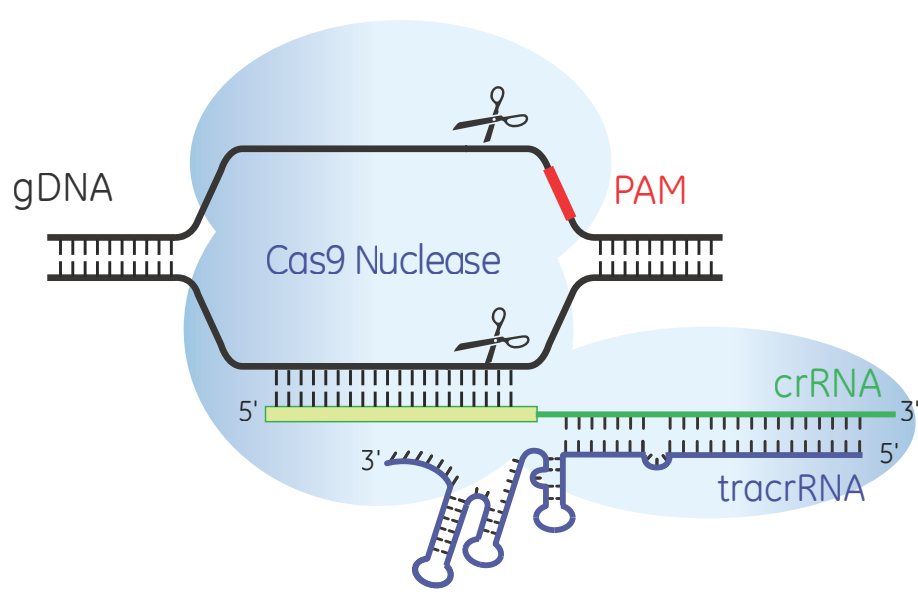# Dharmacon™
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Identification of genes involved in cell cycle regulation using arrayed synthetic CRISPR RNA libraries in a multiparameter high-content assay

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Abstract

Gene knockout using CRISPR-Cas9 has dramatically transformed biological research and has been rapidly applied to loss-of-function screening primarily using pooled lentiviral sgRNA libraries. A synthetic CRISPR RNA (crRNA) approach is amenable to screening in arrayed, well-by-well fashion and expands the types of phenotypic readouts that can be used, including high-content and morphology-based assays. Here, we used a cell cycle reporter cell line to perform an arrayed, synthetic crRNA:tracrRNA screen targeting 169 genes with four crRNAs per gene with high content analysis (HCA) to identify genes that regulate the cell cycle. Multiple parameters were used to classify cells into different cellular states and phases of the cell cycle: cells with irregularly shaped nuclei, cells in G1 phase, cells either in S or G2 phase, cells in mitosis or with condensed chromatin, and cells with multinuclear DNA component. We used a novel statistical method for hit identification and applied multiple strategies including gene expression analysis, confirmation of genomic insertions and deletions, and validation by orthogonal reagents to identify high confidence target genes with roles in cell cycle regulation. Most hits had multiple positive crRNAs per target gene, enabling identification of target genes with high confidence, demonstrating the power of combining synthetic crRNAs libraries with HCA assays in screening for complex cellular phenotypes in an arrayed format. Given the ease of transfecting RNAs into most cell types, we expect many phenotypic assays to be amenable to arrayed screening with synthetic crRNA libraries.

Predesigned crRNA with efficient editing from an algorithm for functional gene knockout

Dharmacon™ Edit-R™ CRISPR-Cas9 synthetic crRNA:tracrRNA mimics the native system and uses two RNA components: the crRNA (green) and the tracrRNA (blue), which form a complex that complexes with Cas9 nuclease (blue circles) then binds to the target genomic DNA (gDNA, black) upstream of a protospacer-adjacent motif (PAM, red), and results in site-specific cleavage.

