APPLICATION NOTE

Orthogonal validation of CRISPR-Cas9 and siRNA generated phenotypes using cell painting.

Key features

- Phenotypic validation of cell cycle gene inhibition: CRISPR-Cas9 knockout and siRNA knockdown with Dharmacon™ reagents
- Phenotypic clustering by dimensionality reduction using High Content Profiler App in Signals™ *VitroVivo*
- Identification of differences in phenotypic response kinetics and heterogeneity in phenotypic prevalence

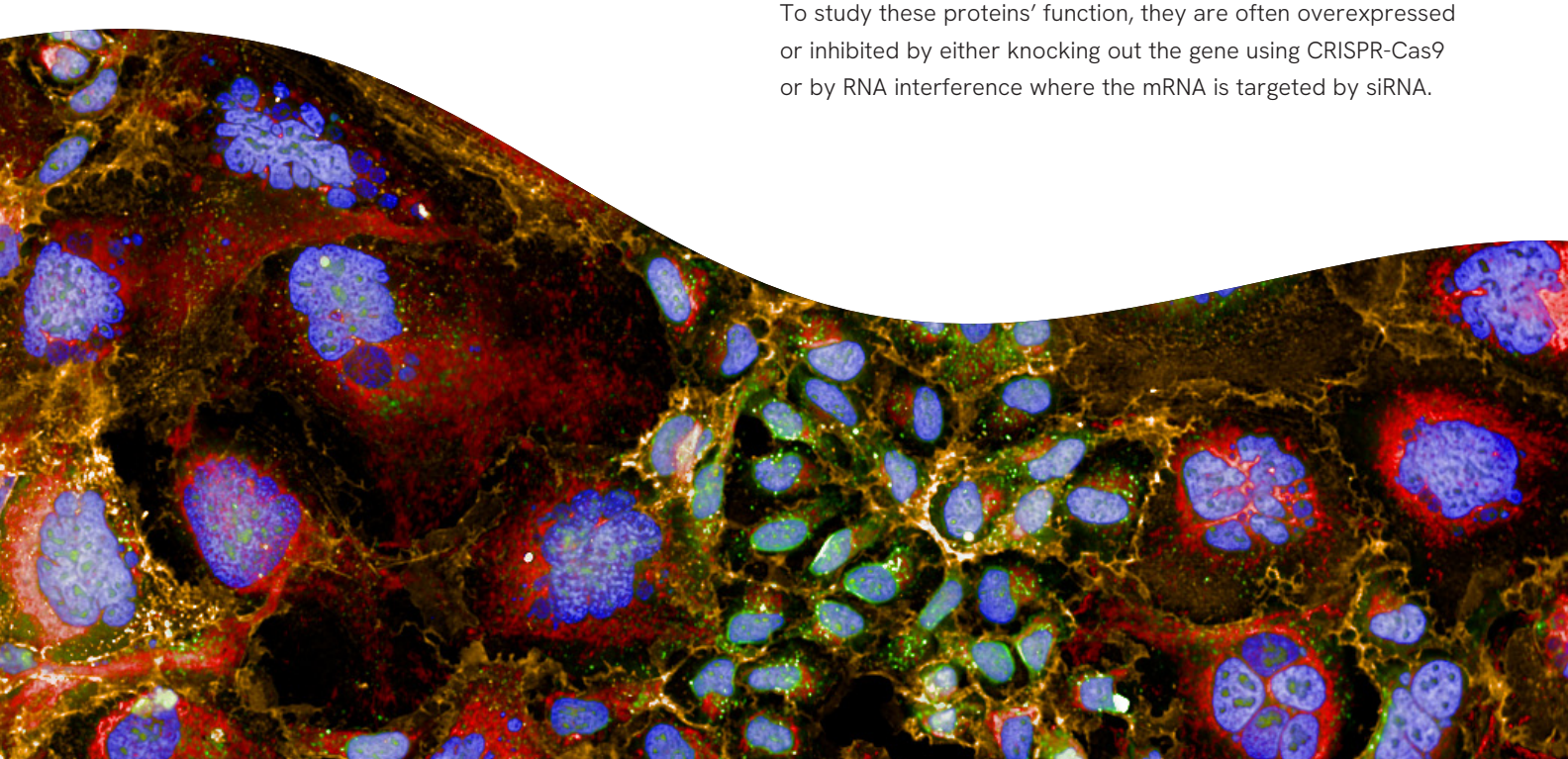
Introduction

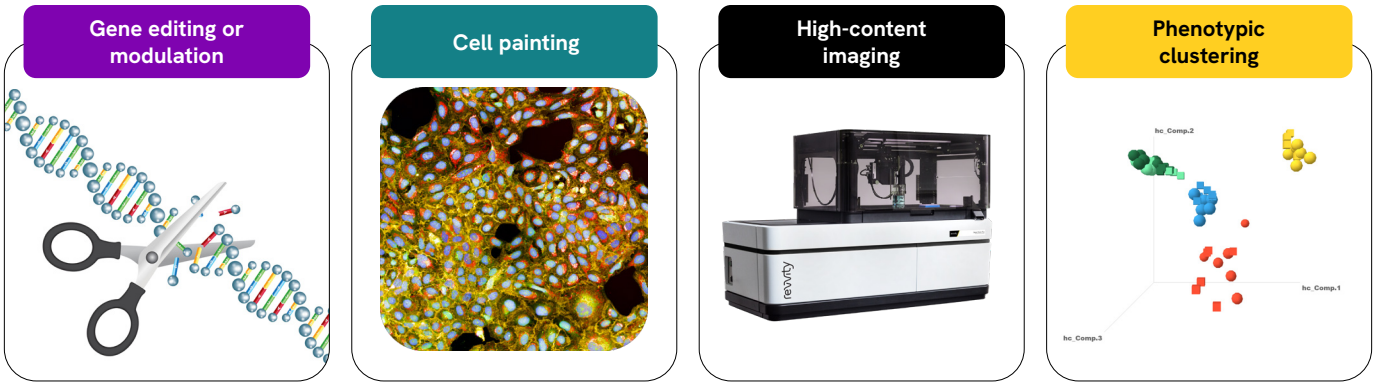
The cell cycle is a fundamental process that cells undergo to divide and replicate. It consists of a series of phases: G1 (growth), S (DNA synthesis), G2 (preparation for mitosis), and M (mitosis), where the cell divides into two daughter cells. Aurora kinase B (AURKB), Geminin (GMNN), and Polo-like kinase 1 (PLK1) are crucial proteins that regulate various stages of the cell cycle. AURKB is involved in chromosome alignment and segregation during mitosis. GMNN prevents DNA from being replicated more than once per cycle, thus maintaining genomic stability. PLK1 is essential for the initiation of mitosis, spindle assembly, and cytokinesis.

