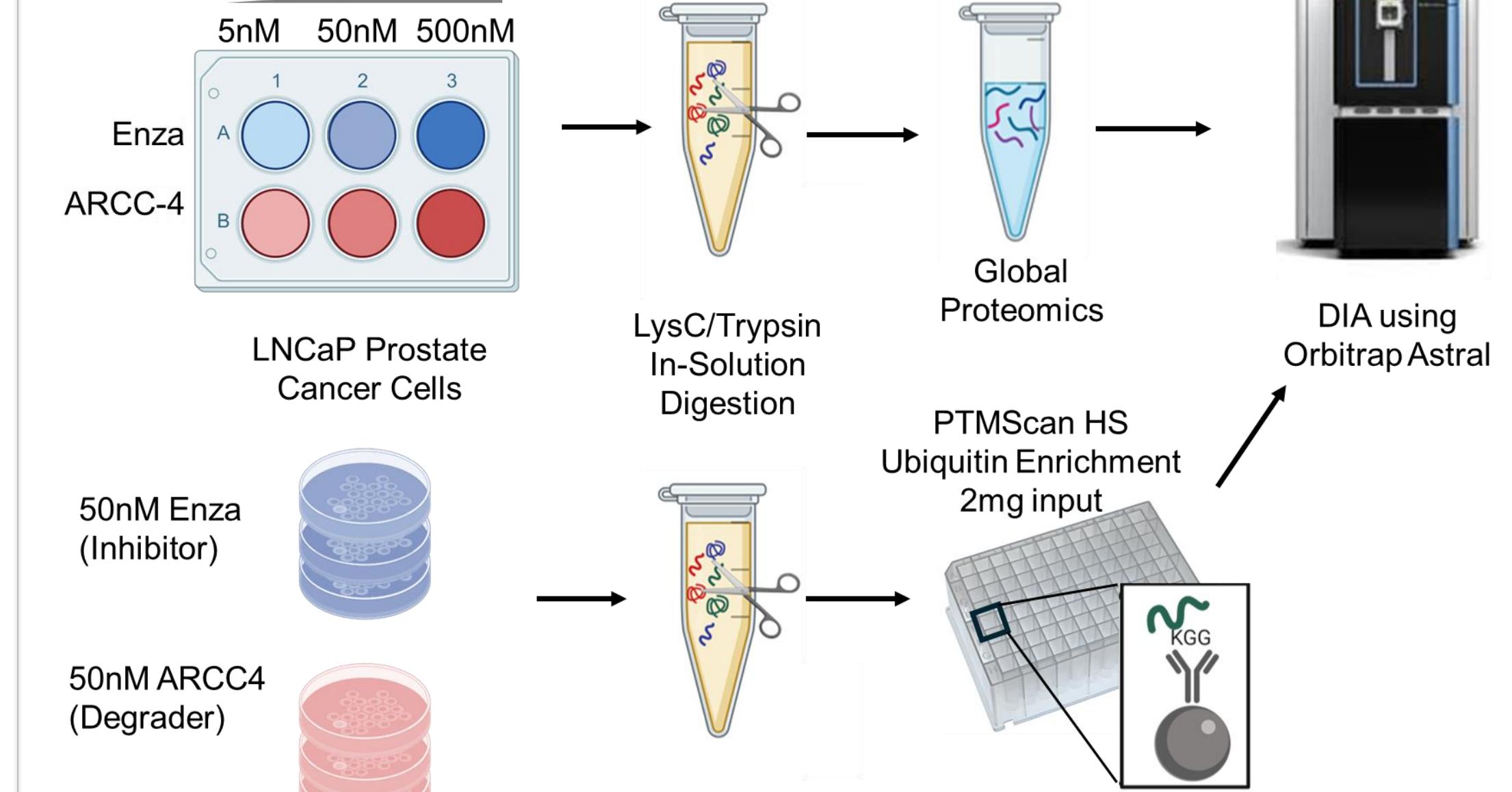
**Figure 1. PTMScan® HS enrichment protocol improves upon classic method**  
 We improved upon older "Classic" PTMScan chemistry for ubiquitin remnant enrichment. The new HS kits were validated against the Classic versions with matched peptide input in multiple tissue types (brain and embryo shown). Unique modified peptide numbers are shown as bars and the percent specificity (number of PTM peptides/number of PTM + unmodified peptides) is shown as a line plot. Classic performance is in gray, and HS is in blue.

