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White Paper

First siRNA Library Screening in Difficult-to-Transfect HUVEC and Jurkat Cells

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High throughput transfection of siRNA libraries has become a valuable tool in target identification and validation. However, such screenings have so far been constrained to mostly easy-to-transfect adherent cell lines. Lonza's 96-well Shuttle® System, based on the well-established Amaxa® Nucleofector® Technology, extends these approaches to primary and difficult-to-transfect cells. Here, we report results of 2 different screening approaches in difficult-to-transfect cell types using Thermo Scientific Dharmacon siGENOME and ON-TARGET*plus* siRNA Libraries. In HUVEC cells, an endothelial cell model for angiogenesis, we performed a loss-of-function screen with a combined protein kinase and a cell-cycle library for studying targets involved in proliferation. Furthermore, we carried out a sensitizer screen in human Jurkat T lymphocytes to identify pathway members involved in FAS-mediated apoptosis.

Introduction

RNAi-based library screening has become a powerful *in vitro* tool to identify drug targets that play a role in disease development and progression[1]. Successful screening experiments using siRNA require efficient delivery of highly functional and specific siRNA molecules into appropriate cells. While lipid-mediated transfection is a common approach for siRNA delivery, many cell types, including suspension cell lines and primary cells, are not compatible with this technology[2]. This limitation prevents analysis of many biologically-relevant cell types and restricts siRNA library screenings mainly to transformed, adherent cell lines[3,4,5] that often exhibit phenotypic and genetic anomalies after extended periods of culturing. Using a cancer cell line e.g., for functional screening can be a valid approach for studying cancer mechanisms. Nevertheless, the diversity of biological questions requires using appropriate cell types, many of them ideally being primary cells. In addition, several of the lipid delivery reagents can cause cytotoxicity and are

capable of inducing a potent interferon response and/or altering gene expression profiles[6,7,8]. These unintended phenotypes can significantly affect experimental outcomes and drastically interfere with understanding a gene's function.

The combination of Dharmacon® siGENOME® and ON-TARGET*plus*® siRNA Libraries with Lonza's Nucleofector® 96-well Shuttle® System overcomes these screening limitations associated with lipid reagent-mediated transfection. The Nucleofector® Technology is optimized for transfection of difficult-to-transfect cell types (in particular, primary cells and suspension cell lines). Furthermore, Dharmacon ON-TARGET*plus*® siRNA reduces the risk of "false positives" by minimizing off-target effects generated by the sense and antisense strands[9].

Here we present the workflow and results of two siRNA screens using the 96-well Shuttle® System to deliver siRNAs to 2 difficult-to-transfect cell types, human umbilical vein endothelial cells (HUVEC) and Jurkats T lymphocytes. HUVEC cells were screened with a library targeting protein kinases and genes associated with the cell-cycle to identify targets genes important for cell viability. In contrast, Jurkat cells were screened with a library of siRNAs targeting apoptosis to identify genes that regulate FAS-mediated cell death.

Material and Methods

siRNA Reagents: All siRNA reagents were Thermo Scientific Dharmacon Products. Negative control: siGENOME® Non-targeting siRNA #1. siRNAs targeting individual genes: siGENOME® siRNA Reagents (SMARTpool® or single duplexes) and ON-TARGET*plus*® siRNA Reagents (pool or single duplexes). siRNA libraries: Human siGENOME® SMARTpool® siRNA Libraries for Protein Kinases (targeting 779 genes) and Cell Cycle Regulation (targeting 111 genes), and Human ON-TARGET*plus*® siRNA Library for Apoptosis (targeting 558 genes).

Transfection: HUVEC cells (Lonza, Cat. No. CC-2519) and Jurkat cells (clone E6-1, ATCC® TIB-152™) were cultured and transfected according to the recommendations in the respective Amaxa® Optimized Protocol for 96-well Nucleofection® (Lonza; HUVEC: standard protocol; Jurkat: automation protocol). Briefly, in each sample, 2×10^4 HUVEC cells or 2×10^5 Jurkat cells were transfected with 20 pmol siRNA (if not noted differently). For optimal assay conditions post-transfection, HUVEC cells were plated in 96-well culture plates at a density of 2×10^3 cells per well in 100 µl, and Jurkat cells were plated at 6×10^4 cells per well in 150 µl culture volume. Outer wells of culture plates were filled with media only and not used for cells in order to avoid edge effects in the phenotypic assays.

