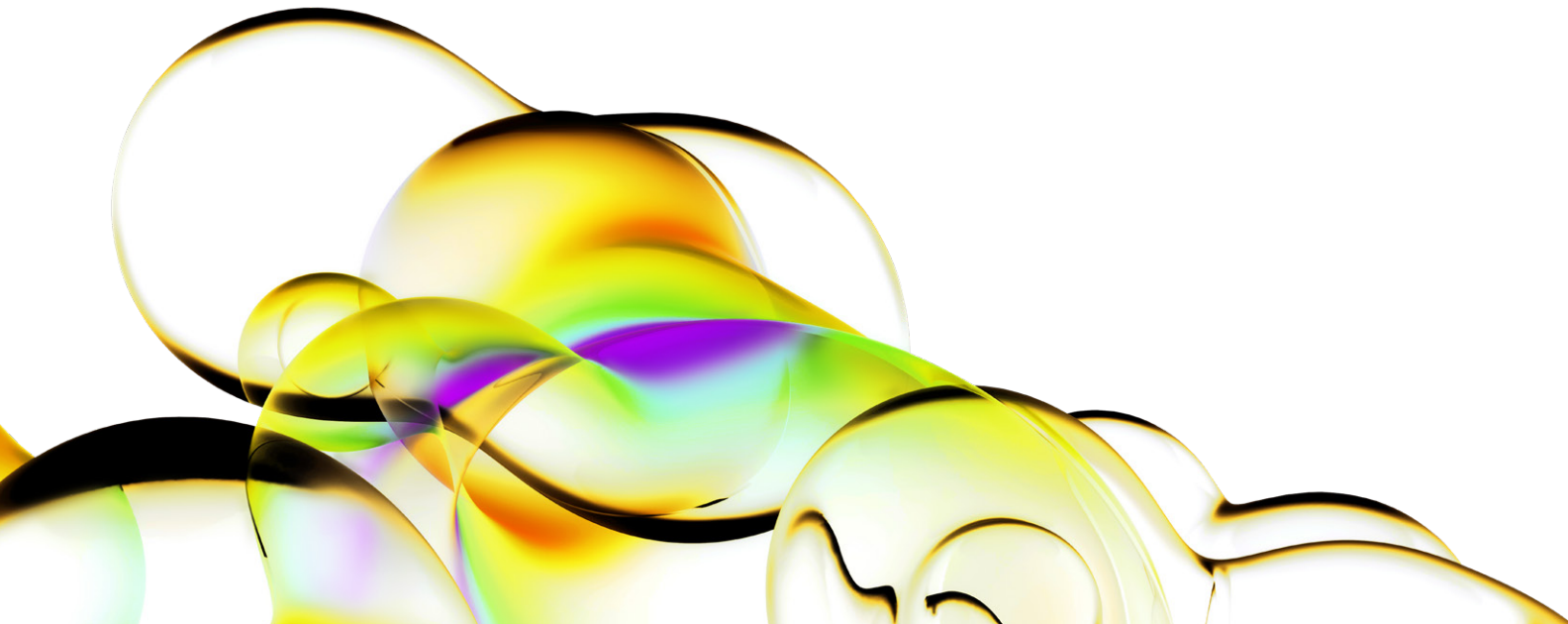
Population penetrance of CRISPRa-guided target overexpression using dCas9-VPR-EGFP mRNA.

Introduction

Gene overexpression experiments in cellular populations enable targeted hypothesis testing, and can complement loss-of-function studies as rescue or stand-alone activation experiments. The CRISPRa platform enables upregulation of specific gene target expression while maintaining the native genomic context, and can be scaled-up for high-throughput assays. A major consideration in these experiments is the penetrance, or fractional population of cells affected by the treatment resulting in an emergent phenotype. At the core of the CRISPRa system is an engineered, catalytically dead Cas9 (dCas9) fused to a triad of transcriptional activator proteins (VPR). This is programmed to target a gene-of-interest by a guide RNA (gRNA), which is designed based on the region immediately upstream of the gene's transcriptional start site (TSS). Following dCas9-VPR sgRNA-guided targeting, transcription is engaged from the gene's native expression cassette, ensuring a robust and specific increase in gene product.

