High-throughput pooled CRISPR screening with high-capacity single cell analysis of the protein kinome using a dual inducible Cas9 system

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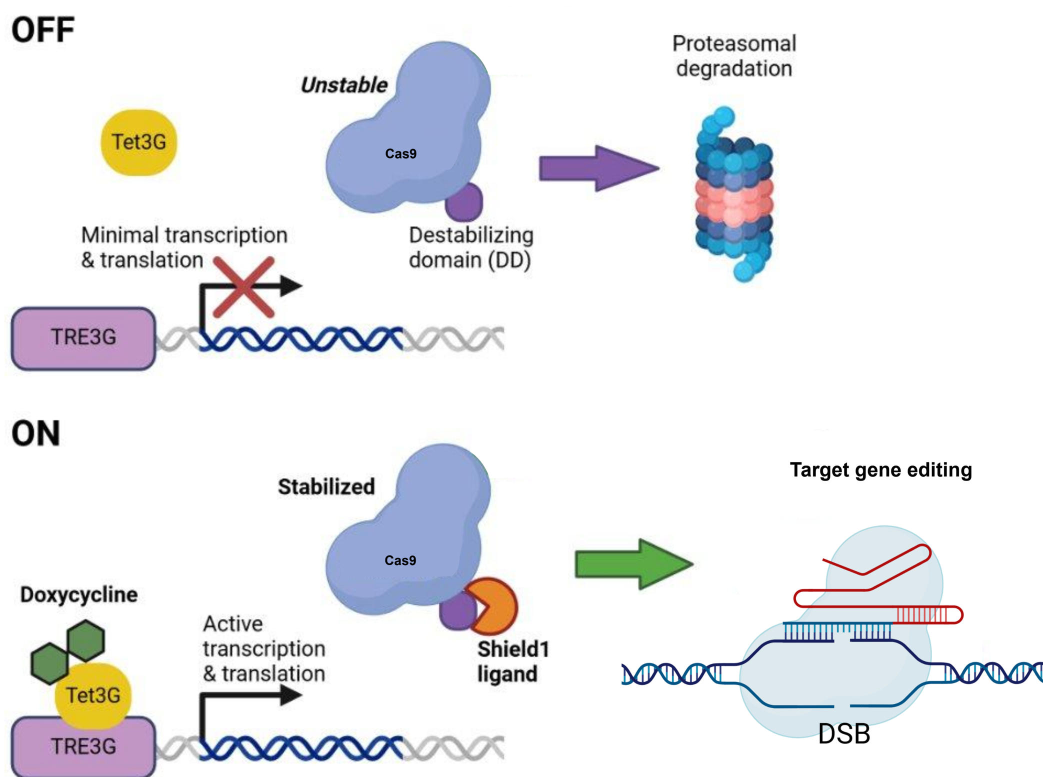
1 Abstract

Kinases are critical regulators of cellular processes such as proliferation, survival, and apoptosis. Dysregulation of kinase activity is implicated in a wide range of diseases, including autoimmune disorders, inflammatory conditions, and cancer, making kinases highly valuable therapeutic targets. Identifying clinically relevant drug targets is a foundational yet complex step in drug discovery. Genomic screening using CRISPR technology addresses this challenge by offering deep genetic insights. Traditional pooled CRISPR screens with phenotypic readouts are often limited to detecting changes that confer a fitness advantage or disadvantage or to sorting cells using proteins or intracellular components labeled with fluorescent dyes, reporters, or antibodies.

Here we developed a unique, dual transcriptionally and post-translationally regulated inducible Cas9 system for rapid small-molecule induction of Cas9 with minimal background expression in the "OFF" state, allowing for precise temporal control of gene editing. We then transduced two cancer cell lines expressing the system with a guide RNA library targeting the protein kinome and employed a microfluidics-free, single cell workflow for unbiased analysis of transcriptional changes resulting from kinase-regulated signaling pathways and protein-protein interactions. This massively scalable approach facilitated the screening of 760 known protein kinases, with 99% of the library recovered in the sequencing analysis and a median of over 5000 genes detected per cell. Critically, it enabled the characterization of many kinases whose knockout do not elicit pronounced growth phenotypes. This proof of concept demonstrates the efficacy of high-throughput combinatorial CRISPR screening with high-capacity single cell analysis for characterizing kinase activity in cancer cell lines.