Phenotypic assays: HUVEC cells were analyzed 72 hours post-transfection for cell viability by CellTiter-Blue® Assay (Promega). For the assay in Jurkat cells, 48 hours post-transfection, 100 µl cell suspension were transferred to a fresh 96-well culture plate. Apoptosis was induced by adding 10 ng cross-linked recombinant soluble FAS-ligand (FasL, Axxora, San Diego, CA). After 2 hours of induction, cell viability was analyzed using the CellTiter-Glo® Assay (Promega) and apoptosis levels were assessed using the Apo-ONE® Assay (Promega) measuring caspase 3/7 activity.

mRNA quantification: The QuantiGene® Branched DNA Assay (Panomics) was utilized to quantitate transcript levels and correlate target knockdown with biological phenotype. Cyclophilin B served as reference mRNA and values were normalized to samples transfected with control siRNA.

Primary screen: For the primary screens (n = 3 independent experiments), cells were transfected with the respective libraries or control siRNAs and analyzed for phenotypic effects (cell viability and/or apoptosis). Data from each screen was analyzed by statistical means: the Z' factors[10] of controls were determined to evaluate the quality of the experiment and robust Z-score calculation[11] was used for hit identification.

Hit validation: For target validation, selected hits were first re-evaluated with a higher number of samples using the siRNA utilized in the primary screen. Samples were randomly arranged across the plate to ensure independence of the phenotype from well positions. Subsequently, hits were further validated by demonstrating multiple knockdown reagents in different formats induced the same phenotypes (e.g., single or pooled siGENOME® or ON-TARGET*plus*® siRNA Reagents).

For more details please refer to: www.lonza.com/rnai-screening

Results and Discussion

General assay setup and screening strategy

Prior to initiating the screens, optimal read-out assay conditions were established using the appropriate controls. We optimized multiple critical parameters that influence the signal strength of the read-out assays including siRNA concentration, cell plating densities and analysis time points (for more details see Box 1).

With the aim to avoid missing potential hits due to "false negatives", 20 pmol (1 µM) of each SMARTpool® siRNA Reagent was chosen for screening. In our setup "false positives" that might be included in a selection of hits due sequence-dependent off-target effects[12] could easily be identified during the subsequent validation steps. We determined optimal cell plating densities, inducer concentrations and incubation times for induction (Jurkat screen only), as well as analysis time points for the selected read-out assays using appropriate controls at the chosen siRNA concentration. In order to determine the extent of the screening window and the reproducibility of the assay for detection of hits in each of the 2 screens, 2 siRNAs directed against known members of the respective pathways were used as positive controls (HUVEC: PLK-1 and CHEK-1, Jurkat: FAS and CASP3). These controls covered both strong and weak positives to provide a screening window that allows for detection of both, strong and weak targets, in the primary screens.

As negative controls, we always included untreated cells (in 96-well solution only), indicating the overall cellular conditions and siGENOME® Non-targeting siRNA #1 for detecting possible nonspecific method-related effects. Pilot screens including all steps and time-frames of the assays were performed to assess the reproducibility of identifying the positive controls, for calculation of Z' factors for the assays and for confirming minimal intra- and inter-plate variation in the screening workflows.

Screen 1: Loss-of-Function Screen for Viability Genes in HUVEC Cells

Viability assay optimization

For the kinase and cell cycle screen in HUVEC cells, siGENOME® SMARTpool® siRNA Reagents targeting Polo-like Kinase 1 (PLK-1) and Cell Cycle Check-point Kinase 1 (CHK-1 or CHEK-1) were selected as positive controls to set up the CellTiter-Blue® Cell Viability Assay. PLK-1 is a key regulator of mitotic progression in mammalian cells and the knockdown of PLK1 is known to induce apoptosis in cancer cells[13,14]. CHEK-1 is involved in the DNA damage response and is also required for cell proliferation and survival. CHEK-1 knockdown by siRNA has been reported to induce mitotic arrest[15]. As such, downregulation of PLK-1 and/or CHEK-1 is expected to decrease cell viability.

Using PLK-1, post-transfection plating densities were adjusted to allow for a significant discrimination of positive and negative control samples on the phenotypic level. This has been achieved by plating HUVEC cells at a low cell density of 2×10^3 per well for 3 – 4 days after transfection (Figure 1A).