Duration or permanence of CRISPRa target overexpression can be modulated to fit the desired assay needs of each researcher. dCas9-VPR-expressing stable cell lines (either pre-made or generated via lentiviral particles) enable extended timepoint assays, but may not be optimal in cases with cells sensitive to constitutive expression of the CRISPRa machinery or exogenous DNA. Alternately, introducing dCas9-VPR mRNA via co-transfection with synthetic CRISPRa crRNA:tracrRNA (guide RNA) provides for a transient overexpression of the gene target (1), and avoids the requisite steps of nuclear entry and transcription when utilizing a plasmid template, thus facilitating an accelerated overexpression milieu (2).



In gain-of-function experiments, it is important to distinguish whether a measurable phenomenon is present in an entire population, or a subsection of cells. Further, low expression of CRISPRa components (dCas9-VPR or guide RNA) or gene targeting should be excluded in experiments with low phenotype penetrance to validate experimental output.

Here we present the population penetrance of CRISPRa components and degree of downstream target protein overexpression, when dCas9-VPR is introduced via mRNA template, along with chemically modified synthetic gRNA.

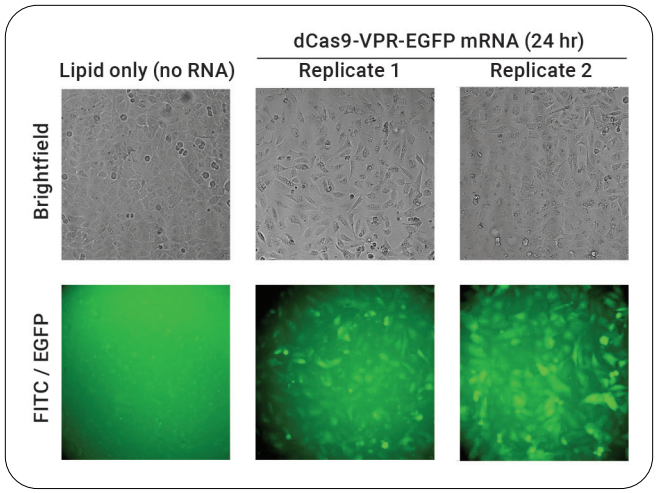
With dCas9-VPR-expressing mRNA, we demonstrate the following:

- Nearly complete EGFP expression (and by proxy, dCas9-VPR) in a population of transfected model cells
- Robust and rapid onset overexpression of target genes
- Nearly complete penetrance of overexpression across cellular populations
- Degree of overexpression is similar when using dCas9-VPR mRNA compared to dCas9-VPR-expressing stable cell lines

Results

dCas9-VPR expression in model cells is highly penetrant

A cancer cell line from an epithelial sarcoma (U2OS) was co-transfected with dCas9-VPR-EGFP mRNA and CRISPRa crRNA:tracrRNA targeting two genes, EGFR and IL1R2, using DharmaFECT™ Duo. The mRNA expression cassette includes an independent peptide sequence allowing for post-translational cleavage of EGFP from dCas9-VPR. In this system, cellular EGFP fluorescence serves as a proxy for dCas9-VPR expression. U2OS cells, 24 hours-post transfection with dCas9-VPR-EGFP mRNA exhibit fluorescence in nearly all observed cells (Figure 1), indicating generation of a complete population for targeted overexpression experiments.



Target overexpression is rapid and robust under CRISPRa control

To enhance targeting efficiency, pooled mixtures of crRNA guides targeting EGFR and IL1R2 were used, each designed to bind an independent locus upstream of the TSS. EGFR and IL1R2 expression was then assessed with quantitative PCR (qPCR). Elevations in target mRNA were immediate and sustained for up to 4 days and reached near-basal levels around days 6-7 post-transfection (Figure 2A). U2OS cells from the same tranfection experiments were processed for immunocytochemical microscopy, and, like trends in target gene RNA levels, acute overexpression of target proteins was measurable within 24 hours of transfection (Figure 2B). Elevation in target proteins persisted until a return to basal levels within 4-6 days post-transfection. Parallel CRISPRa experiments with synthetic CRISPRa crRNA:tracrRNA in stable dCas9-VPR-expressing U2OS cells indicate similar levels of target mRNA and protein overexpression (Figures 2A and 2B).