If AURKB is knocked out, it can lead to errors in chromosome segregation, resulting in aneuploidy, a condition where cells have an abnormal number of chromosomes, which is often associated with cancer. The absence of GMNN can cause uncontrolled DNA replication, leading to genomic instability and potential tumorigenesis. Knocking out PLK1 disrupts the regulation of mitosis, which can halt cell division and lead to apoptosis or programmed cell death.

Understanding the cell cycle and its regulatory mechanisms is crucial for advancements in medical research and treatment strategies. The intricate interplay of these proteins ensures the fidelity of cell division, which is essential for the growth and repair of tissues and the overall health of an organism.

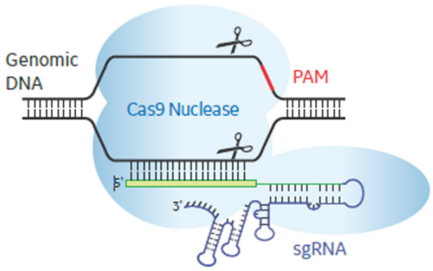
To study these proteins' function, they are often overexpressed or inhibited by either knocking out the gene using CRISPR-Cas9 or by RNA interference where the mRNA is targeted by siRNA.



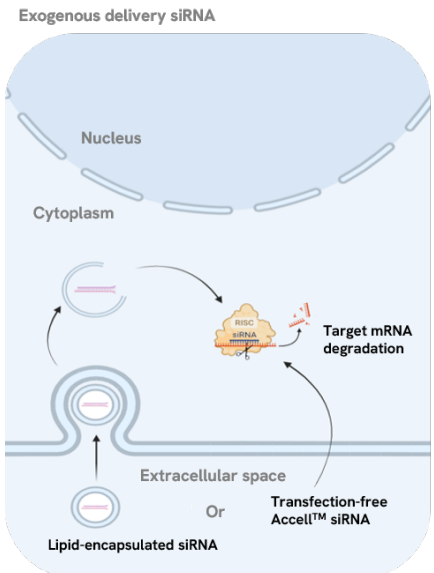


Here, we show how either CRISPR-Cas9 or siRNAs can be used to target the cell cycle regulators AURKB, GMNN, and PLK1, and how the resulting phenotypes from the different methods can be compared using cell painting and high-content imaging on the Opera Phenix™ Plus high-content screening system. Analysis was performed in Harmony™ high-content imaging and analysis software followed by secondary analysis using the High Content Profiler App in Signals™ *VitroVivo*.

Gene knockout



Gene knockdown



Gene knockout via CRISPR-Cas9 (Clustered regularly interspaced short palindromic repeats, CRISPR-associated protein 9) is a revolutionary gene-editing technology that allows for permanent, directed changes to genomic DNA by introducing double-strand breaks in the DNA leading to mutations during repair.

At its core, CRISPR-Cas9 relies on three components: 1. the Cas9 nuclease, 2. CRISPR RNA (crRNA) comprised of targeting and repeat sequences, 3. tracrRNA, which hybridizes to the crRNA through its anti-repeat sequence. Alternatively, the crRNA and tracrRNA can be linked together with a loop sequence for generation of chimeric single guide RNA (sgRNA). The CRISPR-Cas9 complex recognizes DNA targets that are complementary to the crRNA sequence (called spacer) which sits next to the protospacer adjacent motif (PAM). Upon site-specific double-strand DNA cleavage, the cell can repair the break through Non-Homologous End Joining (NHEJ). Because NHEJ is imperfect, it results in small insertions and deletions (indels) that lead to functional gene knockout.

To knockdown the mRNA, small interfering RNAs (siRNAs) have been proven as very effective tools. siRNAs are short, double-stranded RNA molecules transiently silencing gene expression post-transcriptionally by degrading mRNA. They work within the RNA interference (RNAi) pathway by binding to complementary messenger RNA (mRNA) sequences and promoting their degradation, thus preventing the translation of specific proteins. siRNAs are a powerful tool for studying gene function and have potential therapeutic applications in silencing harmful genes.

Both CRISPR-Cas9 and siRNA technologies have opened new avenues for research offering precise and efficient ways to manipulate gene expression, with CRISPR-Cas9 being more efficient and specific, and siRNA less permanent and more suitable for transient studies.

Results and Discussion

Phenotypic analysis using principal component analysis

In the siRNA-mediated knockdown experiment, wild-type U2OS cells were transfected with Dharmacon ON-TARGETplus™ siRNAs that specifically target AURKB, GMNN, and PLK1 (U2OS/siRNA). In the CRISPR-Cas9 mediated knockout experiment, Cas9-stable U2OS cells were transfected with Dharmacon Edit-R™ synthetic single guide RNAs (sgRNAs) that target the respective cell cycle regulators (U2OS/Cas9). Ninety-six hours post transfection, cells were stained using the PhenoVue Cell Painting JUMP Kit followed by imaging and analysis on Opera Phenix Plus with the Harmony software[1].