As shown in Figure 1B, the strength and kinetics of phenotypic effects differ for PLK-1 and CHEK-1, representing potential differences expected for "strong" and "weak" library targets. An analysis time point of 72 hours suited well for both targets. In pilot

Box 1

Parameters to consider for assay development

- Cell type choice
- Choice of siRNA substrates (e.g., single or pool)
- Type of read-out assays
- Suitable negative and positive controls
- Kinetics of phenotypic effects
- Factors influencing signal strength (e.g., condition of cell batch, passage number, cell density before harvesting, donor variance, cell density in assay, concentration of inducers, time point of treatments, time point of analysis)
- Artifact effects with negative impact on hit identification (e.g., edge effects, gradients, patterns with regard to well position)
- Assay robustness (intra-plate, inter-plate, day-to-day, donor-to-donor variations)
- Data analysis (e.g., normalization, Z' factor for screening window, Z scores for hit identification)

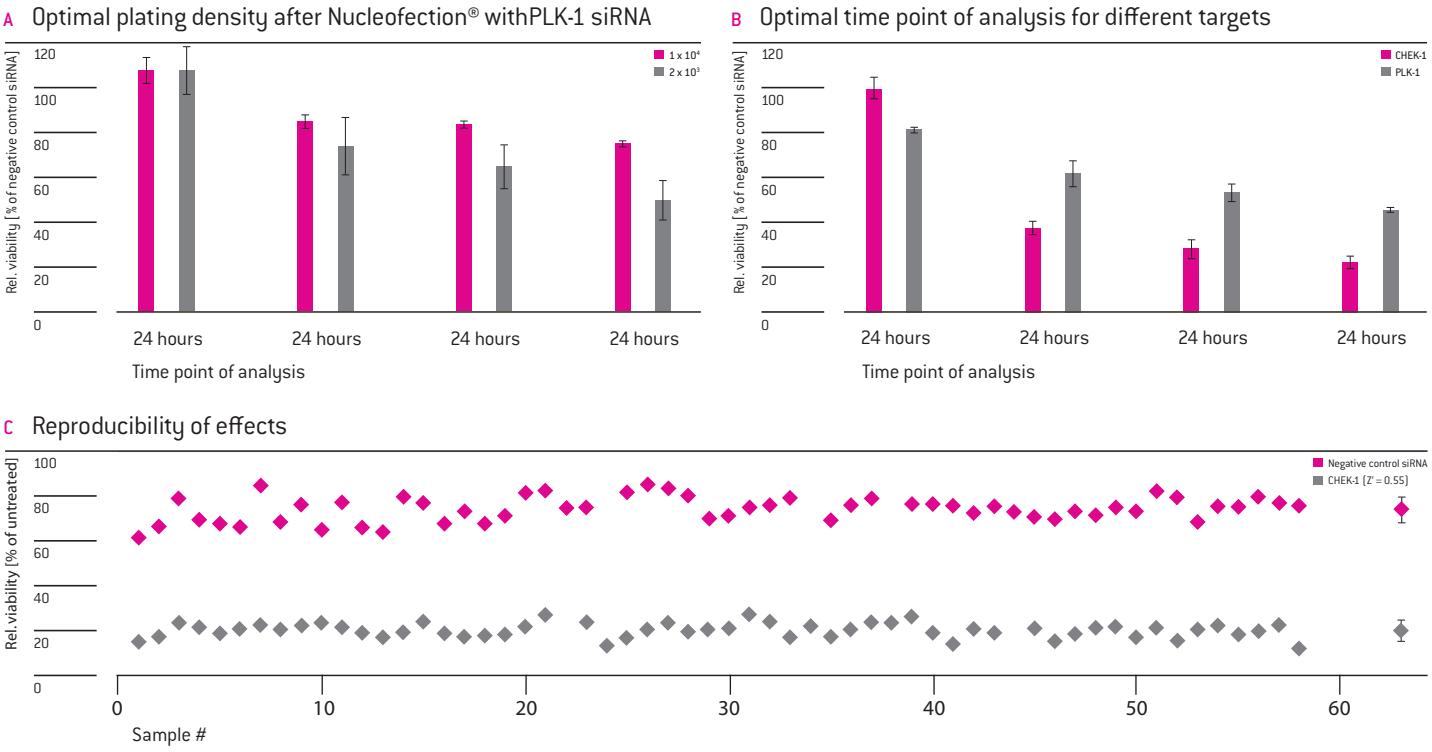


Figure 1: Determination of optimal assay conditions. In 3 independent experiments, HUVEC cells were transfected with 20 pmol SMARTpool® siRNA targeting PLK-1 (A, B) or CHEK-1 (B, C) and siGENOME® Non-targeting Control. Cell viability was analyzed at different time points post Nucleofection® (A/B: 24, 48, 72 and 96 hours; C: 72 hours). Values were normalized to the negative control samples (A, B) or to untreated cells (C). The rightmost dots in C represent the mean and SD of the 60 individual values.

screens for further determination of assay robustness, controls were plated into the central 60 wells of a 96-well culture plate and analyzed for cell viability. Wells in the outer rows were filled with medium only and were not used for cells, thereby avoiding the possibility of edge effects. Z' factors of both positive controls (CHEK-1: 0.55; PLK-1: 0.22; Figure 1C; data for PLK-1 not shown) reflected a suitable window for discrimination of potential hits with different phenotypic strength in the subsequent screen from background.