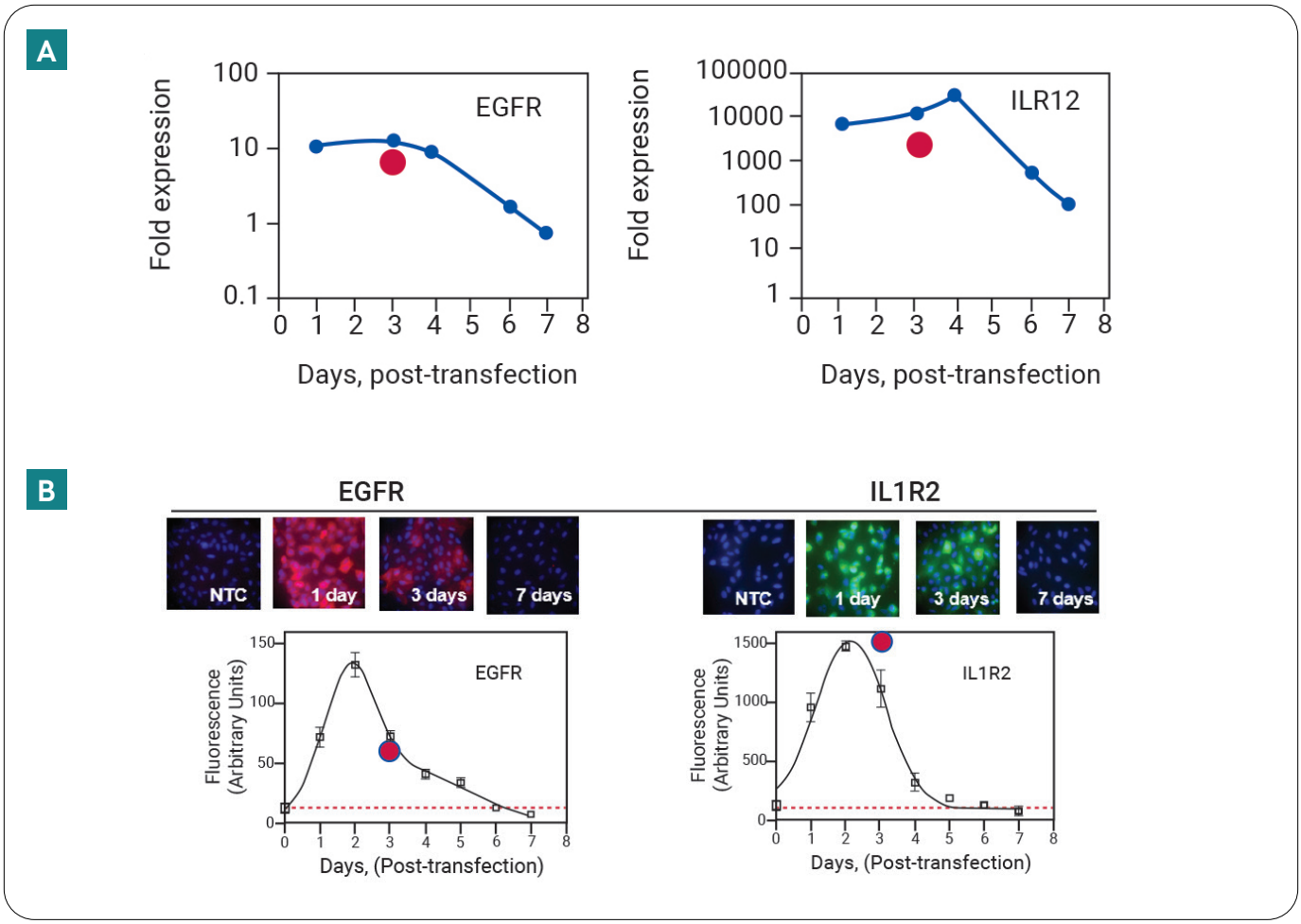


Figure 2: Relative fold target gene RNA and protein expression after dCas9-VPR-EGFP mRNA and CRISPRa crRNA:tracrRNA co-transfection in U2OS cells. Blue trace indicates qPCR signal relative to non-targeting control (1A). Representative fluorescent images of target protein after immunofluorescent microscopy imaging (1B). Traces indicate fluorescent signal relative to non-targeting control (red dotted line) in CRISPRa experiment with dCas9 VPRmRNA in U2OS cells. Red circles indicate corresponding signal in parallel CRISPRa experiments with a stable dCas9-VPR-expressing U2OS cell line.