2 Dharmacon™ Strict-R™ Inducible Cas9 Lentiviral System



OFF

- **Absence** of doxycycline and Shield1

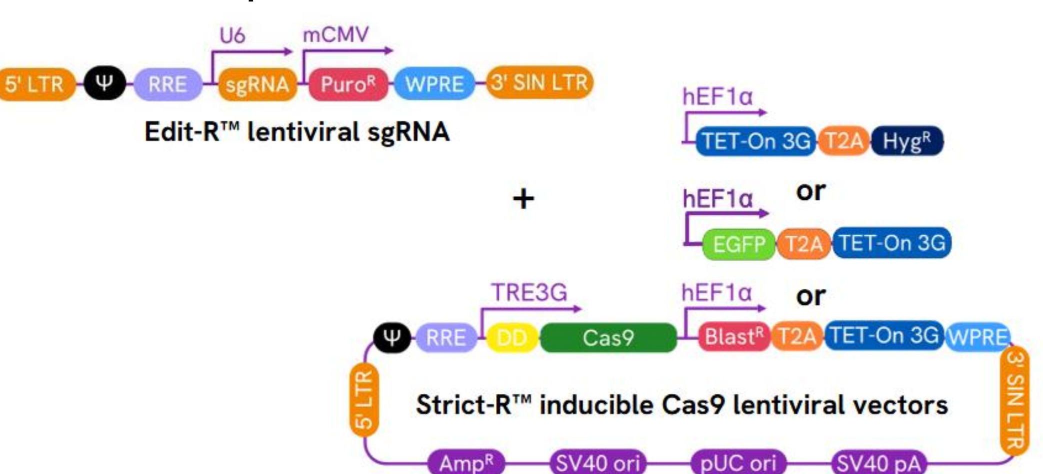
→Leaky bursts of transcription from the TRE3G promoter result in the translation of Cas9 fused to a FKBP12-derived destabilizing domain that tags the protein for rapid proteasomal degradation, minimizing background editing (leakiness).

ON

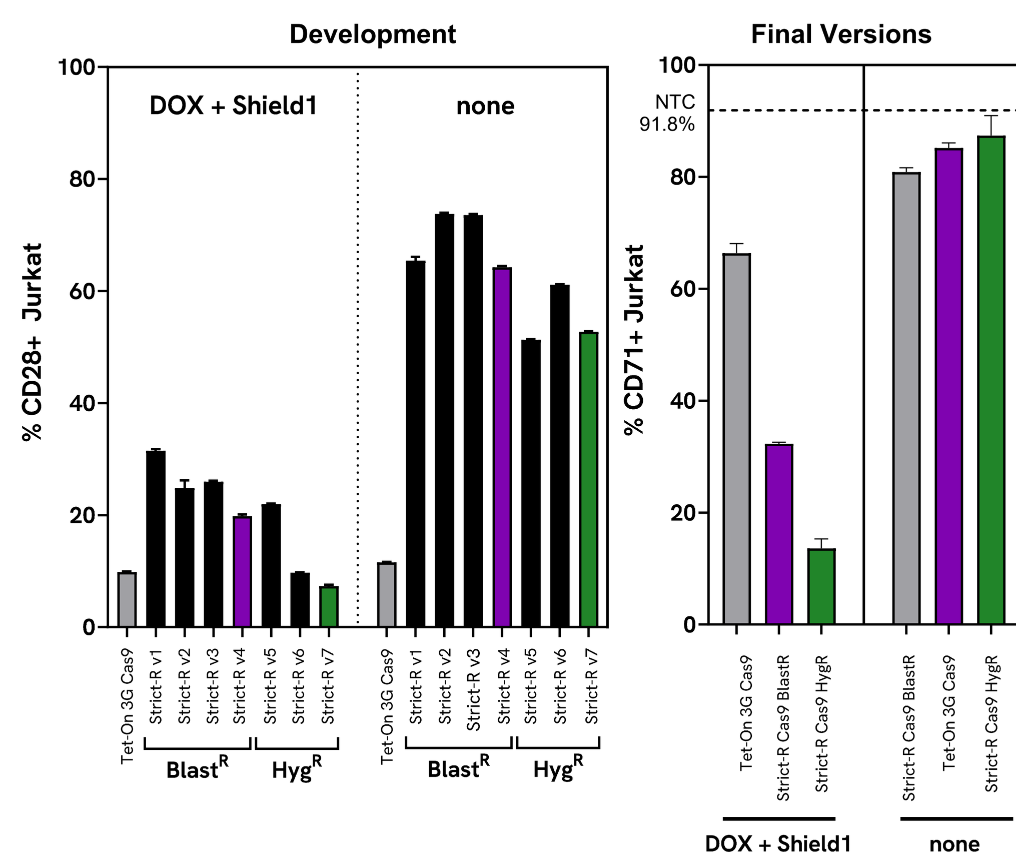
- **Presence** of doxycycline and Shield1