High-scoring crRNAs
93% achieve indels > 40%

Low-scoring crRNAs
33% achieve indels > 40%
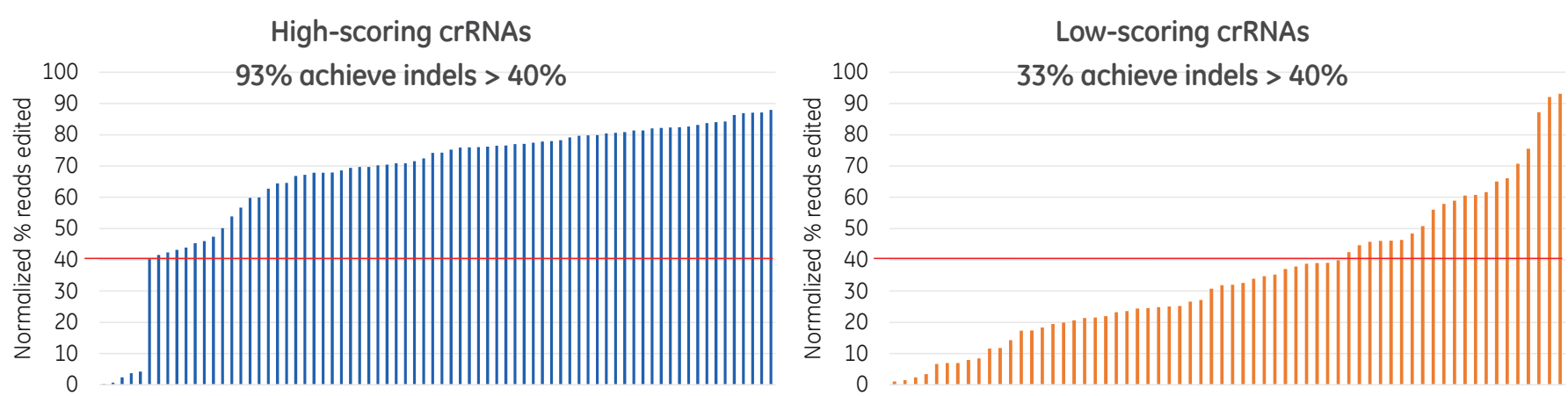
- Ten crRNAs with high functional scores for 10 genes (blue bars) and 10 crRNAs with low functional scores targeting the same genes (orange bars) were analyzed for editing by NGS
- 93% of the high-scoring crRNAs showed > 40% of editing (indel formation) where only 33% of the low-scoring crRNAs had > 40% editing validating the Dharmacon guide RNA algorithm
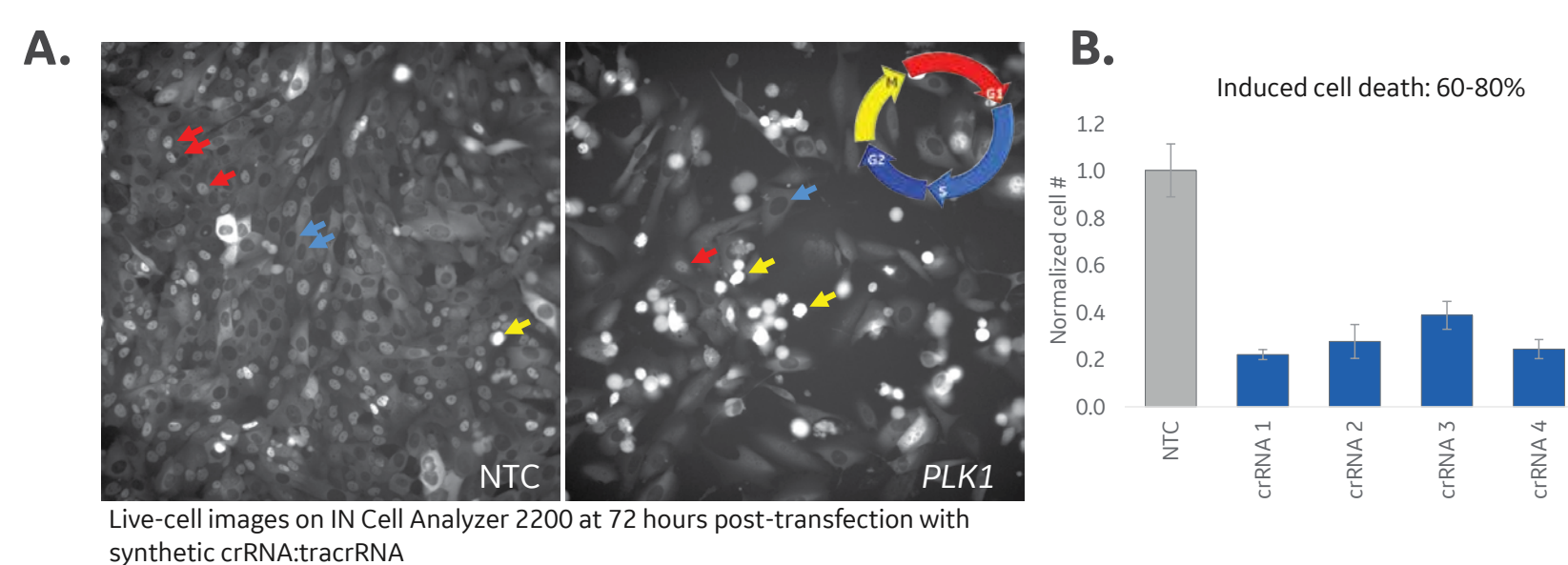
An HEK293T-Cas9 cell line was transfected with 50 nM crRNA:tracrRNA, using 0.25 μL/well of DharmaFECT 1 transfection reagent. Transfected and untransfected cells were lysed 72 h post-transfection and prepped for NGS on a Illumina MiSeq instrument (paired end reads, 2 x 300 length). Percent perfect reads were calculated and normalized to the control untransfected samples; the data is presented as normalized percent edited.