### Androgen Receptor Degrader



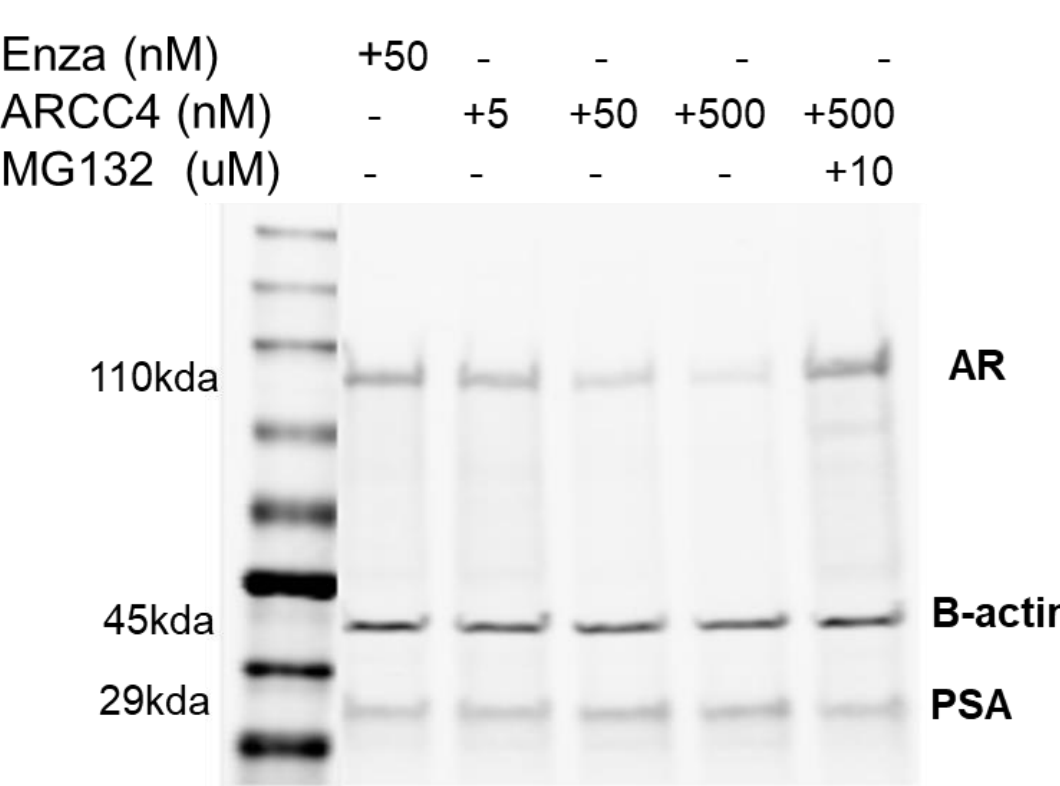
**Figure 2. Androgen receptor degrader drug and control**  
 ARCC4 is a commercially available degrader designed to recruit the VHL E3-ligase to specifically degrade androgen receptor, a key clinical target in prostate cancer(1). Enzalutamide, which is an AR inhibitor, is the standard of care for prostate cancer and does not induce protein degradation.