→Reverse tetracycline-dependent transactivator (Tet3G) binding induces potent transcription from the TRE3G promoter and the addition of Shield1 stabilizes Cas9, thereby enabling robust target gene editing in the presence of a gene-specific sgRNA. *Diagram created with BioRender.com.*

Schematic maps of the Strict-R Inducible Cas9 Lentiviral Vectors

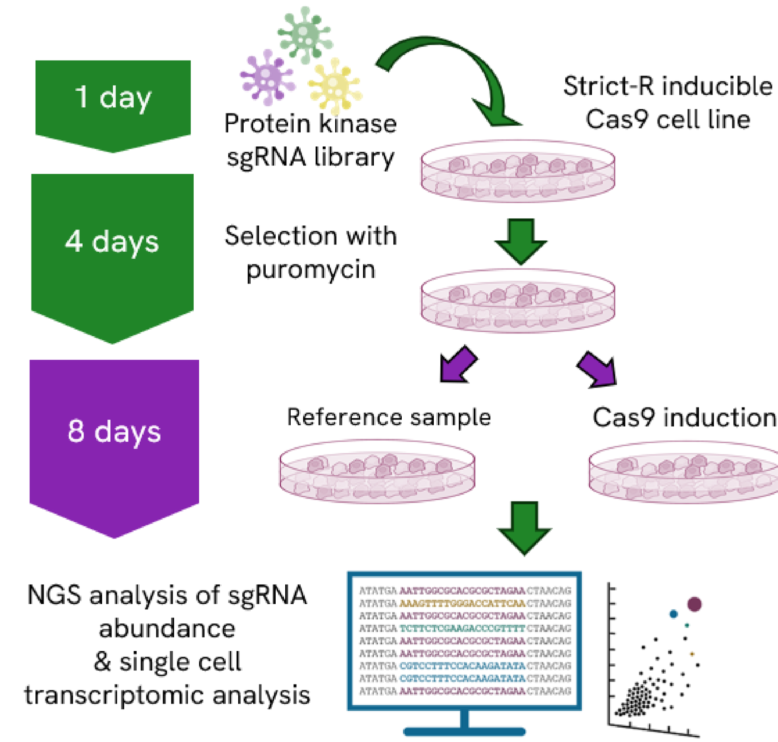


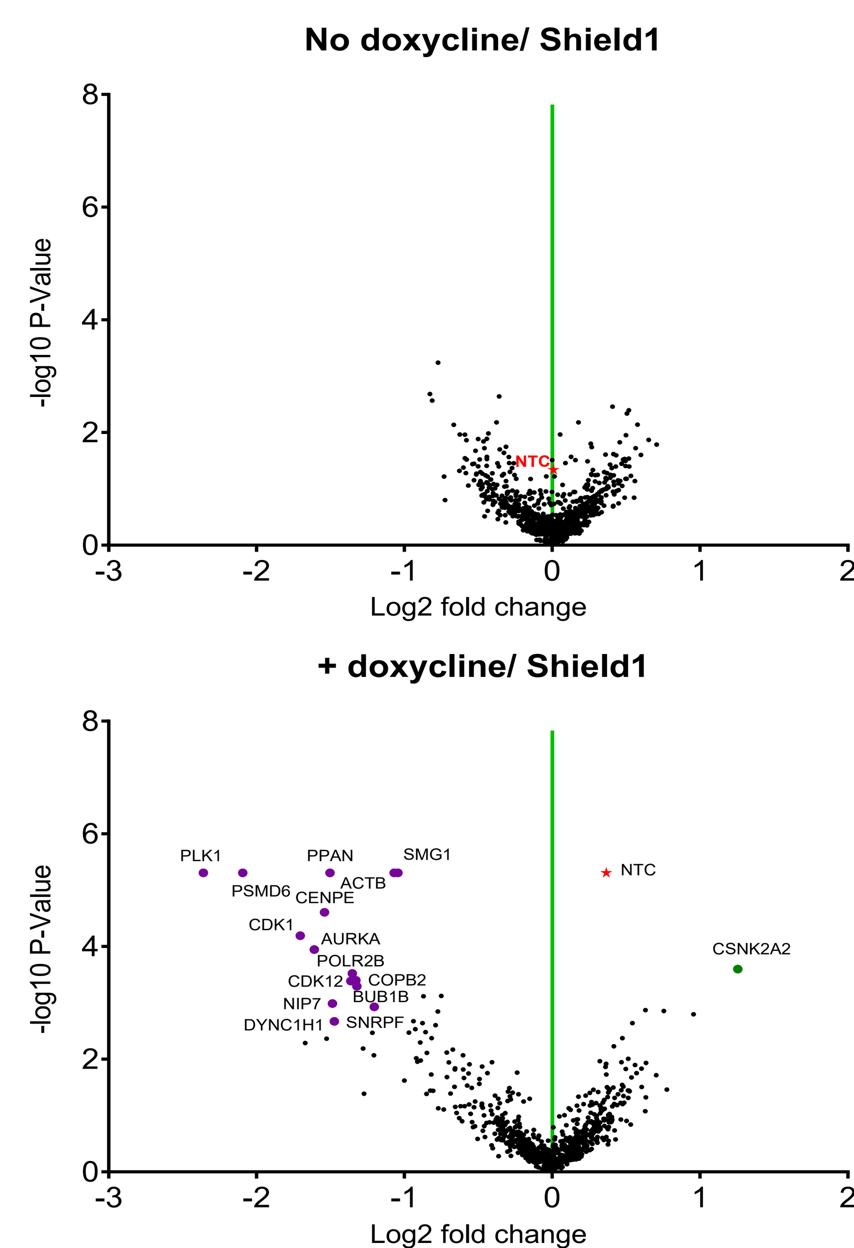
2 Potent editing and reduced leakiness with the Strict-R Inducible Cas9 System



Jurkat cells expressing the listed inducible Cas9 systems were transduced with Edit-R lentiviral sgRNA targeting *CD28* (**left**) or *CD71* (**right**) or Edit-R lentiviral sgRNA non-targeting control (NTC) at MOI 0.3. Cells were selected with 2 μg/mL puromycin for 5 days. Cells were plated in 96-well plates at 50,000 cells/well and stimulated with 500 ng/mL doxycycline and 500 nM Shield1 for 6 days. On day 6, cells were collected and stained for receptor knockout analysis by flow cytometry. Jurkat cells expressing an NTC were used for positive control receptor stain and isotype controls to determine positive and negative receptor gates from the induced media condition.