As anticipated, perturbing any of the targets not only led to changes in phenotype but also to a significant reduction in cell number, in accordance with the gene's profound impact on cell cycle progression (Figure 1).

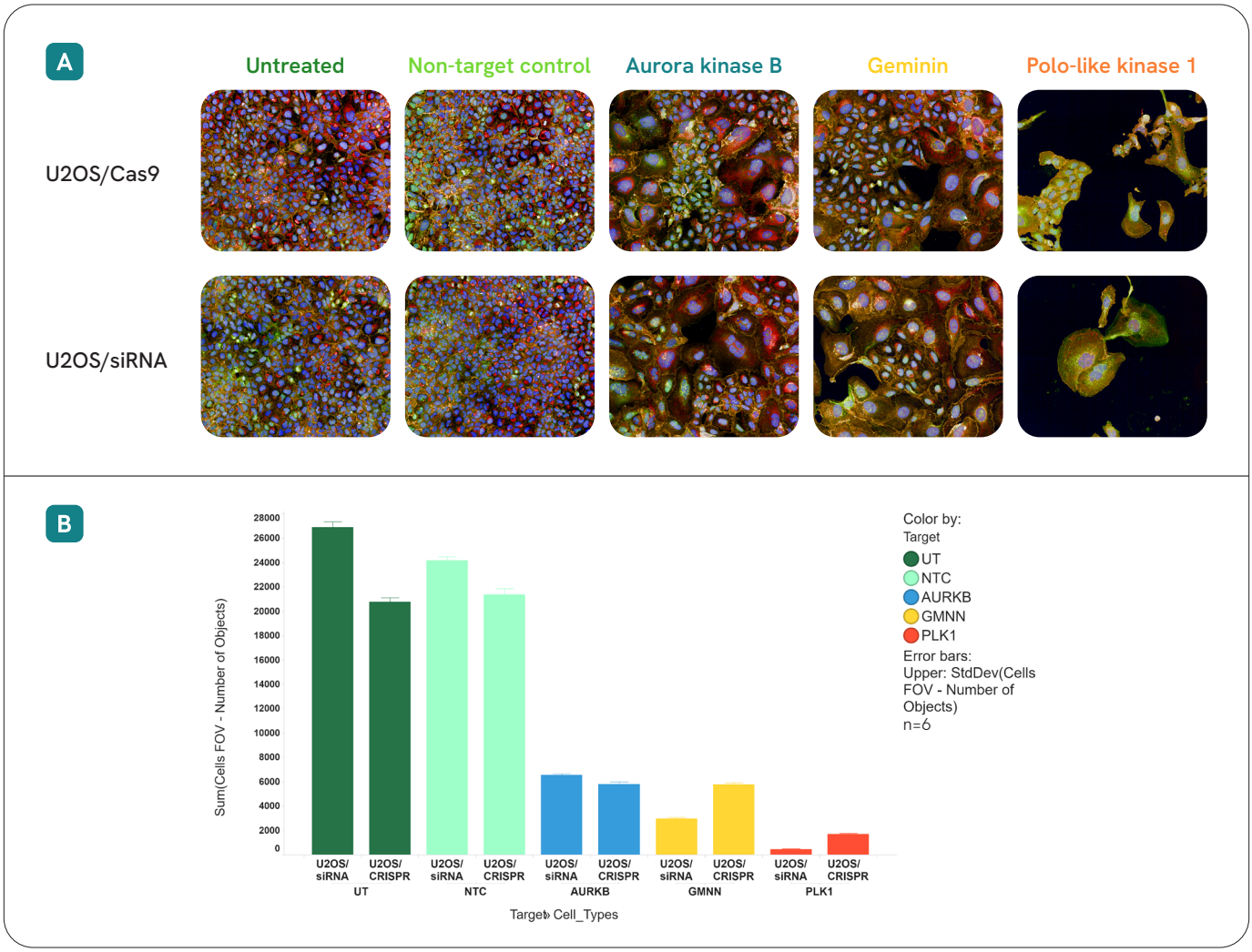


Figure 1: Example images showing phenotypic changes and reduction in cell number 96 hours after inhibition of three cell cycle regulators AURKB, GMNN or PLK1. Perturbed cells were compared to untreated (UT) and non-targeting controls (NTC).

A: Example images of cell-painted cells acquired confocally on Opera Phenix with 20x water immersion lens. Phenotypes of perturbed cells strongly differ from controls e.g. in size, confluency, number of nuclei per cell, for both knockdown and knockout treatments. Each image shows one field of view in a maximum intensity projection of four planes, with a plane distance of 1 µm.

B: The total cell count from six wells across two plates decreases following the knockout or knockdown of genes/mRNA, as all three targets influence cell cycle progression. Moreover, siRNA treatment leads to a comparatively greater reduction in cell numbers for GMNN and PLK1.

To reduce the dimensionality of the data and identify phenotypic clusters, we performed a principal component analysis (PCA) on the data (Figure 2). PCA is a statistical method that helps in identifying patterns and relationships within high-dimensional data while it retains distinguishing information. It accomplishes this by transforming the original data into a new set of uncorrelated variables, known as principal components. Each of these components captures a portion of the total variance in the data, with the first component accounting for the most variance. This technique is commonly used in exploratory data analysis, pattern recognition, and to enhance the efficiency of predictive models.

Controls, AURKB and PLK1 knockouts/knockdowns form distinct clusters independent from the gene modulation method used, suggesting that there is ample variance within the data to differentiate the phenotypes. However, the GMNN phenotypes are more separated: The siRNA-mediated phenotype deviates from the CRISPR-Cas9-mediated phenotype along the first component, which accounts for the most variance.

