APPLICATION NOTE

RNA interference rescue using precision LentiORF collection.

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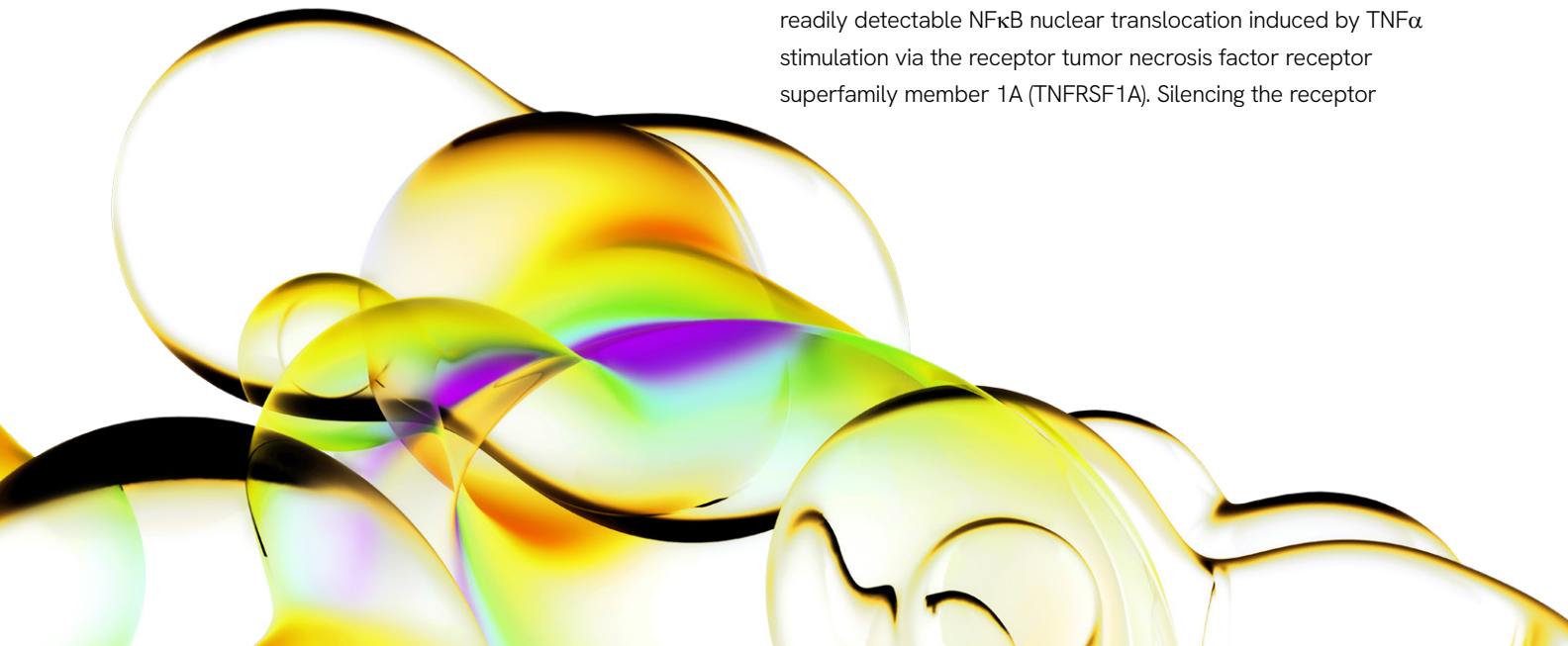
Abstract

The application of siRNA to accomplish RNA interference (RNAi) has emerged as a powerful and useful method to elucidate gene function and pathway analysis. However, in addition to gene-specific silencing through perfect complementarity to the target transcript, siRNA-mediated silencing can also lead to regulation of unintended transcripts through seed-region complementarity. This off-targeted regulation can potentially cause phenotypic effects leading to false interpretations of target gene function. A commonly accepted practice for validating candidate (putative) hits produced from RNAi-based functional analyses includes confirmation with several unique siRNAs, thus building confidence that the phenotype is the result of target mRNA knockdown rather than an off-target event. However, phenotypic rescue with an exogenously expressed siRNA-insensitive transcript is viewed as a more definitive hit validation method.

Revvity's Precision LentiORF™ Collection is a library of expression-ready lentiviral open reading frames (ORFs), that are sequence-validated and guaranteed to express. The collection represents key biological pathways and gene families in the human genome with the long-term goal of creating a genome-scale collection. The ORF constructs only include the protein-coding sequence. So, by transducing cells with these exogenously expressed constructs, researchers can express ORFs that are resistant to cleavage by siRNAs which target the 3'-UTRs of endogenous targets, thus permitting phenotypic rescue as a means of validating gene function.