G1S cell cycle phase marker (CCPM) cell line: a live-cell sensor for different cell cycle phases
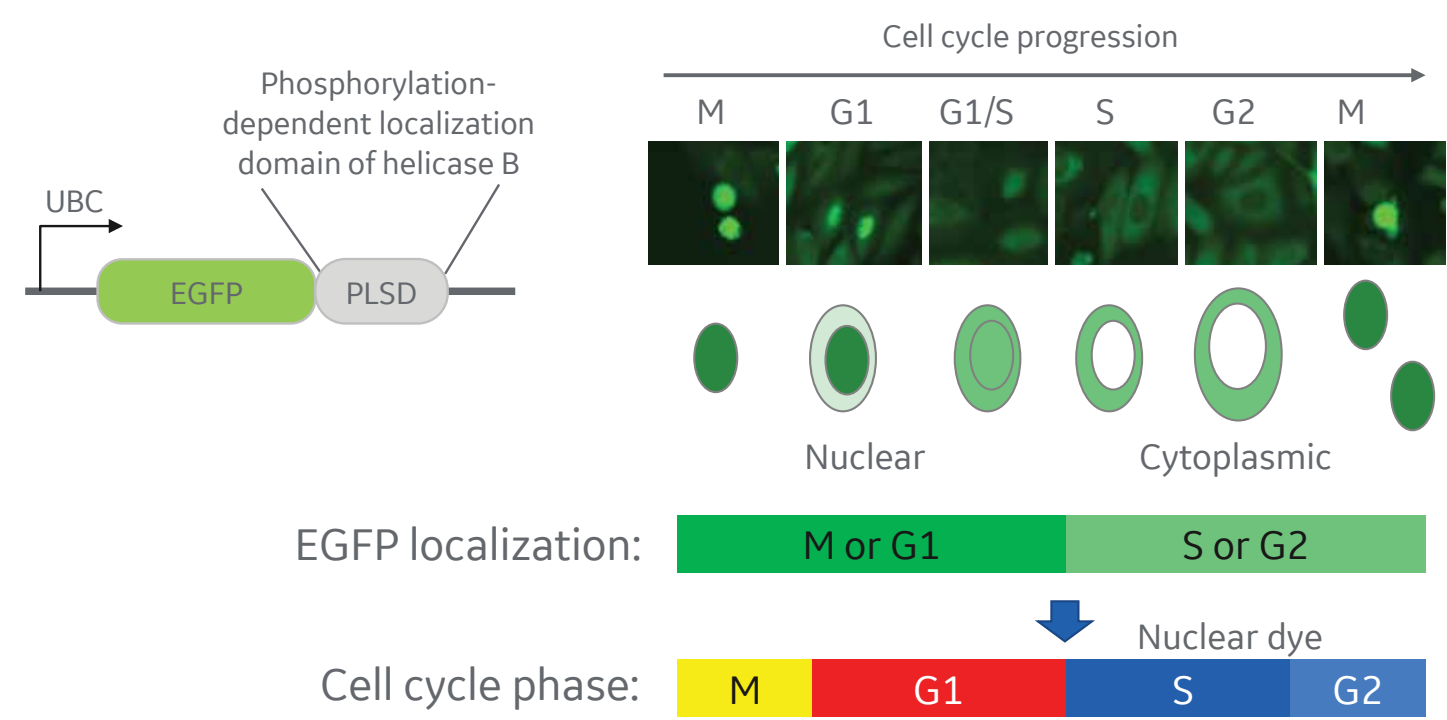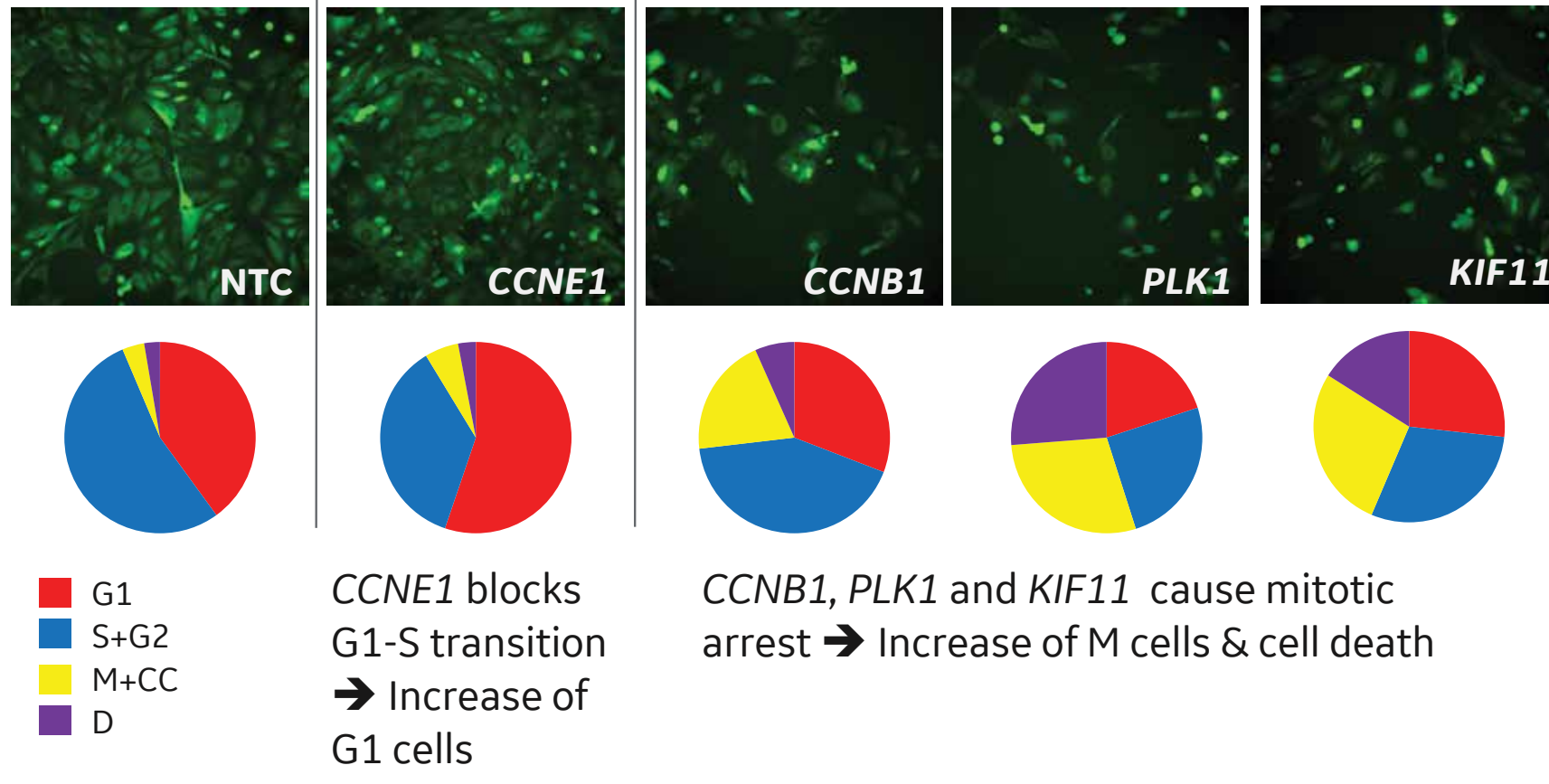