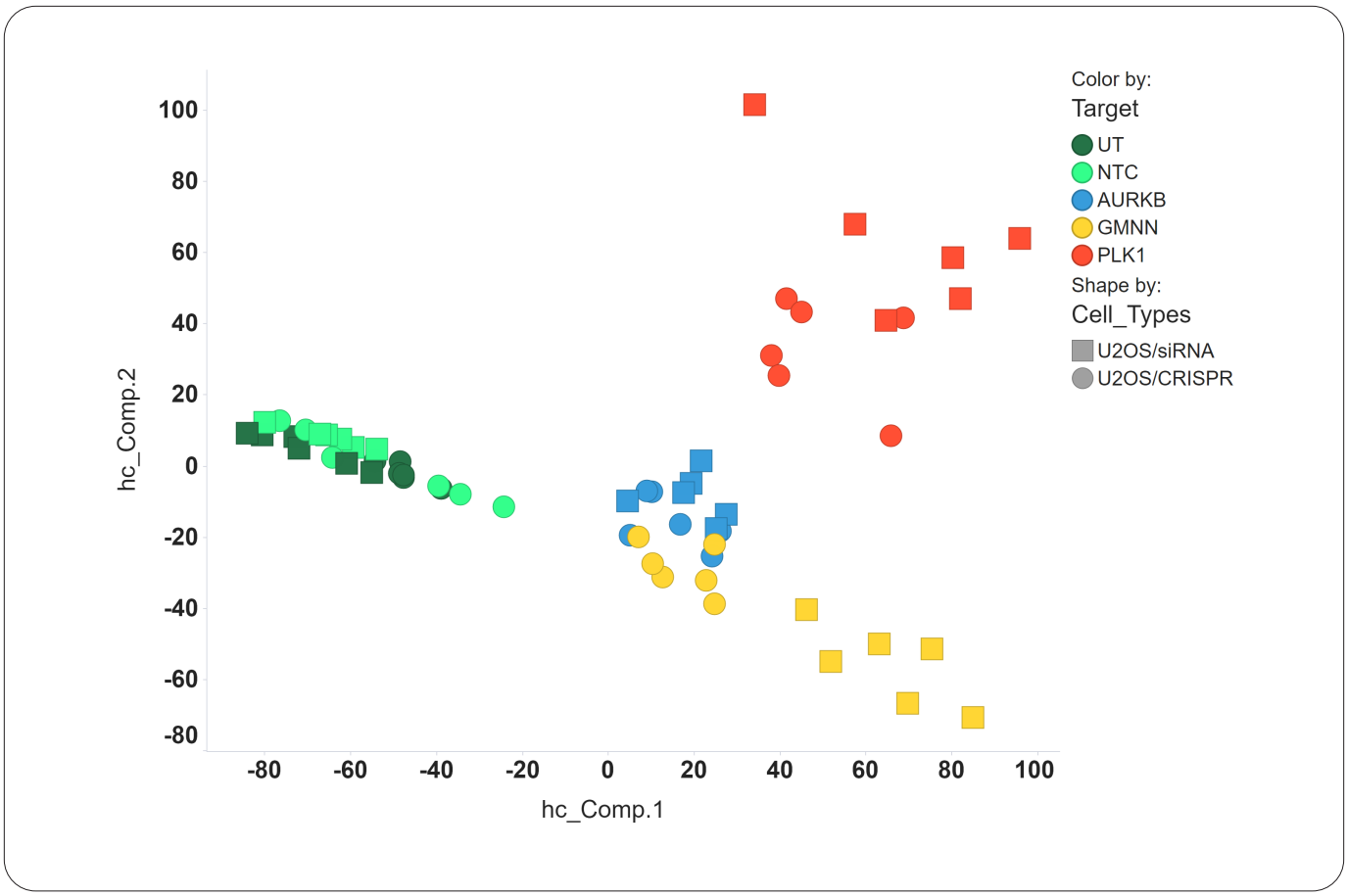
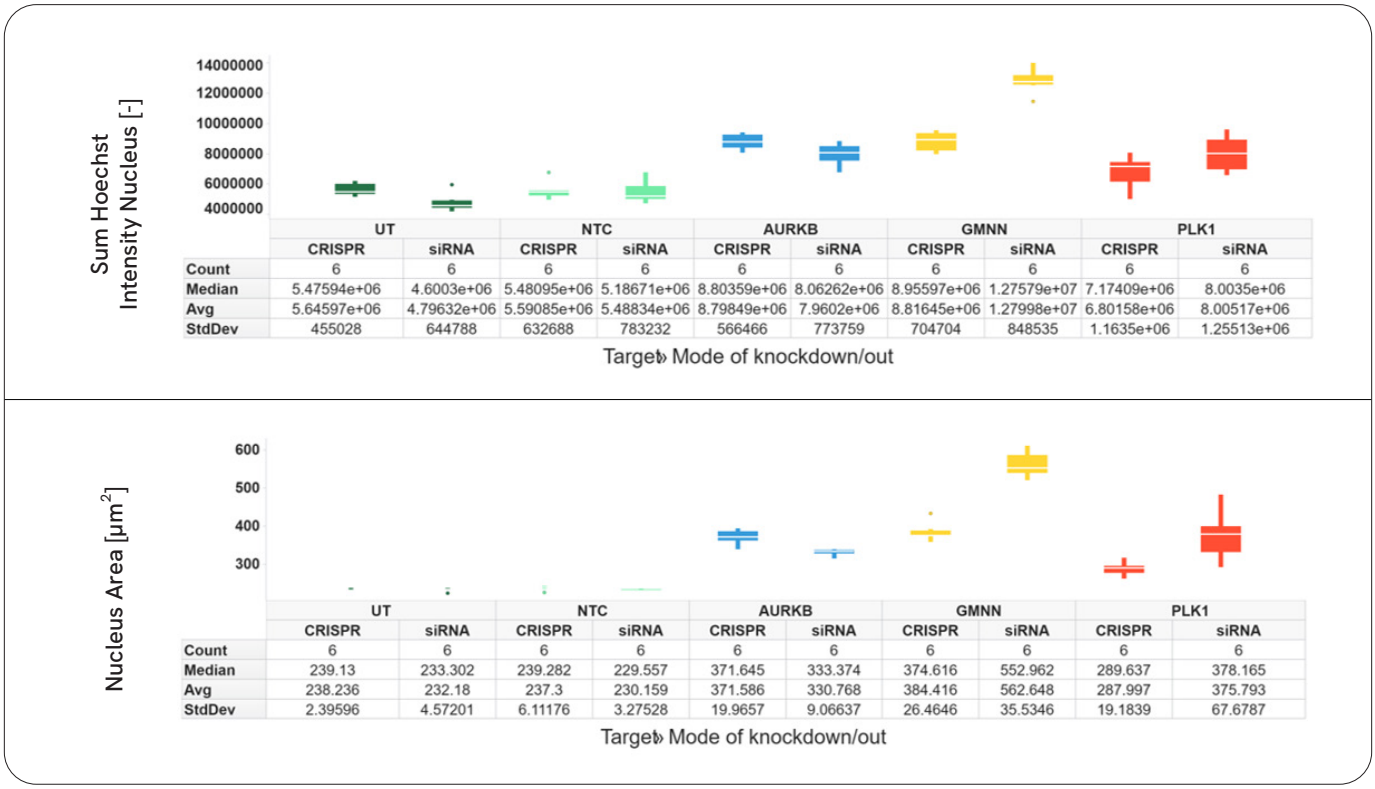