### Experiment Design



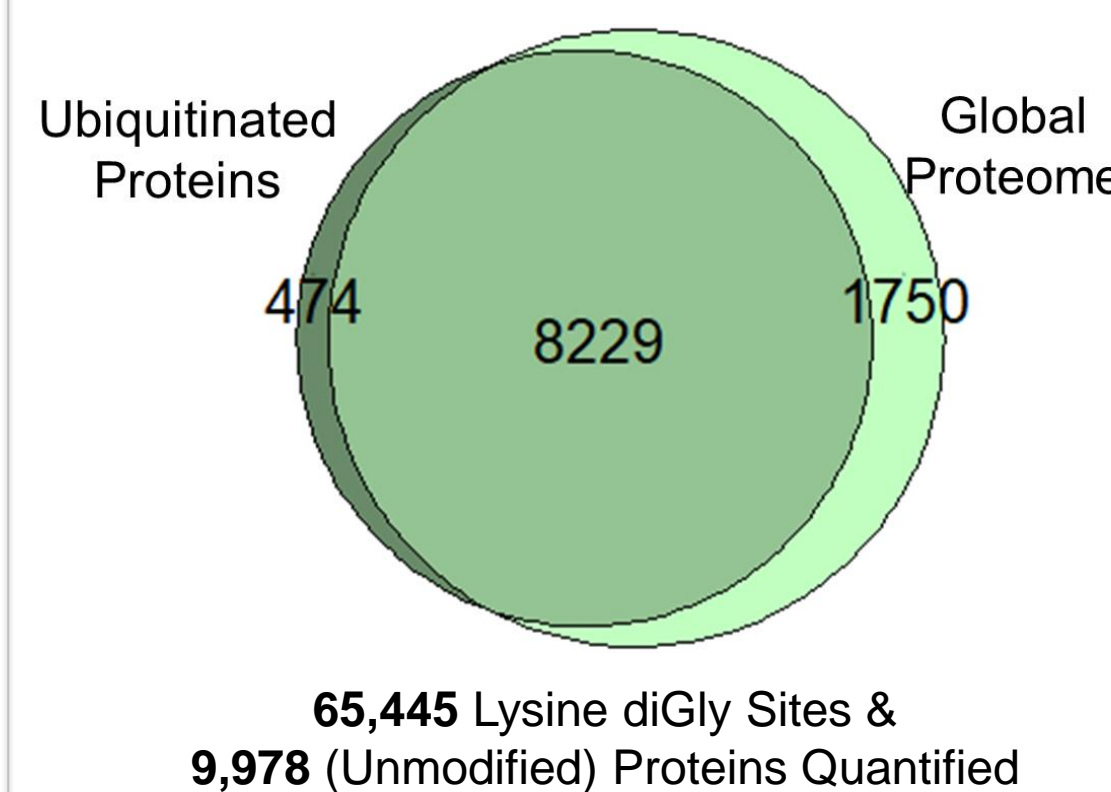
**Figure 3. Proteome and ubiquitinome profiling of AR degrader-treated cells**  
 Human prostate cancer cell line LNCaP FGC was grown in triplicate dishes per condition. ARCC4 (degrader) was dosed at 5nM, 50nM, or 500nM for 4h; control plates were treated with non-degrading Enza at matching concentrations. Lysates were prepared for proteomics analysis by in-solution digestion in urea. DiGly peptides were enriched using PTMScan HS #59322 on a Kingfisher robotic system. All samples were acquired in DIA mode on an Orbitrap Astral. Created with BioRender.com.

### Dose Selection & Confirmation of Activity



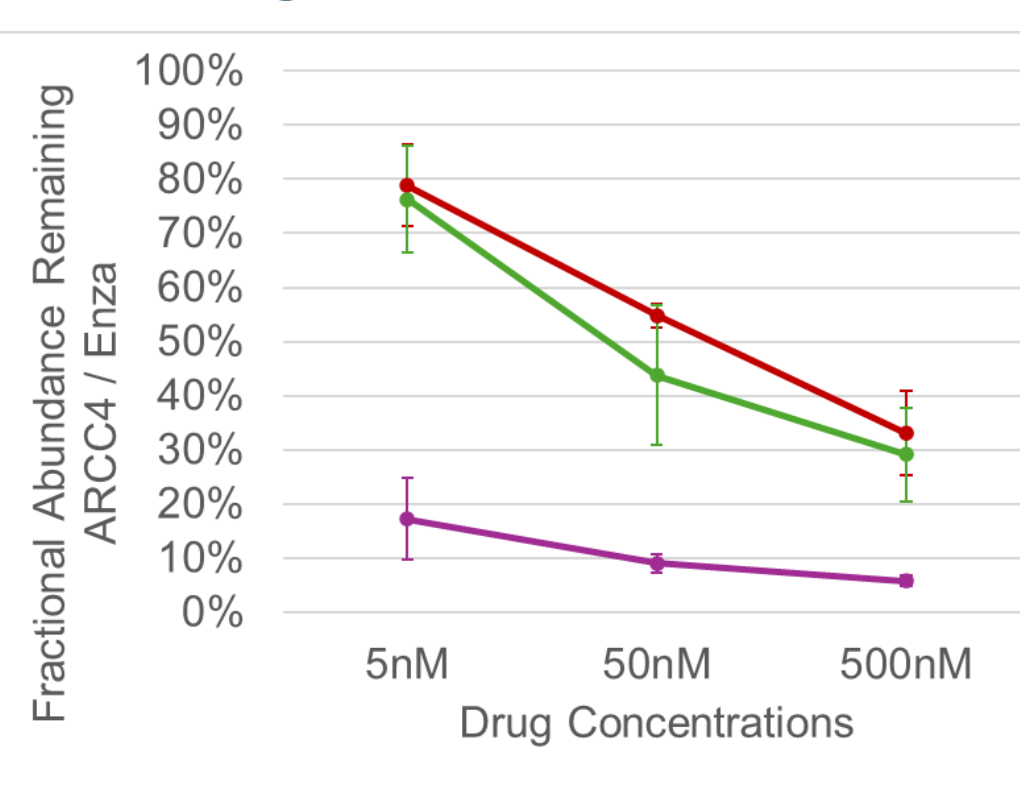
**Figure 4. Western blots confirm ARCC4 activity.**  
 ARCC4 induces dose-dependent degradation of androgen receptor. Proteasome inhibition with MG132 reverses the degradation. Antibodies used: Androgen Receptor cat# 5153; B-actin, cat# 6497; PSA/KLK3, cat#5365.