Primary screen

HUVEC cells were transfected with pools of siRNA targeting individual genes in the Human siGENOME® SMARTpool® siRNA Collection for protein kinases or cell cycle regulation. Multiple independent screening experiments (n = 3) were performed to confirm the reproducibility of individual primary hits. Robust Z-score for cell viability was calculated for each of the 890 targets in the 3 independent experiments. As an example, the robust Z-scores of 1 screening experiment are depicted in Figure 2A. A substantial proportion of targets displayed a MAD (median absolute deviation) below -2 or above 2 (|MAD| > 2), including our positive controls PLK-1 and CHEK-1, which are members of both libraries. 37 targets had a mean |MAD| greater than 2 in the 3 screens and thus were considered as potential hits (Figure 2B). Of the 37 primary hits, 33 have a pro-proliferative/anti-apoptotic function as their downregulation led to increased cell death, while 4 have an anti-proliferative effect as their knockdown allowed for better cell viability. The 16 strongest of the 33 pro-proliferative primary hits were selected for further validation (Table 1).

Hit validation

4 of the 16 selected targets (COPB2, PYCS, CDK4 and MYC) were validated by demonstrating that the phenotype could be reproduced with 3 or 4 of 4 single siGENOME® siRNA Sequences from the original SMARTpool® and with the ON-TARGET*plus*® Pool (Figure 3A). The phenotypes could be nicely correlated to the knockdown on mRNA level (Figure 3B) and to the amount of transfected siRNA (Figure 3C; only COPB2 shown). Both results,