The G1S-CCPM cells express a sensor: an EGFP fused to a phosphorylation-dependent subcellular localization control domain (PLSD) from helicase B, under the UBC promoter for a stable, low level expression. In G1, the EGFP sensor is localized to the nucleus. At the G1/S boundary, upon phosphorylation by the CyclinE/Cdk2 complex, it translocates to the cytoplasm, indicating that cells are in S or G2 phase. This, in combination with staining of the nuclei, can enable identification of cells in all different cell cycle phases.

High-penetrance phenotype in G1S-CCPM-Cas9 reporter cell line

Induced cell death: 60-80%

Live-cell images on IN Cell Analyzer 2200 at 72 hours post-transfection with synthetic crRNA:tracrRNA
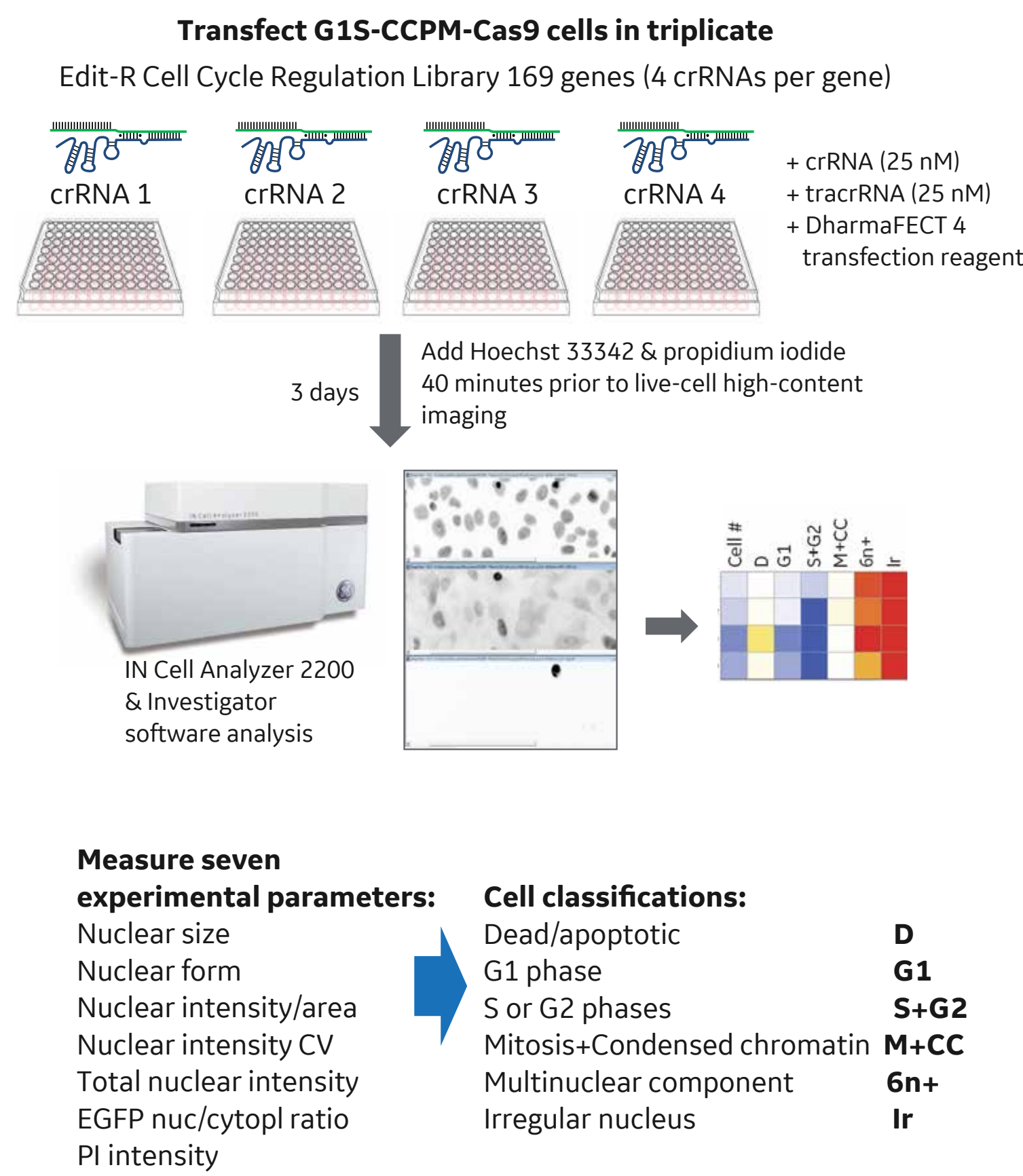
A stable Cas9-expressing version of the G1S-CCPM cell was generated. crRNAs targeting PLK1 or a non-targeting crRNA (NTC) were transfected to validate the phenotype in the G1S-CCPM-Cas9 cells. A. Live-cell images were captured using the IN Cell Analyzer 2200 from cells at 72 hours. In the NTC cell population, most cells are in G1 (red arrows) and S or G2 (blue arrows) with fewer cells in mitosis (yellow arrows), the shortest phase of the cell cycle. When cells are transfected with a crRNA targeting PLK1, there is an increase of rounded cells indicative of mitosis or cell death (yellow arrows) B. A decrease in cell number indicative of the cell death seen with knockout of PLK1.