Figure 2: Principal component analysis of (PCA) controls and cell cycle target knockouts/knockdowns results in the formation of distinct clusters. By applying PCA, we reduce the original large feature data set (5930 features) to a 2-dimensional coordinate system, effectively describing phenotypic variations and patterns. Untreated controls (UT), non-targeting controls (NTC) as well as PLK1 and AURKB exhibit close clusters of similar phenotypic patterns, for both modes of knockdown/out. However, GMNN forms separate clusters of the siRNA-mediated phenotype and the CRISPR-Cas9-mediated one, with the latter closely associated with the AURKB cloud.
Data points are shaped according to the mode of knockdown (□) / knockout (O) and originate from 2x3 wells from 2 independent plates.

Gene editing versus modulation for GMNN inhibition

GMNN serves to prevent DNA re-duplication, and a deficiency in GMNN could lead to the formation of polyploid cells. As a measure of GMNN inhibition, we examined the DNA content and nuclear size in cells where GMNN was inhibited by CRISPR-Cas9 and siRNA and compared these to control cells (Figure 3). The knockdown of GMNN via siRNA resulted in the greatest Hoechst sum intensity in the nucleus and the largest increase in nuclear area, indicating a potent inhibition of GMNN. On the other hand, while the CRISPR-mediated knockout also showed an increase in sum intensity and nuclear area compared to control cells, the effect was less pronounced than with siRNA.



	UT		NTC		AURKB		GMNN		PLK1	
	CRISPR	siRNA	CRISPR	siRNA	CRISPR	siRNA	CRISPR	siRNA	CRISPR	siRNA
Count	6	6	6	6	6	6	6	6	6	6
Median	5.47594e+06	4.6003e+06	5.48095e+06	5.18671e+06	8.80359e+06	8.06262e+06	8.95597e+06	1.27579e+07	7.17409e+06	8.0035e+06
Avg	5.64597e+06	4.79632e+06	5.59085e+06	5.48834e+06	8.79849e+06	7.9602e+06	8.81645e+06	1.27998e+07	6.80158e+06	8.00517e+06
StdDev	455028	644788	632688	783232	566466	773759	704704	848535	1.1635e+06	1.25513e+06

	UT		NTC		AURKB		GMNN		PLK1	
	CRISPR	siRNA	CRISPR	siRNA	CRISPR	siRNA	CRISPR	siRNA	CRISPR	siRNA
Count	6	6	6	6	6	6	6	6	6	6
Median	239.13	233.302	239.282	229.557	371.645	333.374	374.616	552.962	289.637	378.165
Avg	238.236	232.18	237.3	230.159	371.586	330.768	384.416	562.648	287.997	375.793
StdDev	2.39596	4.57201	6.11176	3.27528	19.9657	9.06637	26.4646	35.5346	19.1839	67.6787

Figure 3: Nucleus sum intensities and area after CRISPR knockout and siRNA knockdown: Revealing that GMNN inhibition by siRNA knockdown results in a higher DNA content as well as larger nuclear size compared to GMNN knockout and compared to the other treated cells and controls.

A: Box plot analysis of DNA content (Hoechst sum intensity in the nucleus) from six wells across two independent plates. Regardless of the method used, DNA content increases upon inhibition of cell cycle targets. siRNA mediated knockdown of GMNN shows the strongest increase.

B: Box plot analysis of nuclear size reveals its increase for all three cell cycle targets compared to controls, with the most pronounced effect observed in GMNN knockdown using siRNA.

Upon examining the images of nuclei from cells in which GMNN was inhibited (Figure 4 A), there are fewer cells with larger nuclei in the U2OS/Cas9 cells compared to the siRNA cells. This suggests that the observed differences in intensity and nuclear area do not uniformly apply to all cells. Instead, they originate from a heterogeneous population of larger and smaller nuclei. In the wells where GMNN was knocked down by siRNA, 44.4% of the cells are large (> 600 µm^2), compared to only 18% in cells where GMNN was knocked out by CRISPR (Figure 4 B).