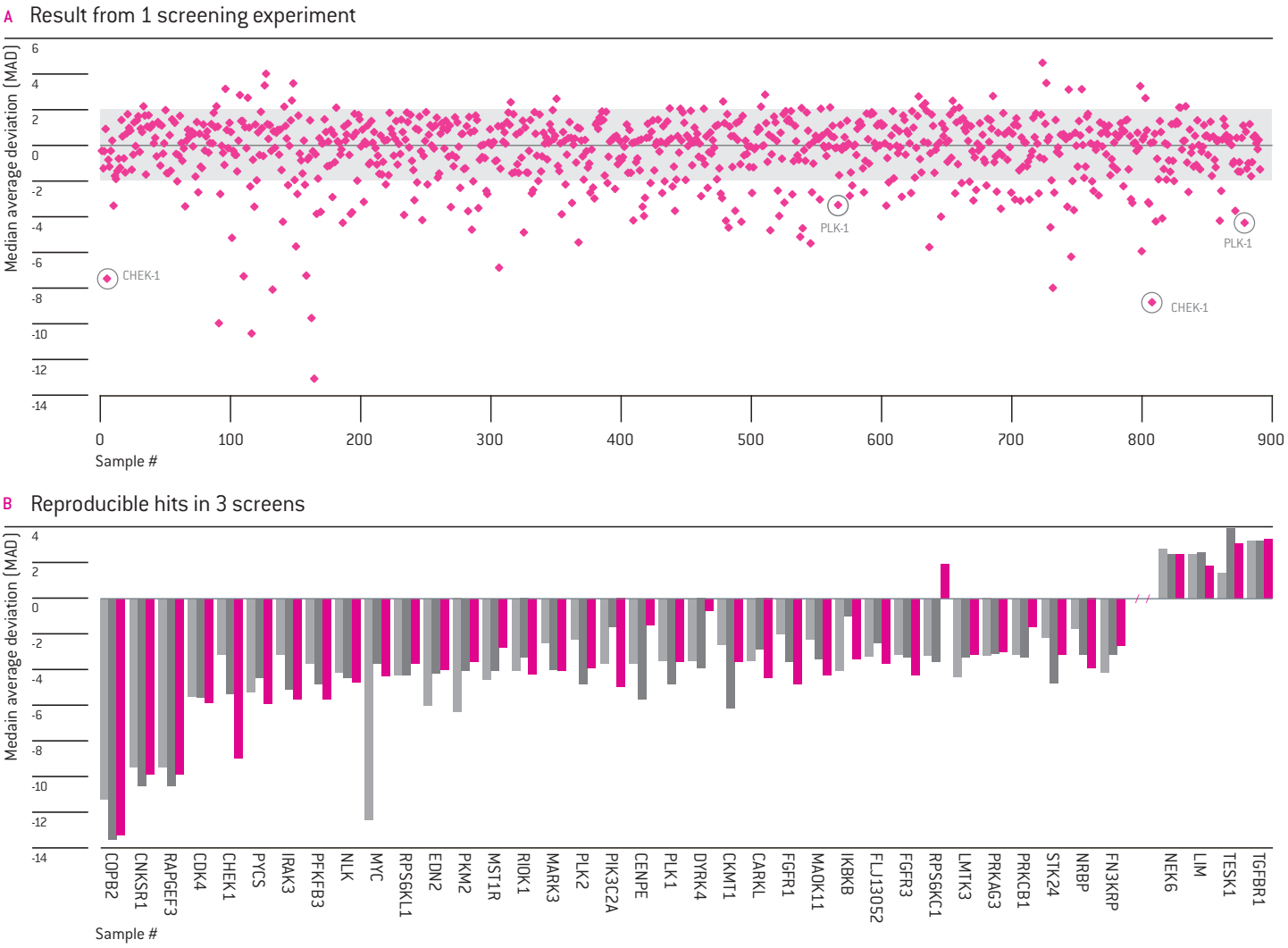


Figure 2: Primary screen. HUVEC cells were transfected with 20 pmol of the combined Human siGENOME® SMARTpool® siRNA Libraries for Kinases (targeting 779 genes) and Cell Cycle Regulators (targeting 111 genes). Cell viability was analyzed 72 hours post Nucleofection®. (**A**) Representation of robust Z-scores of cell viability measures from 1 screening experiment. (**B**) Robust Z-scores of the top 37 primary hits (with an |MAD| > 2) from 3 independent experiments.

redundant effects with independent sequences and proven mRNA knockdown, exclude them from being the result of off-target effects. The relevance of CDK4 and MYC for cell cycle progression has been previously described[16] while no direct relation to cell cycle regulation has been reported for COPB2 or PYCS. COPB2 is a subunit of the so called "coatomer" involved in protein transport between the ER and Golgi compartments[17]. The enzyme PYCS plays a role in amino acid synthesis[18]. Both seem to serve general functions associated with cell growth, such as transport machinery or substrate supply for protein synthesis.

6 of the 16 selected primary hits were confirmed with 2 of 4 single siGENOME® siRNAs but not with the ON-TARGET*plus*® Pool, still suggesting them as potential hits that require further efforts for validation. Only 6 of the 16 selected primary hits are considered "false positives" because neither the ON-TARGET*plus*® Pool nor more than 1 of 4 single siGENOME® siRNAs reproduced the phenotype seen with the original siGENOME® SMARTpool®. Most likely these are the result of off-target effects of individual siRNA sequences.

Screen 2: Sensitizer Screen for FAS-induced Apoptosis in Jurkat Cells

Viability and apoptosis assay optimization

FAS receptor and CASP3 are known members of the apoptosis pathway in Jurkat cells[19]. For this reason, ON-TARGET*plus*® siRNA Pools targeting both genes were used to optimize the assay conditions, e.g., cell plating density, time of apoptosis induction by FAS-ligand and analysis time point, similarly to the approach described above for the HUVEC screen. Results demonstrated that FAS receptor knockdown provided the most prominent effects in the viability assay as opposed to the caspase assay. In contrast, CASP3 exhibited the opposite behavior, thus we employed both readout assays during screening.

Primary screen

Jurkat cells were transfected in 3 independent experiments with the Human ON-TARGET*plus*® siRNA Library for Apoptosis. Based on robust Z-scores for both, cell viability and apoptosis assays (mean of 3 experiments), we selected 10 targets with a |MAD| of at least 5 as potential hits, that included the 2 positive controls FAS receptor and CASP3 re-identified from the library (Figure 4, Table 1). One target, SPATA3, was considered a "false positive" as it showed a very strong effect in only one experiment. Most of the