### Deep Proteome and Ubiquitinome Coverage



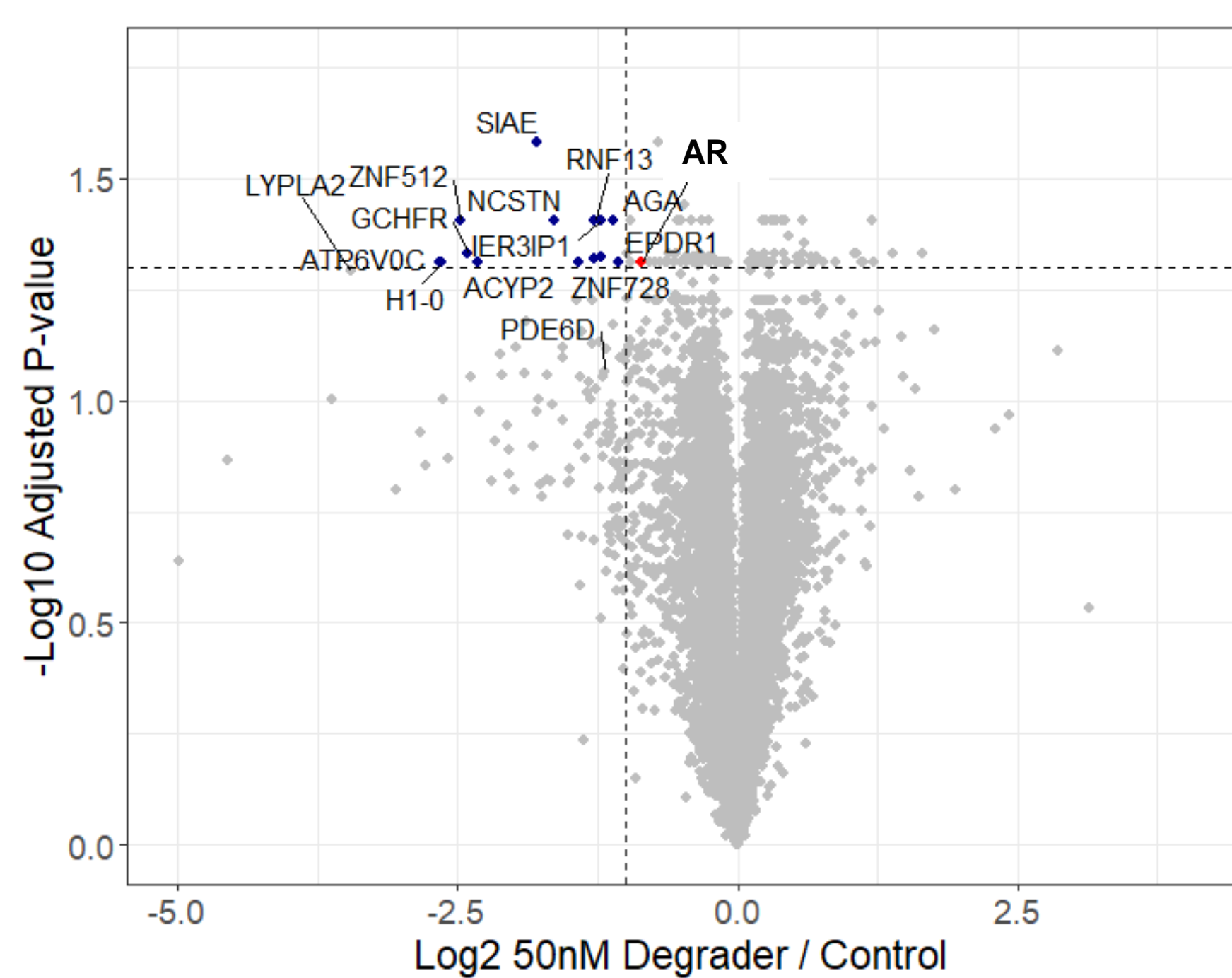
**Figure 5. Deep proteome coverage enables comparison between ubiquitin and protein measurements.**  
 In the global proteome samples, 9978 proteins were quantified with complete profiles across all injections. PTMScan HS enriched 72,507 ubiquitinated peptides from 8704 proteins that could be quantified with up to 50% missing values imputed using background signal. DIA with the Astral enabled nearly 80% overlap at the protein level between these two datasets.