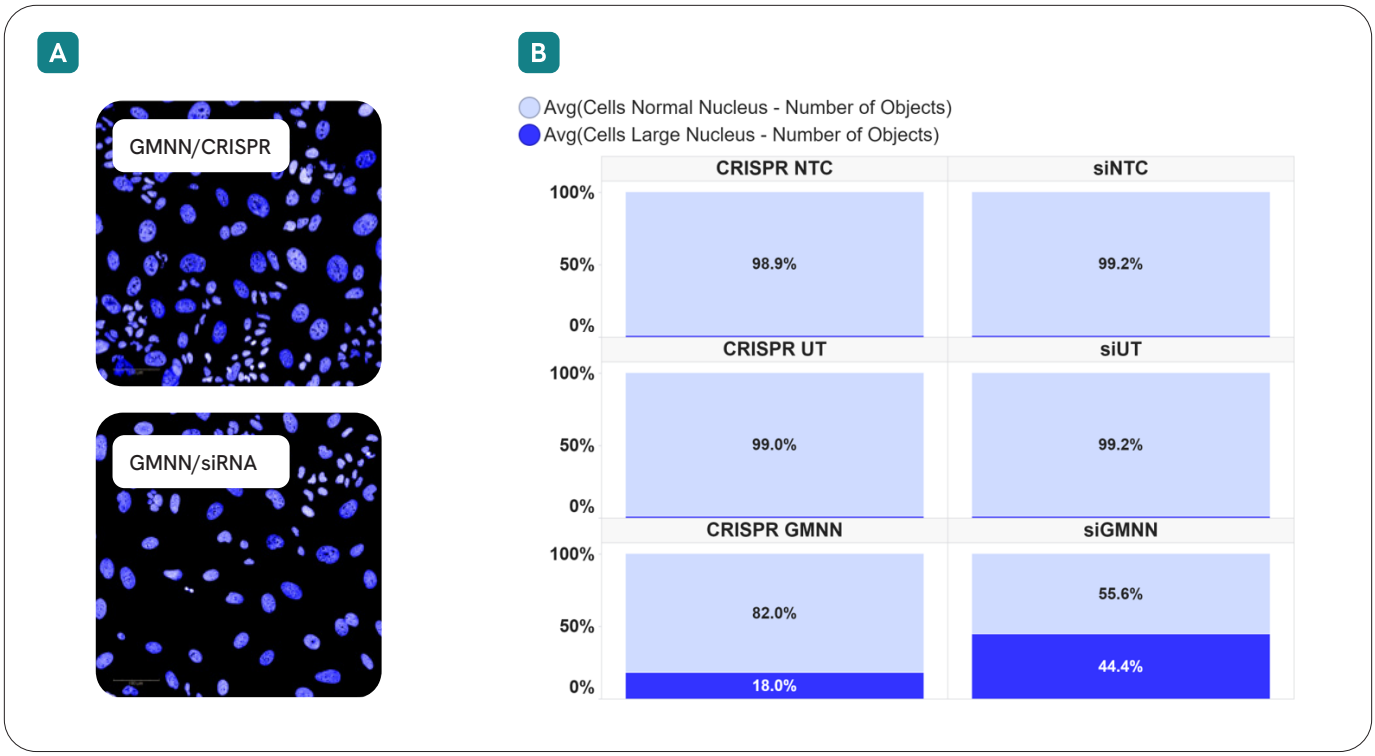


Figure 4: Prevalence of larger nuclei in GMNN-targeted cells: The size of nuclei is heterogeneously distributed, with siRNA-treated cells showing a higher proportion of larger nuclei (> 600 μm^2).

A: Images show varying ratios of larger (> 600 μm^2) to smaller nuclei in cells where GMNN is knocked out or knocked down. The proportion of large nuclei is relatively higher in the siRNA-treated cell population.

B: Percentage of large nuclei in controls (non-targeting and untreated) vs. GMNN-targeted cells: 44.4% of nuclei are large (> 600 μm^2) when GMNN is knocked down by siRNA, compared to only 18% in cells where GMNN was knocked out by CRISPR.

We re-evaluated the data from the GMNN knockout experiment, this time focusing on the cell painting properties of nuclei that exhibited either the large or small phenotype. When we amalgamated the findings from the large GMNN CRISPR knockouts with the results from all cells in other conditions, the PCA-shift between the siRNA and CRISPR-mediated GMNN inhibition was eliminated, leading to a unified clustering of the wells (Figure 5A top vs. 5B top).

This prompted us to question whether the small cells are more akin to the control phenotype or represent a different form of the GMNN knockout/knockdown phenotype.

The PCA for the small GMNN-perturbed cells shows that they are clearly distinct from the control cells, with CRISPR and siRNA being closer together as well but not as separated from the other targets when compared to the large cells (Figure 5C top). This points to the fact that these cells are also GMNN-inhibited but display a different phenotype.

Overall, this shows that both ways to inhibit GMNN led to the same phenotypic changes with siRNA having the faster kinetics for this distinct target.

Hierarchical clustering

An alternative approach to allocate phenotypes to clusters is hierarchical clustering. In this case, we employed the Ward's method for the clustering of both columns (encompassing all 5930 features) and rows (with row numbers serving as identifiers for the different wells). Ward's method is an agglomerative technique that aims to minimize the variance within the data. It initiates with each data point in an individual cluster, which are subsequently merged to reduce the variance. This method is particularly beneficial for quantitative variables.

Figure 5A compares the result of this clustering with the PCA analysis from figure 2 for identical data sets. When considering all cells in the GMNN knockout/knockdown experiments (Figure 5A bottom), it is observed that the cells modified by CRISPR are distinct from those modified by siRNA. Interestingly, these CRISPR-modified cells cluster more closely with the AURKB knockout/knockdown cells. However, when we focus only on the "large"-nucleated cells in the GMNN knockout/knockdown experiments (Figure 5B bottom), these cells form a tight cluster. A similar pattern is observed when analyzing only the "small" sized nuclei (Figure 5C bottom).