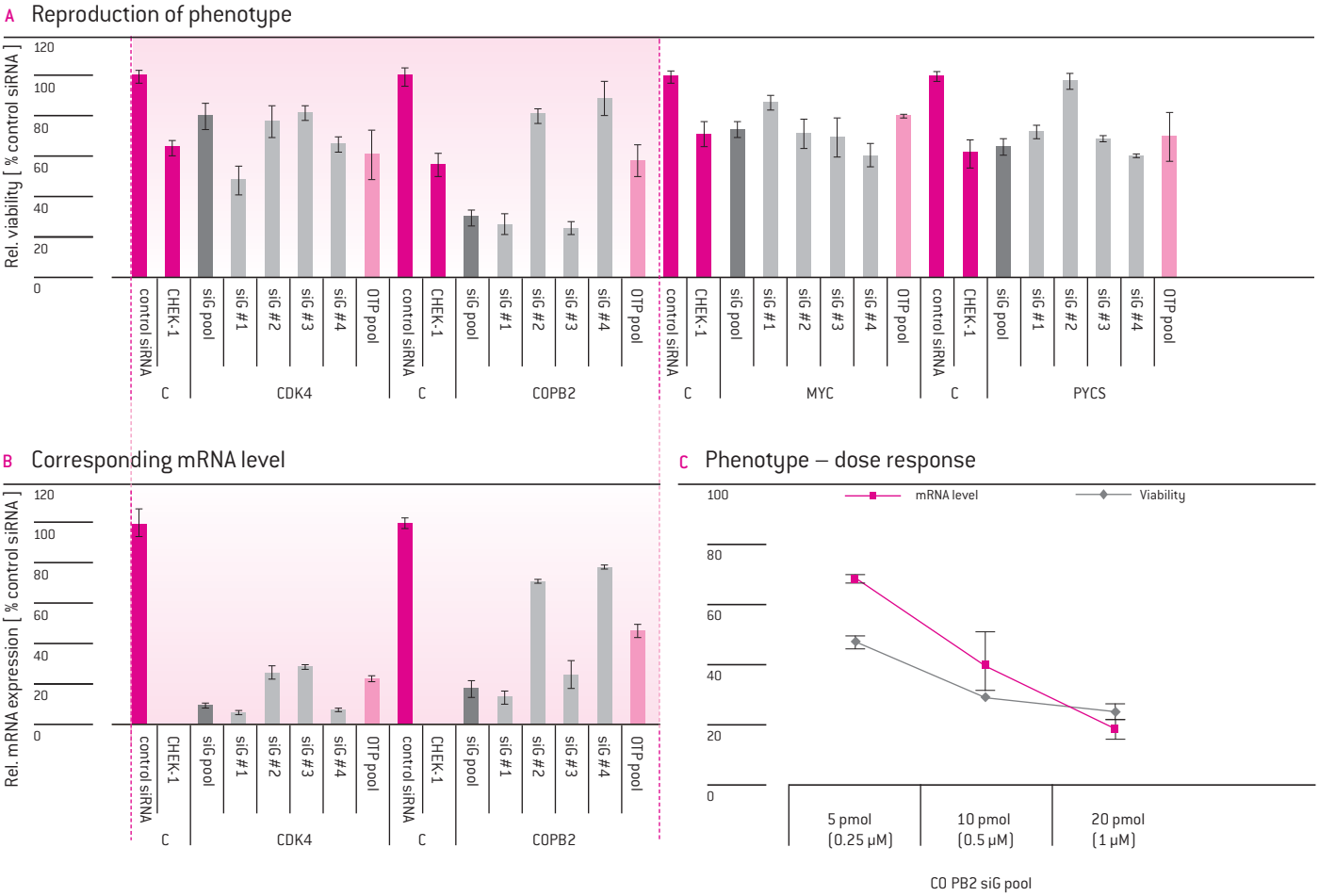


Figure 3. Hit validation. HUVEC cells were transfected with 20 pmol (if not indicated differently) siGENOME® (siG) SMARTpool® or single siRNA #1 – 4 (from the de-convoluted pool) targeting CDK4, COPB2, MYC or PYCS. CHEK-1 and siGENOME® Non-targeting siRNA #1 (siC) served as controls. 72 hours post Nucleofection®, cell viability was analyzed and normalized to negative control siRNA (A, C) and mRNA levels were determined for CDK4 (B) and COPB2 (B, C) and normalized to cyclophilin B mRNA and negative control siRNA.

potential hits identified by the viability assay were not revealed by the apoptosis assay and vice versa, exemplified by FADD and CASP3 (Figure 5A/B and data not shown).