Penetrance measurements indicate high degree of over expression amongst cell populations in CRISPRa experiments

To assess measures of target overexpression amongst cell populations, an immunocytofluorescent image analysis pipeline was generated using CellProfiler 4 (3). Once assembled, the semi-automatic pipeline applies masks to cells based on user-defined parameters (object diameter, average pixel threshold, etc) and generates an output comprised of mean intensity per object (cells or nuclei under the applied object diameter settings), number of cells per image and other data was not germane to this application (object location, edge intensity, etc). At 72-hours post-transfection, fluorescent cell intensity data for EGFR and IL1R2 targets, non-targeting transfections and autofluorescent controls were binned and fit to a Gaussian model using Graphpad Prism 9 (Figure 3A).

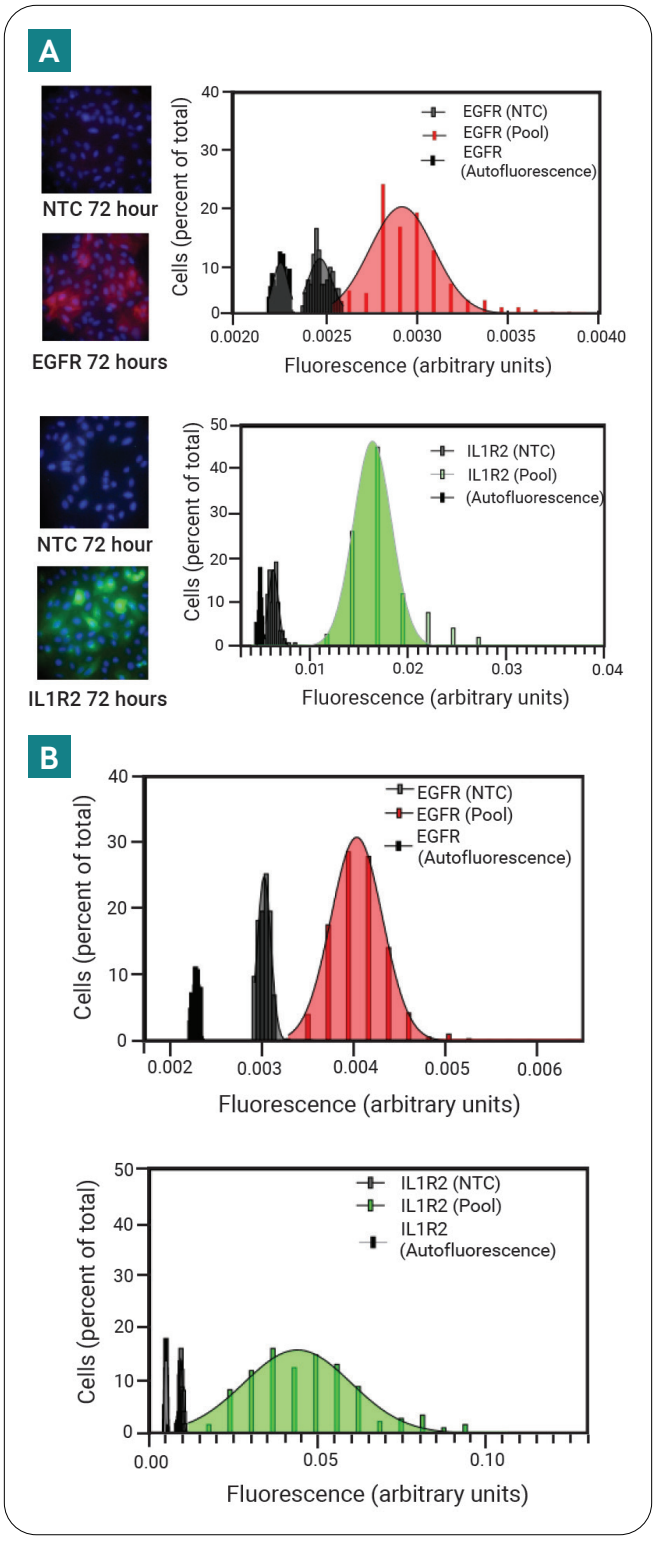


Figure 3: Population analysis of immunocytochemical images from CRISPRa experiments in U2OS cells. Representative fluorescent images of cells transiently transfected with dCas9-VPR and either target gRNA or non-targeting control gRNA (NTC) and subsequent population analysis (Figure 2A). Corresponding analysis in parallel experiments in stable dCas9-VPR-expressing U2OS cells (Figure 2B).