It is important to note that these "normal" cells with "smaller" nuclear phenotype are distinctly clustered away from the control wells. This suggests that they already exhibit a phenotype consistent with GMNN knockout/ knockdown.

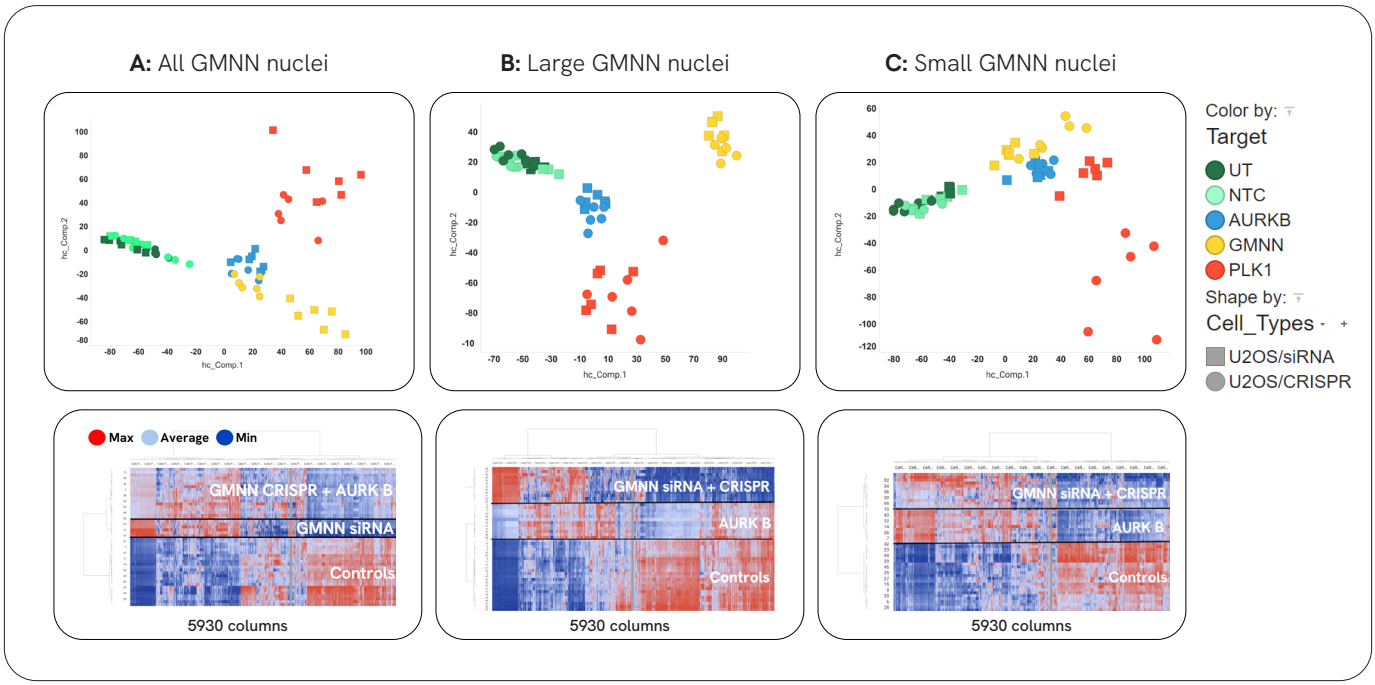


Figure 5: The selection of a nuclear size-dependent subset of GMNN-phenotypes influences the clustering by PCA and changes the pattern of hierarchical clustering. GMNN-inhibited phenotypes generated by siRNA knockdown and CRISPR knockout cluster closer together when selecting the phenotype-subsets „small" or „large" nuclei as shown by PCA (top) and similarity-patterns in hierarchical clustering using the Ward 's method (bottom). Identical data sets were used in both methods, excluding PLK1 in hierarchical clusters for improved readability.

A Top: GMNN forms separate clusters for siRNA- vs. CRISPR-Cas9-mediated phenotypes when all sizes of nuclei are considered. AURKB and GMNN-CRISPR cells seem to have similar phenotypes.

A Bottom: The same effect can be seen in the similarity-based pattern after hierarchical clustering (focusing on AURKB and GMNN for improved visualization).

B Top: The PCA shift between the siRNA and CRISPR-mediated GMNN inhibition is eliminated, when for PCA only larger nuclei of the GMNN population are considered (but all sizes of nuclei for all other conditions) leading to a unified clustering of the GMNN wells.

B Bottom: GMNN-patterns uniformly cluster when cells with "large" nuclei in the GMNN knock-out/knock-down population are selected.

C Top: When the PCA is performed for only small GMNN nuclei, CRISPR and siRNA clouds are closer together and not as separated from the other targets.

C Bottom: This effect is confirmed in the pattern created for the "small" sized GMNN-nuclei.

Conclusions

In summary, both siRNA-mediated knockdown and CRISPR-Cas9-mediated knockout of AURKB, GMNN, and PLK1 in U2OS cells resulted in pronounced phenotypic changes and a reduction in cell number, underscoring the crucial roles of these genes in cell cycle progression. Disregulating these proteins can lead to profound implications, including developmental abnormalities, diseases, and various forms of cancer. Therefore, these proteins are not only pivotal in understanding cellular biology but also serve as potential targets for therapeutic interventions in diseases characterized by uncontrolled cell proliferation.

To analyze changes resulting from gene modifications, we applied a Principal Component Analysis (PCA) to 5930 phenotypic features extracted through cell painting analysis. This revealed distinct clusters for control and treated cells. Notably, the separation of GMNN phenotypes along the first principal component indicated a variance between siRNA and CRISPR-Cas9-mediated GMNN inhibition.