Hit validation

We started validation experiments for FADD and our former positive control CASP3 by using the single ON-TARGET*plus*® siRNA Sequences from the original pool. For FADD, viability phenotypes could be reproduced by 2 of 4 single ON-TARGET*plus*® siRNAs while for CASP3 only 1 of 4 showed an effect (Figure 5A). In conformity with the primary screening results, apoptosis phenotype (caspase 3/7 activity) was clearly reproduced for CASP3 with 2 of 4 single siRNA sequences, but not for FADD (Figure 5B). Observed phenotypes could be clearly correlated to mRNA knockdown (Figure 5C). FADD and CASP3, the two hits we validated to date, are known members of the FAS-mediated apoptosis pathway [19,20]. The importance of the remaining hits for this pathway has yet to be proven.

Screen 1: Protein Kinases and Cell Cycle Regulators in HUVEC Cells				
COPB2*	CHEK-1*,C	NLK	PKM2	CENPE
CNKSR1	PYCS*	MYC*	MST1R	PLK-1*,C
RAPGEF3	IRAK3	RPS6KL1	RIOK1	
CDK4*	PFKFB3	EDN2	MARK3	
Screen 2: FAS-induced Apoptosis in Jurkat Cells				
FASC	BAK1	PARP1	CASP3*,C	TRIM35
FADD*	DFFB	APAF1	TNFRSF1A	CASP8

Table 1: Top hits selected from primary screens. Hits are sorted in descending order of MADs. Hits marked with * have been already validated. C indicates the controls used for assay optimization.

Conclusion

Meaningful siRNA screening results strongly depend on efficient delivery of the siRNA into the selected cell type and appropriate assay establishment prior to starting the screen. Here, we have combined the highly functional Dharmacon siGENOME® or ON-TARGET*plus*® siRNA Reagents with the 96-well Shuttle® System to perform RNAi screens in difficult-to-transfect cell types. The screens in Jurkat and HUVEC cells are the first successful examples in these cell types relevant for research in immunology and angiogenesis, respectively. The combination of these 2 technologies is particularly powerful in that both cell types are refractory to lipid reagent-based transfection methods. This demonstrates that identification and validation of gene targets can now be conducted in more biologically-relevant cell types, e.g., primary cells, as the selection of cell types is not limited to those accessible by lipid-mediated transfection anymore.

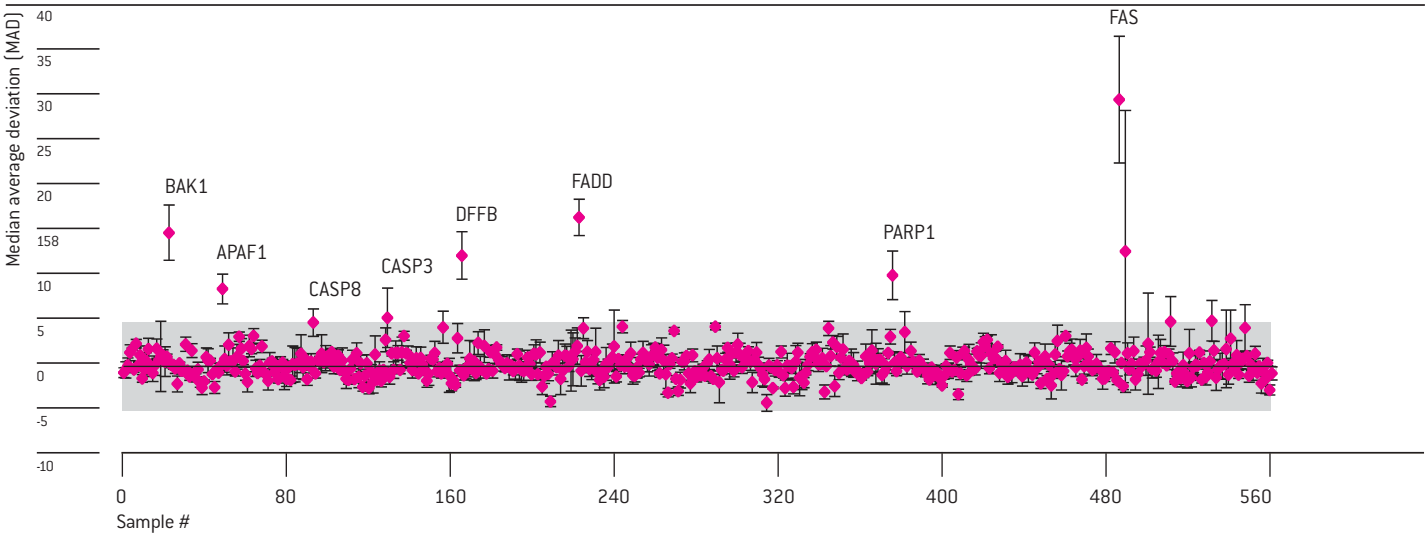
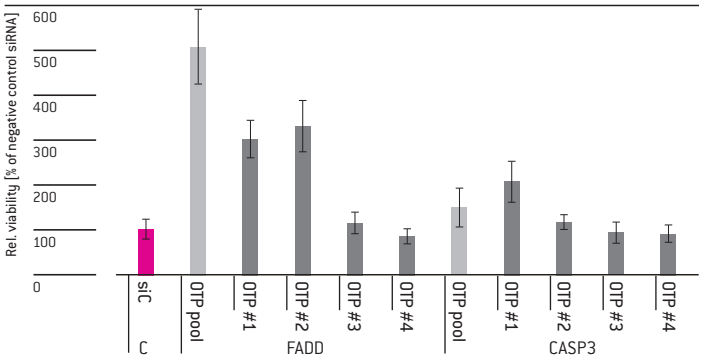
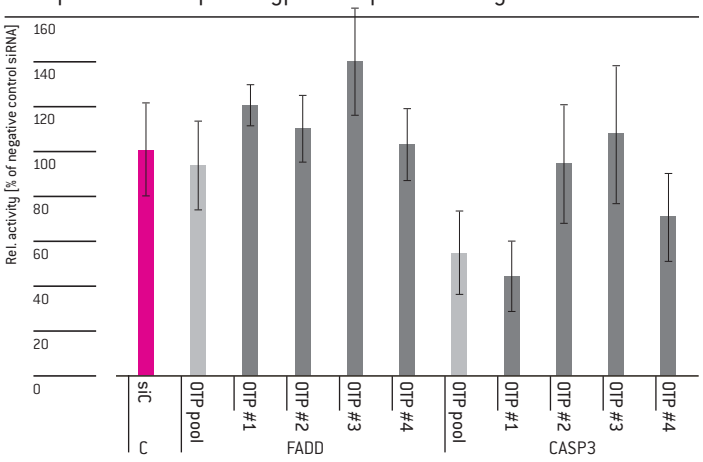