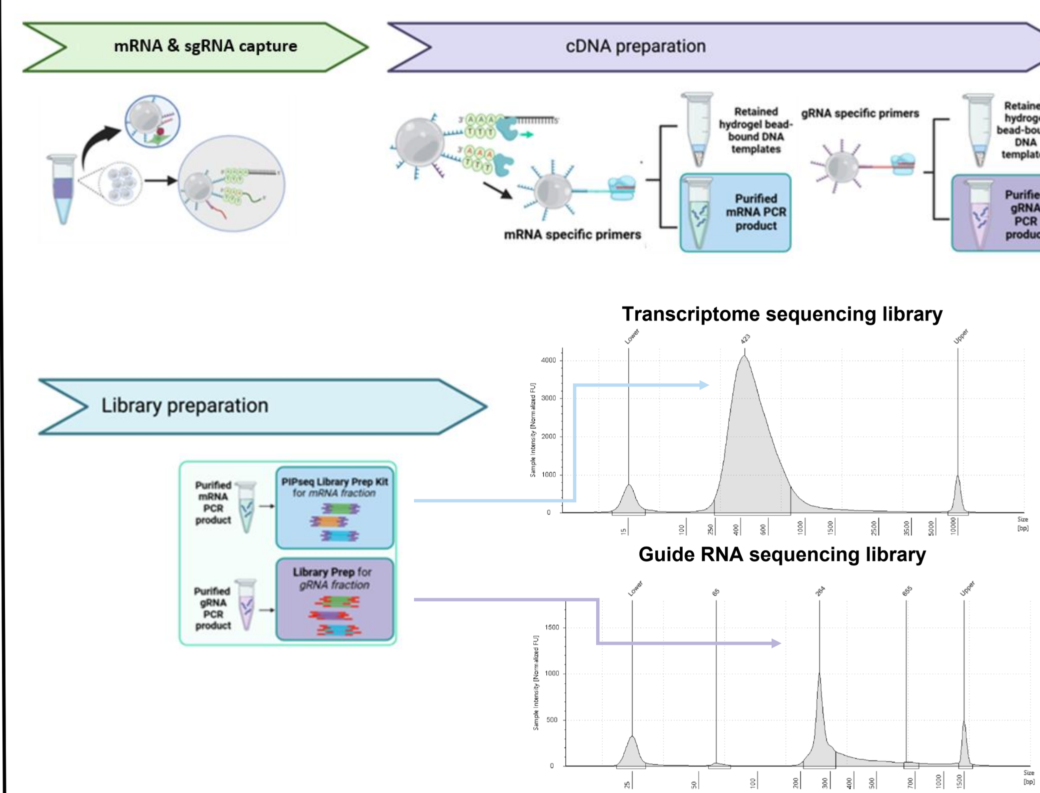
3 Exquisitely regulated Cas9 expression for pooled CRISPR screening



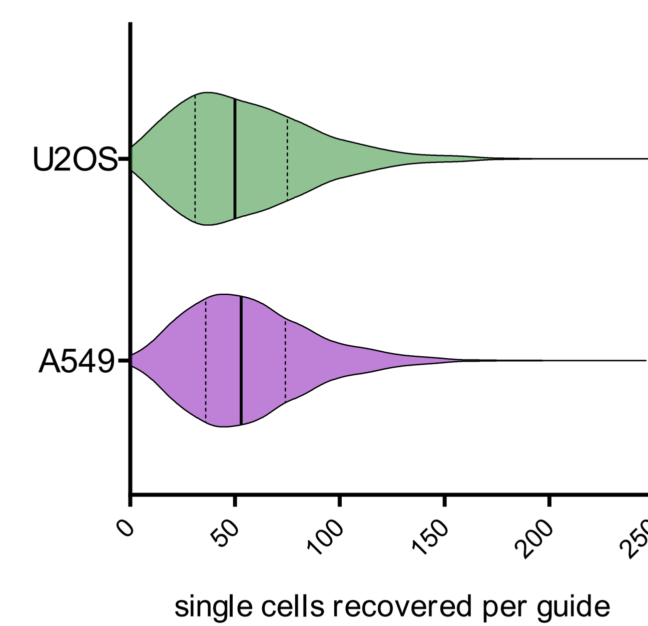


A pooled lentiviral library comprised of 3240 Edit-R sgRNAs targeting 760 human kinases, a small panel of essential genes, and 100 non-targeting controls (NTCs) was transduced into Strict-R inducible Cas9-expressing U2OS and A549 cell lines at MOI of 0.3 and 300-fold sgRNA representation. At 24 hours post-transduction, cells were selected with 2 μg/mL puromycin for 4 days. The reference (T0) sample was harvested, and matched samples were stimulated with 500 ng/μL doxycycline/ 500 nM Shield1 or cultured in standard growth media for 8 days. Genomic DNA was harvested from the T0, uninduced and induced samples and PCR-amplified for high-throughput sequencing on an Illumina platform. Log2 fold change of induced U2OS cells compared to T0 is plotted vs. –log10 MAGeCK P-value. The sgRNAs that were significantly (FDR ≤ 0.10) higher and lower abundance that also showed > |2|-fold abundance change are in green and purple, respectively. NTC abundance is starred in red, and the green line indicates zero-fold abundance change.