### Androgen Receptor Dose-Dependent Degradation



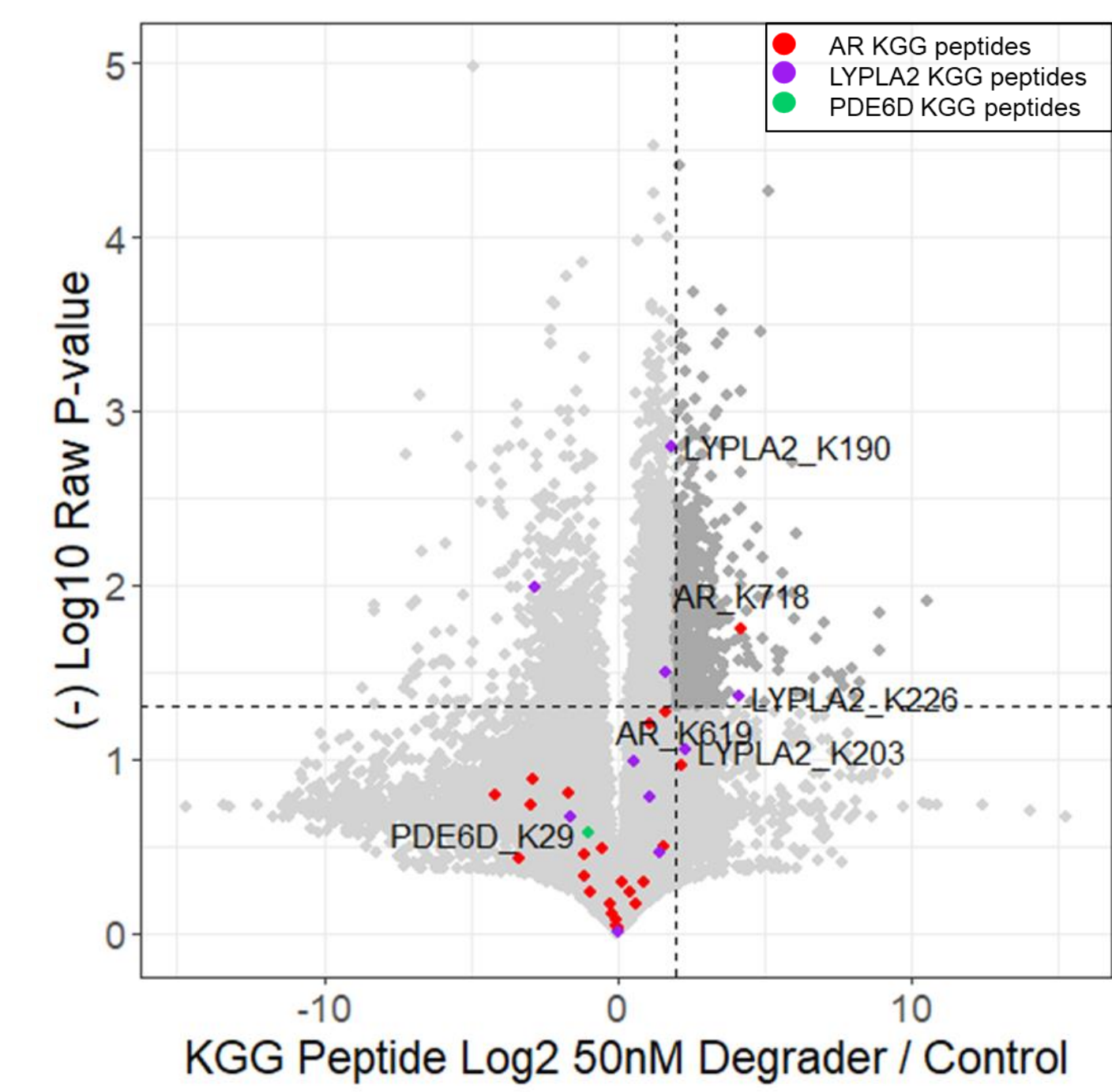
**Figure 6. AR and off-target substrates exhibit dose-dependent response to ARCC4.**  
 In the global proteome samples, proteins that decreased in abundance between Enza and ARCC4 conditions and continued trending downwards with increasing dosage were identified. At 500nM ARCC4, AR decreased to 33% of its abundance with 500nM Enza. PDE6D and LYPLA2 also were degraded in a dose-dependent manner. Error bars indicate 1 sd.