Phenotypic assay validation with positive control genes

| ■ G1 |
| ■ S+G2 |
| ■ M+CC |
| ■ D |

CCNE1 blocks G1-S transition → Increase of G1 cells

CCNB1, PLK1 and KIF11 cause mitotic arrest → Increase of M cells & cell death
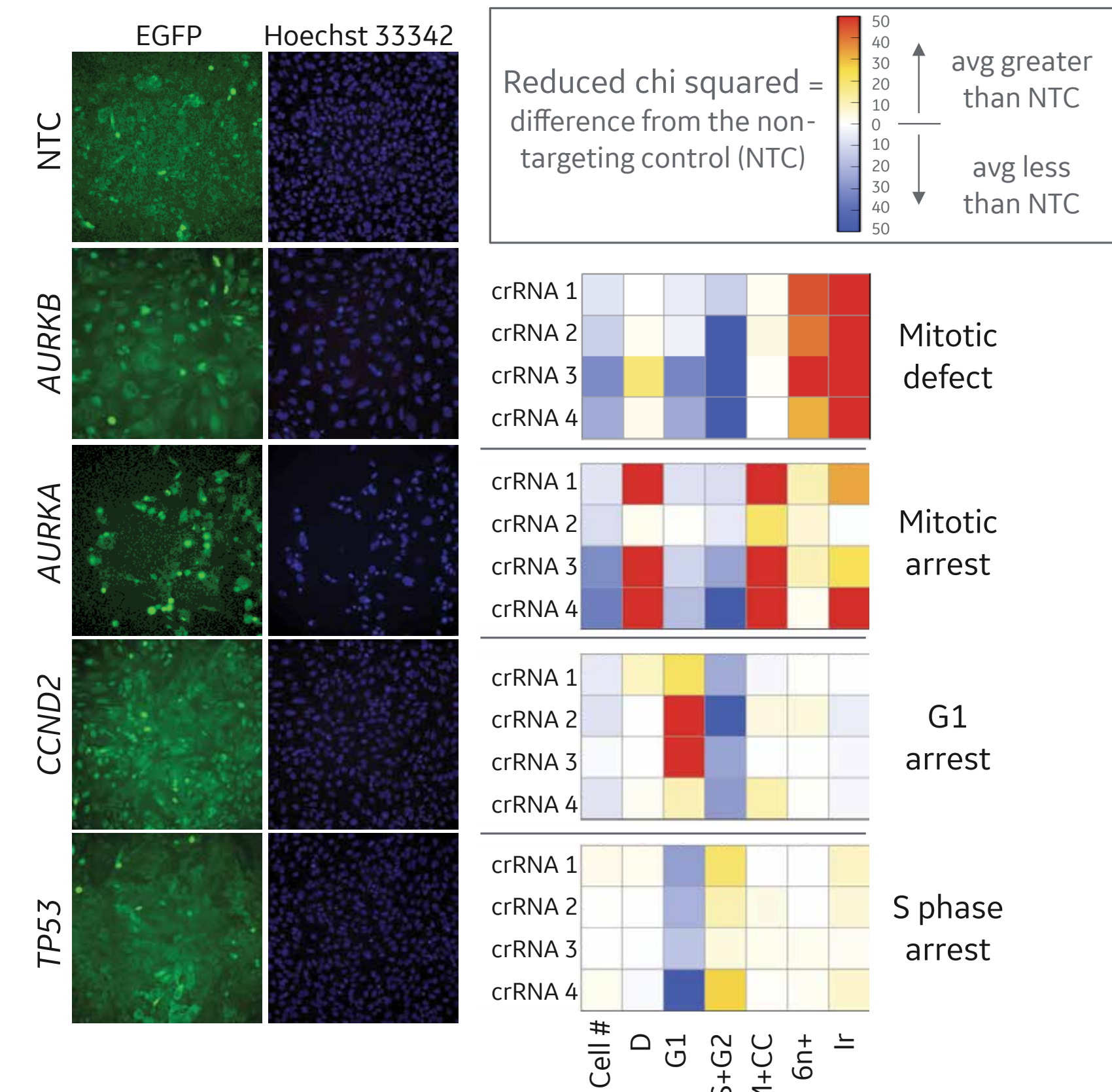
Assay validation was carried out with crRNA targeting genes known to affect cell cycle transition. Images show the EGFP localization and the pie charts indicate the cell cycle analysis. Compared to the non-targeting control (NTC), CCNE1 knockout results in an increase in G1 cells, consistent with its role in the regulation of G1-S transition. Knockout of CCNB1, PLK1, and KIF11, which all play a role in mitosis, results in an increase of cells in M phase.