Per this analysis, nearly the entire population of imaged, transfected cells exhibited a greater degree of fluorescence compared to non-transfected control populations. Thus, nearly 100% of the overexpression phenotype was penetrant (penetrance values as percent of total population of transfectants: EGFR ~ 90%, IL1R2 ~100%).

This analysis also supports evaluation of overexpression heterogeneity amongst a cell population. By determining Gaussian curve span at ½ height, these models offer an evaluation of population variability. Consequently, a narrower Gaussian span suggests a population of cells exhibiting homogenous target protein expression. According to present qualitative analysis of variability, overexpression appears to be heterogenous, likely due to expected differences in cytoplasmic mRNA, gRNA and dCas9-VPR molecule partitioning (4) and /or a heterogenous proteome in cancer cell populations (5).

Parallel CRISPRa experiments with synthetic CRISPRa crRNA:tracrRNA and stable dCas9-VPR-expressing U2OS cells indicate comparable penetrance and heterogeneity of protein overexpression (Figure 3). Comparing immunocytochemical analyses of stable cells with cells co-transfected with dCas9-VPR-EGFP mRNA indicates nearly complete penetrance of overexpression in both cases, along with similar degrees of heterogeneity.

Discussion

Revvity's CRISPRa system can be customized for the duration of target gene overexpression and sensitivity of cell line to exogenous DNA. Here we demonstrate the use of CRISPRa dCas9-VPR-EGFP mRNA for transient overexpression experiments where a rapid rise in endogenous target protein levels can be initiated throughout a cellular population by co-transfecting with synthetic CRISPRa crRNA:tracrRNA). In addition, we establish that overexpression penetrance and efficacy when using dCas9-VPR-EGFP mRNA is comparable to using stable cell lines expressing dCas9-VPR.

By analyzing immunocytochemical microscopy images, we find that these CRISPRa components, whether transiently or stably expressed, can induce target protein overexpression throughout a cellular population. Further, estimated expression heterogeneity indicates a similar range of protein expression in cells, regardless of whether dCas9-VPR is expressed in a transient or stable background.

Transient target gene overexpression can be ideal where experimental conditions require rapid target amplification followed by a return to basal expression conditions. In addition, due to the ease with which overexpression can be initiated in a cell population following a simple mRNA and crRNA:tracrRNA co-transfection, this platform can enable scaling up towards arrayed high-content screening experiments.

Materials and methods

Tissue culture

U2OS cells (ATCC, HTB-96) and stable dCas9-VPR U2OS cells (Revvity, dCas9-VPR-012) were maintained in established media (high glucose DMEM with 10% (v/v) FBS and 2 mM L-glutamine) at 37 °C and 5% CO2. Cells were passaged by rinsing with calcium-free PBS, incubating with 0.25% trypsin for 5 minutes at 37 °C, then diluting with full media and reseeding on fresh 96-well plates.

Transfection

Transient dCas9-VPR experiments: U2OS cells were seeded in 96-well, glass bottom plates at 10,000 cells/well one day prior to transfection. Cells were transfected with crRNA:tracrRNA (Revvity, EGFR crRNA SMARTpool: P-003114-01; IL1R2 crRNA SMARTpool, P-007690-01 and EDIT-R tracrRNA, U-002005)and dCas9-VPR-EGFP mRNA (Revvity, CAS12212) at final concentrations of 25 mM and 200 ng/well, respectively. Synthetic crRNA:tracrRNA and dCas9-CPR-EGFP mRNA were complexed with 0.5 uL/well DharmaFECT Duo Transfection Reagent (Revvity, T-2010) in serum free media for 20 minutes at room temperature. Cell growth media was removed and replaced with complexed transfection mixtures, after being diluted 5-fold into complete growth media. Each condition was replicated eightfold to enable replicate measures and propagation through the experimental timeline.