### Proteins Degraded by 50nM ARCC4



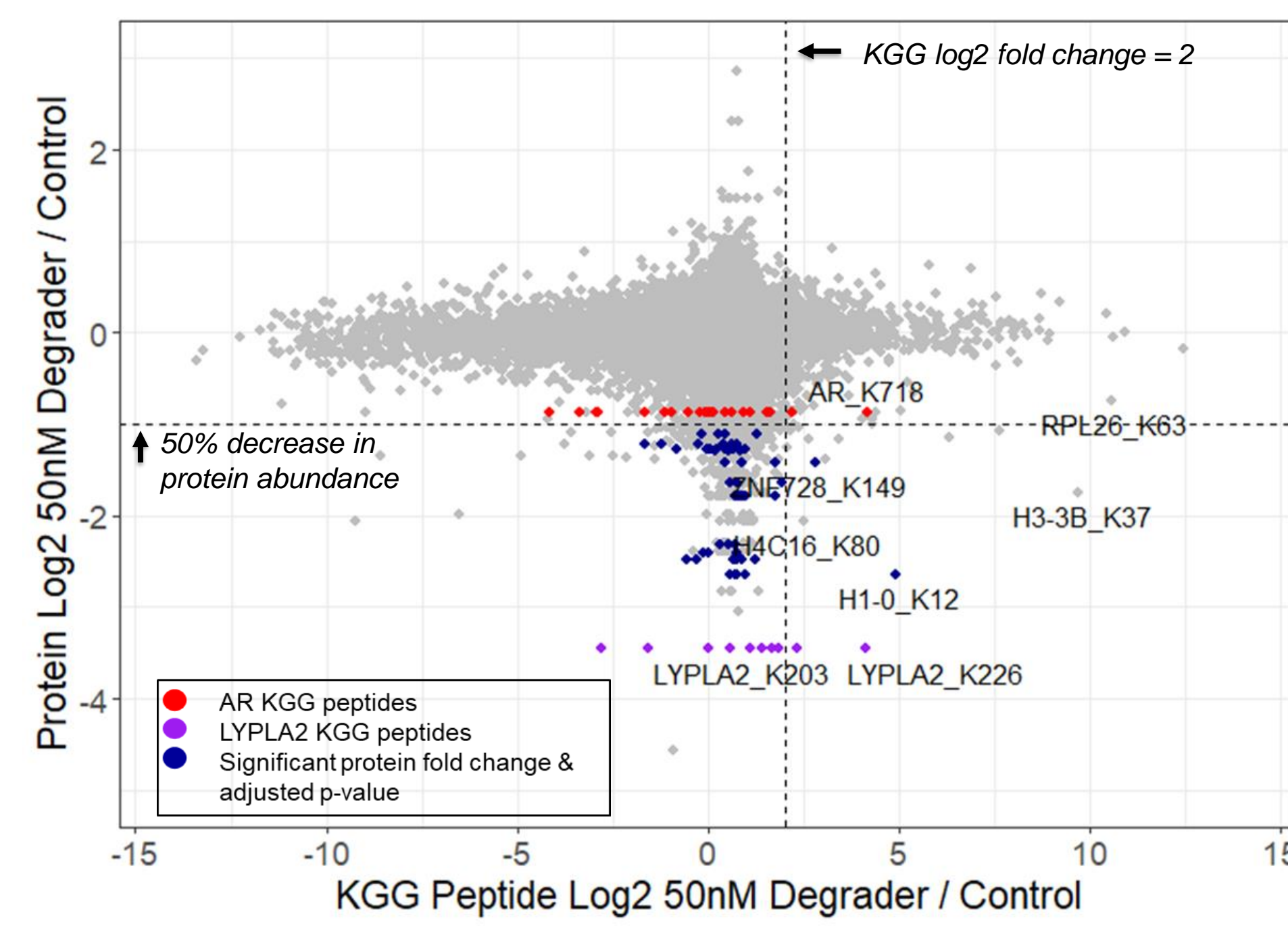
**Figure 7. Multiple proteins degraded by ARCC4.**  
 Dotted lines indicate where the adjusted p-value=0.05 (y-axis) and where protein abundance decreased by 50% (x-axis). AR is indicated with a red dot. Proteins indicated by blue points represent off-target activity of the drug. In the 5nM and 500nM comparisons, no proteins reached significance, though many of the same proteins decreased in abundance.