Screening workflow with arrayed synthetic crRNA in the G1S-CCPM-Cas9 cells

Transfect G1S-CCPM-Cas9 cells in triplicate
Edit-R Cell Cycle Regulation Library 169 genes (4 crRNAs per gene)

crRNA 1   crRNA 2   crRNA 3   crRNA 4

+ crRNA (25 nM)
+ tracrRNA (25 nM)
+ DharmaFECT 4 transfection reagent

Add Hoechst 33342 & propidium iodide 40 minutes prior to live-cell imaging

3 days

IN Cell Analyzer 2200 & Investigator software analysis

Measure seven experimental parameters:
Nuclear size
Nuclear form
Nuclear intensity/area
Nuclear intensity CV
Total nuclear intensity
EGFP nuc/cytopl ratio
PI intensity

Cell classifications:
|	
Dead/apoptotic	D
G1 phase	G1
S or G2 phases	S+G2
Mitosis+Condensed chromatin	M+CC
Multinuclear component	6n+
Irregular nucleus	Ir

The Edit-R Cell Cycle Regulation library (Cat #GC-003200-01) consists of four different crRNAs in separate wells targeting 169 genes. G1S-CCPM reporter cells stably expressing Cas9 were transfected in triplicate with synthetic crRNA:tracrRNA using DharmaFECT 4 transfection reagent (Cat #T-2004-01). After 72 hours, live-cells were imaged on the IN Cell Analyzer 2200 (GE Healthcare), adding Hoechst nuclear stain and the live/dead stain propidium iodide 40 minutes prior to scanning. Images were analyzed using the IN Cell Investigator software. Each plate had multiple negative controls [NTC #1 (Cat #U-007501-01-05) and NTC #2 (Cat #U-007502-01-05) each in triplicate] as well as validated positive controls.

High-content analysis demonstrates robust multiparametric phenotypes for representative genes

EGFP   Hoechst 33342

Reduced chi squared = difference from the non-targeting control (NTC)

avg greater than NTC

avg less than NTC

NTC

AURKB — crRNA 1 / crRNA 2 / crRNA 3 / crRNA 4 — Mitotic defect

AURKA — crRNA 1 / crRNA 2 / crRNA 3 / crRNA 4 — Mitotic arrest

CCND2 — crRNA 1 / crRNA 2 / crRNA 3 / crRNA 4 — G1 arrest

TP53 — crRNA 1 / crRNA 2 / crRNA 3 / crRNA 4 — S phase arrest

Cell # / D / G1 / S+G2 / M+CC / 6n+ / Ir

Cell images of EGFP localization and nuclear staining shown for representative genes with roles in different phases of the cell cycle show visible differences in cell density, morphology and EGFP localization. Reduced chi squared statistical method was used as a measure of the difference of the HCA parameters for the crRNA from the negative controls and visualized as heat maps, with red as significantly above and blue as significantly below the NTC control average. Knockout of AURKB leads to increase of large cells with multinuclear DNA component. Knockout of AURKA leads to an increase of cells with condensed chromatin, indicative of mitotic block. Knockout of CCND2, leads to an increase of cells in G1 and a decrease of cells in S+G2, indicative of G1 arrest. Knockout of TP53 leads to a decrease of cells in G1 with concomitant increase of cells in S+G2, indicative of S phase arrest.

Multiparametric results from an arrayed screen with the Edit-R crRNA Cell Cycle Regulation library