4 Direct capture of large CRISPR libraries with Particle-templated Instant Partitions (PIPs)

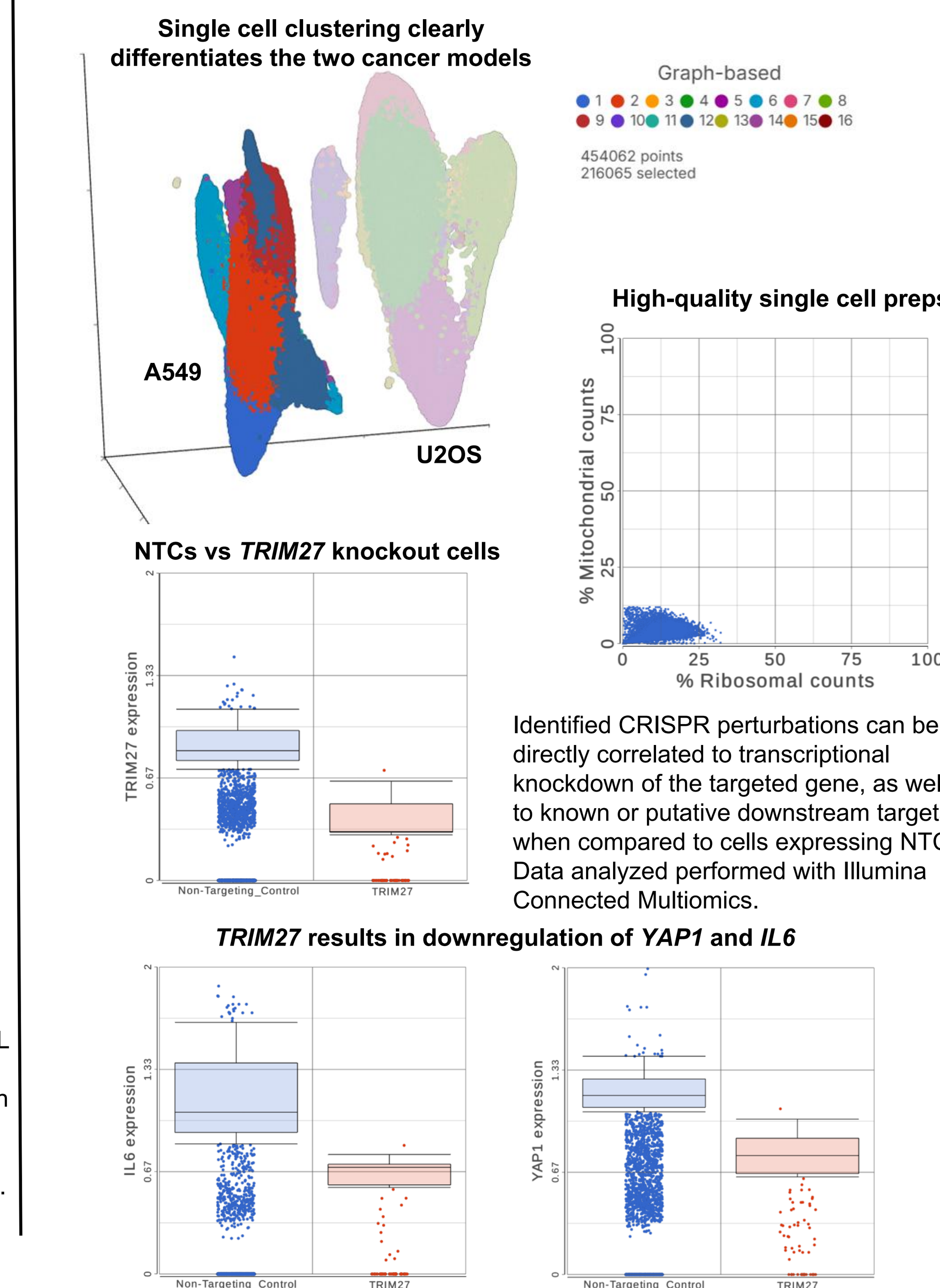


Sample	# Cells Captured	Median Genes/ Cell	# Cells with gRNA reads	Cells with 1 gRNA	Median gRNA (Read) Counts per Cell	% Guides Detected
A549_1	112,629	5,134	102,268	68,071	1,159	100.0
A549_2	103,436	5,194	92,039	61,827	1,099	100.0
U2OS_1	109,254	5,753	88,081	53,015	467	99.8
U2OS_2	128,743	5,098	107,694	63,332	459	99.8