Figure 4: Reproducible hits from all 3 screens. Jurkat cells (clone E6-1, ATCC TIB-152™) were transfected in 3 independent experiments with the Human ON-TARGET*plus*® siRNA Library for Apoptosis (targeting 558 genes). Apoptosis was induced by adding 10 ng FAS-L to the cells 48 hours post Nucleofection®. Cell viability was analyzed after 2 hours. The mean of robust Z-scores of cell viability measures was calculated for 3 independent experiments. Targets with an |MAD| of at least 5 are marked as potential hits.

A Reproduction of phenotype – viability



B Reproduction of phenotype – caspase activity



C Corresponding mRNA levels

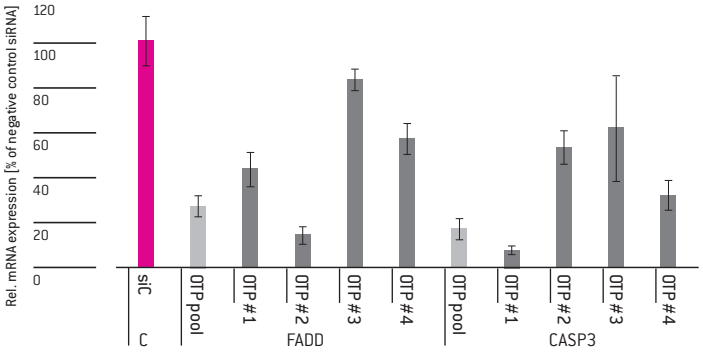


Figure 5: Hit validation. Jurkat cells (clone E6-1, ATCC® TIB-152™) were transfected with 20 pmol ON-TARGET*plus*® (OTP) pool (light grey bars) or single siRNA #1 – 4 (from the de convoluted pool; dark grey bars) targeting FADD or CASP3. siGENOME® Non-targeting siRNA #1 (siC) served as negative control (red bars). Apoptosis was induced by adding 10 ng FAS-L to the cells 48 hours post-transfection. Cell viability (A) and caspase activity (B) were analyzed 2 hours post-induction and normalized to negative control siRNA. mRNA evels (C) were determined 24 hours post Nucleofection®. Values were individually normalized to cyclophilin B mRNA and set in correlation to negative control siRNA (100%).

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