avg greater than NTC

avg less than NTC
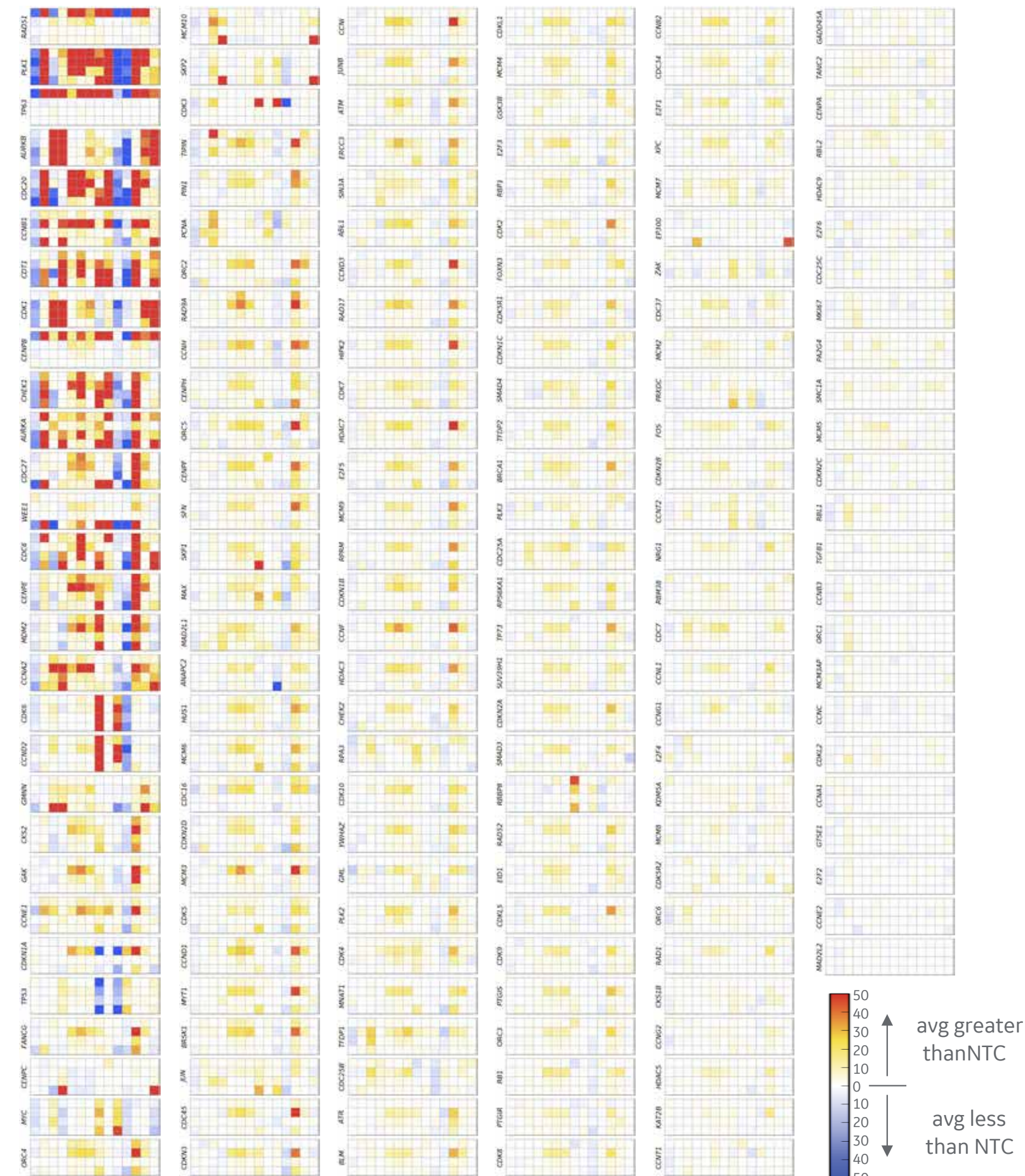
- Heat maps were generated for each parameter based on the reduced chi squared value and ordered from highest to lowest for all 169 genes in the Edit-R crRNA Cell Cycle Regulation library.
- Each gene has four rows, one for each crRNA. Forty-one hits were identified on the basis of reduced chi squared value ≥ 6.
- Further hit analysis (RNA-seq expression data and gene annotation analysis) identified 33 hits for evaluation in a secondary (confirmation) screen using the same crRNAs.

Strong primary screen hits confirm with multiple crRNAs

Confirmation screen
- ● Strong
- ● Medium
- ● Nonspecific crRNA
- ○ Not confirmed
- ● Non-hits from primary screen

All 169 genes from the primary screen and 33 genes from the confirmation screen are plotted as the number of crRNAs with positive phenotype v. the phenotype intensity.

- 24 of 33 confirmed robust phenotype in repeat experiment
- 19 of 24 confirmed hits with ≥ 3 crRNAs
- Only 3 hits with single crRNA per gene suggesting low off-targeting

Summary & conclusions

Algorithm-designed, synthetic crRNAs can be successfully implemented in an arrayed screening application using multiparametric high-content analysis

An arrayed screen identified genes with different roles in cell cycle regulation with high confidence due to:
- high reproducibility among replicate plates
- multiple crRNAs per gene causing the phenotype
- very few hits with single crRNA per gene, suggesting low off-targeting

Multifaceted hit stratification workflow was implemented that included: expression analysis and gene annotation analysis, ranking hits based on redundancy and phenotypic strength, confirmation of phenotype in an independent experiment and validation with orthogonal reagents

Reference

Ž. Strezoska, M.R. Perkett, E.T. Chou, E. Maksimova, E.M. Anderson, S. McClelland, M.L. Kelley, A. Vermeulen and A. van Brabant Smith. High-content analysis screening for cell cycle regulators using arrayed synthetic crRNA libraries. Journal of Biotechnology. 251, 189–200 (2017). doi.org/10.1016/j.jbiotec.2017.04.017

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