Further examination of nuclear size and intensity in the GMNN population, where inhibition typically leads to polyploid cells, showed that siRNA-treated cells had a higher percentage of large nuclei compared to CRISPR-treated cells. This suggests variability in the kinetics of gene inhibition. Based on these findings, we re-ran the PCA and incorporated hierarchical clustering using the Ward 's method, considering nuclear size. Focusing separately on large and small nuclei revealed that small nuclei still exhibited GMNN inhibition but with a milder phenotype, while focusing on large nuclei led to a unified clustering of both GMNN populations. This could indicate that siRNA-mediated knockdown has a faster inhibitory effect on GMNN.

In summary, using two or more orthogonal technologies that perturb gene function can strengthen the results and validate phenotypic data. Furthermore, this type of analysis shows that differences in the kinetics of target inhibition can result in different phenotypes when data is analyzed on well level only. This might be even more prominent when using small molecules which directly act on the target without the delay of either DNA editing or mRNA depletion and which additionally will have concentration-dependent off-target effects.

Materials and methods

Table 1: List of materials, instruments, and software.

Cells	U2OS, HTB-96, ATCC U2OS cells stably expressing Cas9 (Revvity # HD Cas9-012)
Medium	MEM-RS medium (Cytiva # SH30564.01) DMEM high glucose (Cytiva # SH30081.FS) 10% Fetal bovine serum (Cytiva # SH30071.03) L-Glutamine (Gibco # 25030081)
CRISPR-Cas9 knockout	DharmaFECT 4 transfection reagent (Revvity # T-2004-03) Dharmacon Edit-R™ synthetic single guide RNAs (sgRNAs) targeting cell cycle genes or controls (Revvity catalog #) ▪ Non-targeting negative control 2 (# U-009502-01-05) ▪ AURKB (# SQ-003326-01-0002) ▪ GMNN (# SQ-003270-01-0002) ▪ PLK1 (#SQ-003290-01-0002)
siRNA knockdown	Dharmacon ON-TARGETplus™ siRNAs targeting cell cycle genes or controls (Revvity #) ▪ ON-TARGETplus non-targeting control pool (# D-001810-10-05) ▪ AURKB (# L-003326-00-0005) ▪ GMNN (# L-003270-00-0005) ▪ PLK1 (# L-003290-00-0005)
Microplates	PhenoPlate™ 96-well (Revvity # 6055300)
Cell painting kit	PhenoVue Cell Painting JUMP Kit - 1 x 384 wells (Revvity # PING 21)
Image acquisition and Analysis	Opera Phenix™ Plus high-content screening system (Revvity, # HH14001000) Harmony™ high-content imaging and analysis software (Revvity)
Secondary analysis	High Content Profiler App in Signals™ *VitroVivo* (Revvity Signals)

Cell culture, CRISPR-Cas9 knockout, siRNA knockdown

Knockout and knockdown: Adherent wild-type U2OS (osteosarcoma) cells were transduced with Edit-R lentiviral Cas9 nuclease expression vector to generate a cell line stably expressing Cas9 (CRISPR associated protein 9) under a hEF1α (human elongation factor - 1α) promoter (cell line HD Cas9-012 or lentiviral particles VCAS10126).

Next, cells were plated at 750 cells/well in 100 µL growth medium into PhenoPlate-96-well microplates. On the day of transfection, for each well, 0.0125 µL of DharmaFECT 4 was added to 9.98 µL MEM-RS medium. Liposome complexes were allowed to form undisturbed for five minutes at room temperature, at which point the transfection mixture was added either to pooled sgRNAs or siRNA diluted in MEM-RS (sgRNA or siRNA final concentration of 25 nM/well). Transfection mixture was incubated undisturbed for an additional twenty minutes at room temperature before diluting with growth medium and adding to the cells. Cells were cultured for an additional 96 hours, at which point samples were used for T7EI endonuclease assay to confirm indel formation by CRISPR-Cas9 or used for RT-qPCR to confirm siRNA knockdown or used for the cell painting protocol (data not shown).

Cell painting

Cell painting was performed according to instructions provided with the PhenoVue Cell Painting JUMP kit. In brief, live cells were stained with 500 nM PhenoVue 641 mitochondrial stain, followed by fixation with 4% PFA (paraformaldehyde). After washing, a mix of 0.1% Triton X-100, 1.5 µg/ml PhenoVue Fluor 555 – WGA, 5 µg/ml PhenoVue Fluor 488 - concanavalin A, 8.25 nM PhenoVue Fluor 568 – phalloidin, 1.62 µM PhenoVue Hoechst 33342 nuclear stain, and 6 µM PhenoVue 512 nucleic acid stain was applied to the cells. Eight cell components (DNA, cytoplasmic RNA, nucleoli, actin, Golgi apparatus, plasma membrane, endoplasmic reticulum, and mitochondria) are stained with 6 dyes. Finally, plates were heat sealed and stored at 4°C until measurement.

Image acquisition

6 cell painting stains were measured in 5 channels on the Opera Phenix Plus high-content screening system in confocal mode using a 20x water immersion objective. 9 fields of view were acquired per well, each with a 1 µm-spaced stack of 4 planes.

Feature extraction and secondary analysis

To extract phenotypic features (such as morphology, texture, intensity) from different cell regions, *Maximum Intensity Projection* images were analyzed in Harmony® software. The analysis involved segmenting the nuclei and cytoplasm, removing objects that touch the image borders, and extracting 5930 cellular features using the dedicated building block *Calculate Cell Painting Properties* in extensive setting (for more details, see 1).

Downstream analysis of morphological profiles was done by importing feature data into the *High Content Profiler* app of Signals *VitroVivo*. A reduction of dimensionality was achieved with a principal component analysis (PCA) and hierarchical clustering.

References

1. Application Note: Phenotypic analysis of CRISPR-Cas9 cell-cycle knockouts using cell painting (Revvity.com)

Authors

Alexander Schreiner
Angelika Foitzik
Barbara Sonnenberg
Žaklina Strezoska
Josien Levenga

Revvity, Inc.