### Ubiquitin Peptides Induced by 50nM ARCC4



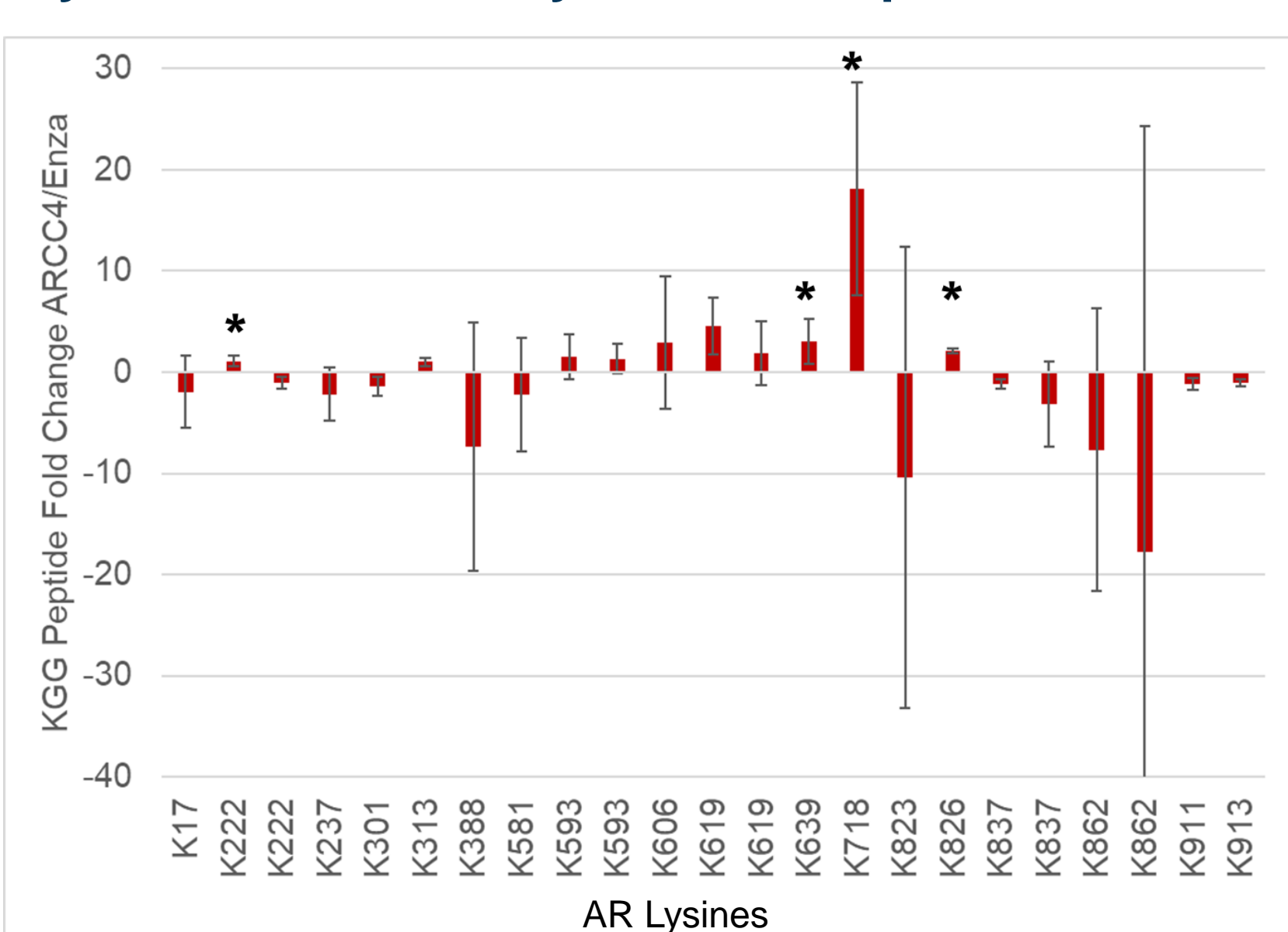
**Figure 8. ARCC4 induces ubiquitination changes.**  
 Dotted lines indicate where the raw p-value=0.05 (y-axis) and where KGG peptide abundance decreased by 4-fold (x-axis). AR is indicated with red points. No change in PDE6D ubiquitination suggests this is not a substrate of ARCC4 despite the trend in protein abundance.