Stable dCas9-VPR experiments: Parallel experiments with U2OS cells engineered to stably express dCas9-VPR protein (1) were carried out in a similar manner as above, but without the inclusion of dCas9-VPR mRNA.

RT-qPCR

Relative target and control gene expression was determined at 24-hour timepoints from 24-168 hours post-transfection. Total RNA was isolated from cell lysates using an SV 96 Total RNA Isolation System (Promega, Z3500). From RNA isolates, cDNA was synthesized using a Maxima First Strand cDNA Synthesis Kit (Thermo, K1672). 7.5 uL TaqMan Master Mix (Thermo, 4304437) was mixed with 2.0 uL undiluted cDNA and 0.5 uL of appropriate TaqMan probe (Thermo, GAPDH, 02786624_g1; EGFR, 01076090_m1; IL1R2, 00174759_m1). Samples were mixed and 10 uL of each reaction was transferred to individual wells on a 384-well plate and loaded onto a Roche LightCycler 480 II for thermal cycling and analysis. Each sample was performed in technical duplicate. The relative expression of each target gene was calculated with the ΔΔCq method using GAPDH as the housekeeping gene and normalized to a non-targeting control.

Fluorescence microscopy and analysis

For immunocytochemical analysis, cells were processed on the 96-well glass culture support. After removing media, 4% paraformaldehyde (PFA) fixative was added for 30 minutes at room temperature, then washed twice with PBS. Cells were permeabilized by incubating with with 0.5% (v/v) Triton X-100 in PBS for 15 minutes at room temperature and washed three times with PBS. After blocking cells with 3% (w/v) BSA in PBS for 30 minutes at room temperature, 5 ug/mL mouse-derived anti-EGFR (Abcam ab30) and 5 ug/mL goat-derived anti-IL1R2 (Novus, AF-263) were added to blocking solution for 1 hour incubation at room temperature with gentle rotation. Cells were then washed three times with PBS with 0.1% (w/v) BSA and 0.1% (v/v) Triton X-100, then incubated with fluorescent secondary detection antibodies (1:1000 dilutions of each goat anti-mouse conjugated to AF555 [Thermo, A32727] and donkey anti-goat conjugated to AF488 [Thermo, A32814]) in PBS. After three washes with PBS with 0.1% (w/v) BSA and 0.1% (v/v) Triton X-100, 1 ug/mL Hoeschst 33342 in PBS was added for 15 minutes at room temperature followed by three washes in PBS. Wells were left in 100 uL PBS and sealed for imaging. Fluorescent images were captured at 20X magnification using a Nikon Eclipse Ti and processed with CellProfiler image analysis software and Prism (GraphPad).

References

1. Strezoska, Ž., Dickerson, S. M., Maksimova, E., Chou, E., Gross, M. M., Hemphill, K., Hardcastle, T., Perkett, M., Stombaugh, J., Miller, G. W., Anderson, E. M., Vermeulen, A., & Smith, A. (2020). CRISPR-mediated transcriptional activation with synthetic guide RNA. Journal of biotechnology, 319, 25–35.

2. Leonhardt, C., Schwake, G., Stögbauer, T. R., Rappl, S., Kuhr, J. T., Ligon, T. S., & Rädler, J. O. (2014). Single-cell mRNA transfection studies: delivery, kinetics and statistics by numbers. Nanomedicine : nanotechnology, biology, and medicine, 10(4), 679–688.

3. Stirling, D. R., Swain-Bowden, M. J., Lucas, A. M., Carpenter, A. E., Cimini, B. A., & Goodman, A. (2021). CellProfiler 4: improvements in speed, utility and usability. BMC bioinformatics, 22(1), 433.

4. Huh, D., & Paulsson, J. (2011). Random partitioning of molecules at cell division. Proceedings of the National Academy of Sciences of the United States of America, 108(36), 15004–15009.

5. Mahdessian, D., Cesnik, A. J., Gnann, C., Danielsson, F., Stenström, L., Arif, M., Zhang, C., Le, T., Johansson, F., Shutten, R., Bäckström, A., Axelsson, U., Thul, P., Cho, N. H., Carja, O., Uhlén, M., Mardinoglu, A., Stadler, C., Lindskog, C., Ayoglu, B., … Lundberg, E. (2021). Spatiotemporal dissection of the cell cycle with single-cell proteogenomics. Nature, 590(7847), 649–654.

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