In this study, we selected a well-characterized pathway involving readily detectable NFκB nuclear translocation induced by TNFα stimulation via the receptor tumor necrosis factor receptor superfamily member 1A (TNFRSF1A). Silencing the receptor



prevents NFκB nuclear translocation — a phenotype which can then be rescued by the corresponding LentiORF construct. This example highlights the necessary controls and optimization required for an RNAi rescue experiment and serves as a proof-of-principle for the utility of these constructs as powerful complementary tools in RNAi-based strategies to validate phenotypes associated with siRNA-mediated silencing.

Introduction

Synthetic siRNA libraries targeting whole genomes are powerful tools for large-scale functional screens in cellular models through RNA interference (RNAi). However, it has been clearly established that in addition to target-specific effects, siRNAs may also induce a range of off-target effects, usually through seed-mediated complementarity within the 3′ untranslated region (3′-UTR) of mRNA transcripts (Figure 1)[1-4]. These off-target effects can lead to misinterpretation of data generated by large-scale siRNA screening.

A variety of validation strategies can be employed to determine the target specificity of an siRNA-induced phenotype. Testing multiple unique siRNAs is the most common validation practice, especially in follow-up analysis of candidate hits derived from high throughput screens. However, functional rescue experiments have historically been considered the 'gold standard' in loss-of-function studies[5]. Specifically, rescuing the siRNA-induced phenotype by expressing the target gene in an RNAi-resistant form is a decisive way to validate the target gene's role in the observed phenotype[6].

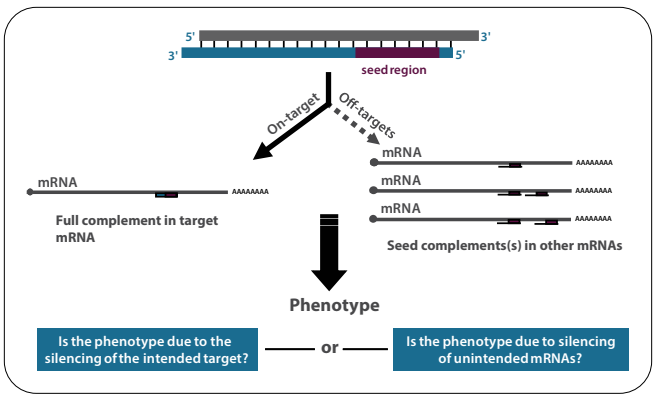


Figure 1: Validation of target specificity of siRNA phenotype is necessary for accurate data interpretation in RNAi screening. siRNA phenotypes may result from target-mediated effects or effects derived from partial seed-mediated complementarity to unintended targets, which can lead to false positive interpretations of RNAi screening results.

One approach to generating RNAi-resistant constructs involves the creation of silent point mutations in the siRNA target site[7-9]; however, the cloning procedures required to introduce silent mutations in the siRNA target sequence can be time consuming, expensive and mutations might not necessarily result in resistance to the siRNA. Another strategy to circumvent these difficulties employs cross-species cDNAs as RNAi-resistant rescue constructs due to DNA sequence divergence. Some of the challenges for this approach include highly similar or identical target sequence across conserved genes or gene function(s) not being conserved across species[10]. A third approach, demonstrated herein, can be used to validate 3′ UTR-targeting siRNAs by exogenously expressing the ORF of the target gene with a transduced expression construct (Figure 2).

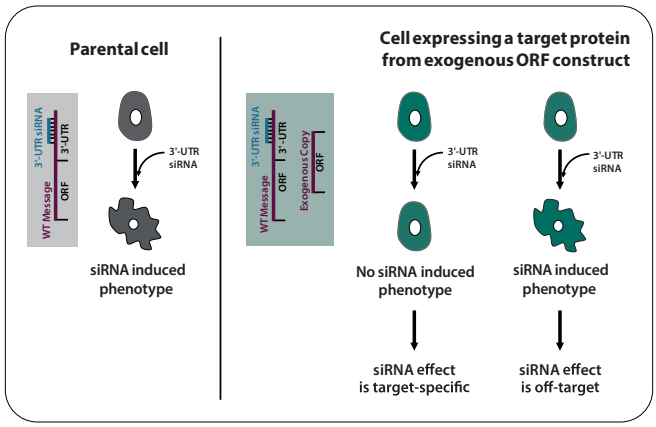
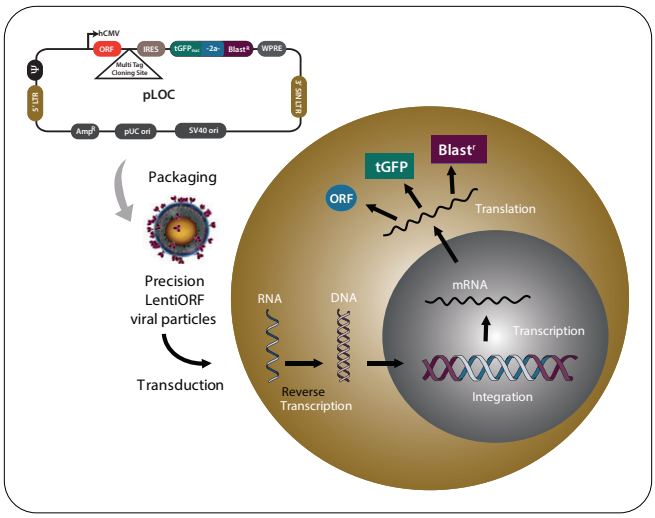