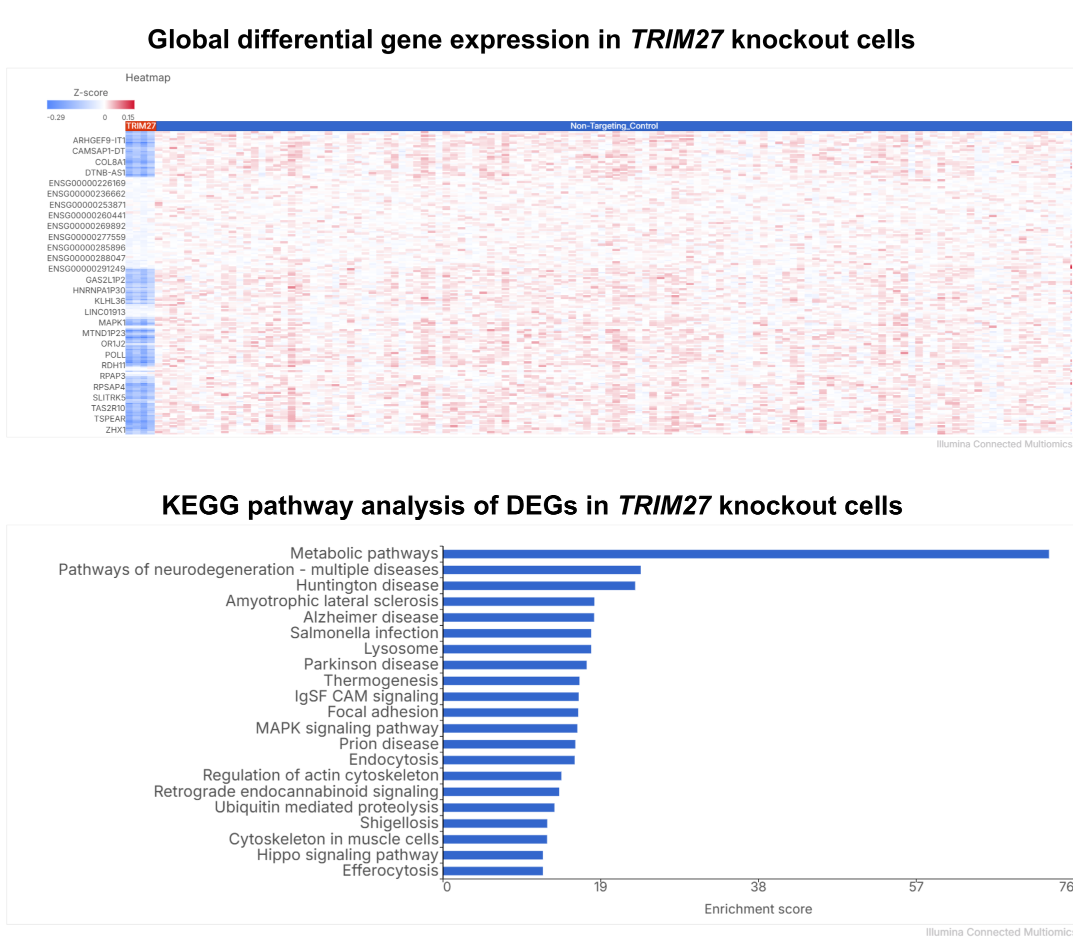
400,000 edited A549 and U2OS cells from the previously described screen were loaded in duplicate to Illumina Single Cell CRISPR Prep T100 kits. Libraries were sequenced on a NovaSeq X™ 25B flow cell at a read depth of ~50,000 reads/cell for the gene expression prep and ~15,000 reads/cell for the CRISPR prep. Samples were processed with DRAGEN™ v4.5. Guide assignments were performed using a custom implementation of gaussian mixture modeling with gRNA counts (Braunger *et al.*, *Bioinformatics*, 2024). Nearly every sgRNA is the library was recovered with a mean of **57** A549 cells and **55** U2OS cells per targeting sgRNA design after filtering.