### Ubiquitin Peptide Abundance Compared to Protein



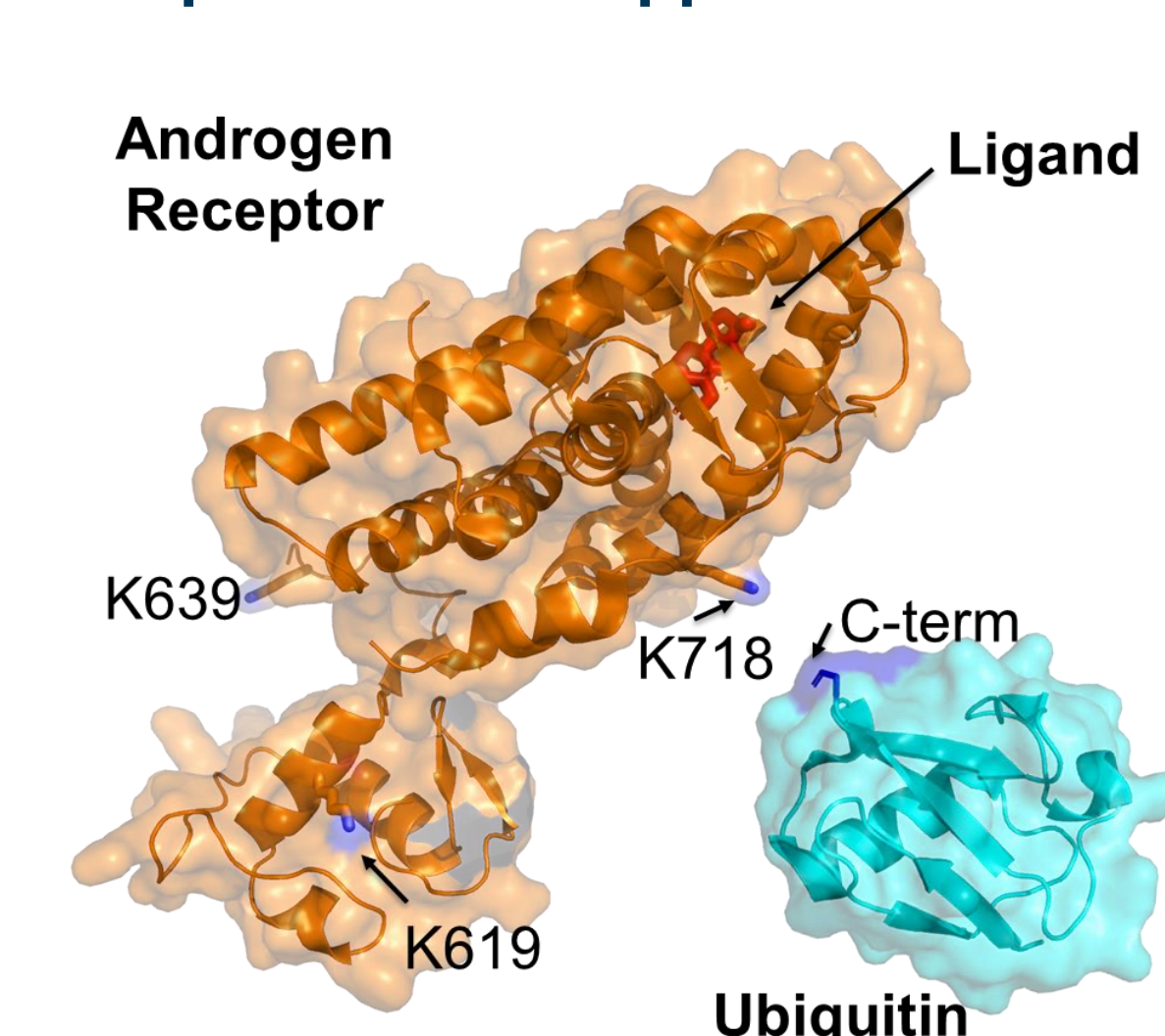
**Figure 8. Ubiquitination measurements compared to protein.**  
 Many proteins appeared to be off-target substrates based on the global proteome measurement alone, but only a subset of those proteins also had a lysine with a substantial increase in ubiquitination. LYPLA2, H1-0, and ZNF728 show the most severe off-target degradation. Dotted lines indicate where the KGG peptide abundance increased by 4-fold and where protein abundance decreased by 50% (y-axis).

### Lys718 is the Primary Site of Ubiquitination on AR



**Figure 10. Ubiquitinated Peptides of AR.**  
 Bars indicate linear peptide fold-changes between the treatments. Some lysines are covered by more than one peptide, typically due to digestion variants. Error bars indicate one standard deviation in the ratio measurement. Stars (\*) indicate peptides that had unadjusted p-values <0.05. Ubiquitination at Lys718 increased 18-fold, far above other AR sites. This site likely is primarily responsible for the degradation of AR by ARCC4.

### Ubiquitin Sites Mapped to AR Structure



**Figure 11. Ubiquitinated Mapped to AR Structure.**  
 PDB structure 1E3G covering the ligand binding domain was supplemented with an Alpha Fold model of part of the DNA-binding domain. A bound ligand molecule shown in red illustrates the buried binding pocket that ARCC4 likely uses. The structure of ubiquitin is shown for scale. Modeling the ubiquitination sites of the substrate protein provides the starting point for a structure-based optimization of the drug molecule. The model also enables structural predictions for how the PROTAC would recruit the E3 ligase complex and leave room for elongating a polyubiquitin chain.



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