5 Unbiased protein kinome perturbation atlases in two cancer models



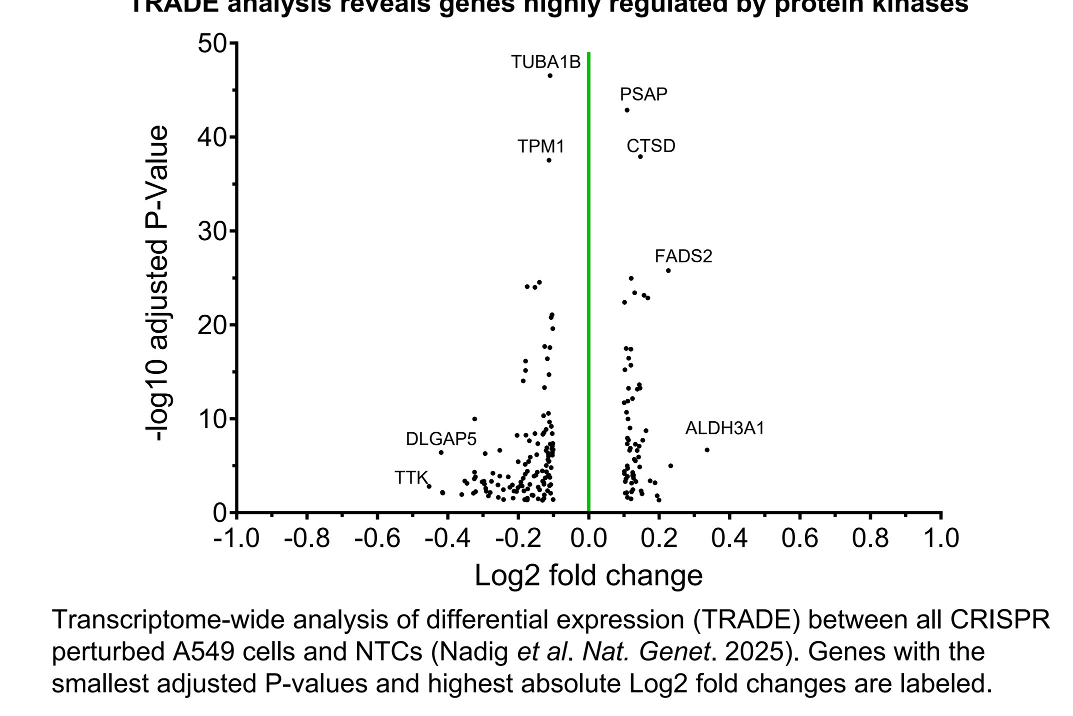
Identified CRISPR perturbations can be directly correlated to transcriptional knockdown of the targeted gene, as well as to known or putative downstream targets, when compared to cells expressing NTCs. Data analyzed performed with Illumina Connected Multiomics.

Global differential gene expression in *TRIM27* knockout cells



Identified CRISPR perturbations can also be correlated to global transcriptional changes, as visualized with the above heatmap. KEGG pathway analysis of the differentially expressed genes (DEGs) in *TRIM27* knockout cells. TRIM27 knockdown has been shown to inhibit the Hippo signaling pathway in other cancer types (Yao *et al.*, *Pathol Res Pract.* 2020).

TRADE analysis reveals genes highly regulated by protein kinases



Transcriptome-wide analysis of differential expression (TRADE) between all CRISPR perturbed A549 cells and NTCs (Nadig *et al. Nat. Genet.* 2025). Genes with the smallest adjusted P-values and highest absolute Log2 fold changes are labeled.

6 Summary

- The combination of the Tet-On system and the FKBP12-derived destabilizing domain greatly reduces leakiness while enabling potent CRISPR gene editing with the addition of two highly cell-permeable small molecules.
- Dual transcriptional and post-translational regulation of Cas9 expression provides precise temporal control over CRISPR knockout in pooled screening experiments.
- Dual sgRNA and mRNA capture with particle-templated instant partitions enables efficient single cell sequencing of large pooled CRISPR screens.
- This highly scalable, vortexer-based single cell workflow eliminates the need for specialized vectors or complex microfluidic equipment.
- Comprehensive perturbation atlases were created from the knockout of 760 protein kinases in human lung adenocarcinoma A549 cells and human osteosarcoma U2OS cells that can be used to uncover regulatory gene networks and resolve disease-relevant pathways.

Scan here to learn more about the Strict-R™ inducible system!

Scan here to check out Dharmacon™ CRISPR screening libraries

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