Figure 2: ORF expression can be used as a rescue strategy for validation of target-specific effects of 3′-UTR-targeting siRNAs. siRNA targeting the 3′-UTR of a target mRNA results in a phenotype (schematically represented as a morphology change). This siRNA-induced phenotype could either be target-specific or due to off-targeting effects. Exogenously expressing the ORF of the gene of interest provides the cell with an RNAi-resistant target for an siRNA targeting the 3′-UTR of the gene and could be used to rescue the siRNA-induced phenotype. If this results in phenotype rescue, it is an affirmation that the phenotype is due to target-specific effects of the siRNA. However, if the phenotype is still observed, one must also consider other sources for the aberrant phenotype including unintended or non-specific silencing.

The exogenous ORF transcript lacks the 3′-UTR sequence targeted by the siRNA, therefore, the siRNA will silence endogenous, but not exogenous expression, permitting rescue of the target-specific siRNA phenotype. The major challenges of this approach include availability of fully sequenced cDNA clones and time consuming cloning procedures to remove the 3′-UTR region. The Precision LentiORF Collection simplifies this approach by taking advantage of the fully sequenced ORFeome Collaboration

Collection (orfeomecollaboration.org) in a lentiviral vector to create a library of expression-ready ORFs. The vector is uniquely designed for the expression of a given ORF and two markers (TurboGFP, Evrogen, Moscow, Russia, and *Blasticidin S* resistance gene) under the CMV promoter, providing visual and selectable markers for ORF expression (Figure 3). While these constructs could be transfected as plasmids, they can also be transduced as lentiviral particles allowing for expression in an expanded range of cells including those that are refractory to transfection. In addition, the expression level of the ORF can be modulated by varying the ratio of functional viral particles to cells (multiplicity of infection; MOI) to approximate single or multi-copy gene expression.



Fgure 3: The Precision LentiORF Collection is a lentiviral genome-scale library of validated and expression-ready ORFs. Fully sequenced and expression-ready constructs are packaged in high-titer, purified lentiviral particles for expression in many cell types, including difficult-to-transfect cells. The construct's single transcript is expressed under the CMV promoter and includes the ORF, fluorescent reporter, and mammalian selection marker (TurboGFP, Evrogen, Moscow, Russia, and *Blasticidin S* resistance gene).

To demonstrate the utility of Precision LentiORF constructs in RNAi rescue and to provide a workflow for the experimental design of a rescue, a signaling pathway with well-studied components and a well-characterized phenotype was desired. For this reason, we selected the signaling pathway which involves the tumor necrosis factor receptor superfamily member 1A (TNFRSF1A) and the cascade of events that lead to nuclear translocation of the transcription factor, NFκB, upon receptor stimulation. By silencing expression of the receptor, we were able to test the ability of the corresponding LentiORF construct to restore the phenotype.

TNFRSF1A[11] is a receptor for tumor necrosis factor alpha (TNFα), a multifunctional proinflammatory cytokine that is involved in the regulation of a wide spectrum of biological processes. In addition to its primary role in immune cell regulation, TNFα has been shown to regulate cell proliferation, differentiation, apoptosis, inflammation, tumorigenesis, viral replication, and lipid metabolism[12, 13]. The binding of TNFα to TNFRSF1A causes a conformational change in the receptor, leading to multiple cell signaling events. One of the major signaling events is NFκB activation (Figure 4A)[14, 15]. NFκB is a heterodimeric transcription factor that is normally localized to the cytoplasm, bound and inhibited by IκBα. Upon TNFα binding to TNFRSF1A, major signaling events are initiated, among which is IκBα phosphorylation. Phosphorylated IκBα is rapidly degraded, releasing NFκB to translocate to the nucleus where it mediates the transcription of a vast array of genes involved in cell survival and proliferation, inflammatory responses, and anti-apoptotic factors.

In wild type cells treated with TNFα, NFκB translocates from a predominantly cytoplasmic localized state to the nuclei (Figure 4B). NFκB localization can be measured by high-content imaging analysis using standardized protocols and software. However, upon inhibition or down-regulation of TNFRSF1A, as with TNFRSF1A siRNA treatment, the events leading to translocation of NFκB in response to TNFα treatment are inhibited and NFκB remains localized to the cytoplasm. Figure 4C demonstrates the effect of an siRNA targeting TNFRSF1A on the cellular localization of NFκB upon TNFα stimulation in HeLa cells. Briefly, NFκB translocated to the nucleus upon TNFα treatment in untreated cells (UT) or cells transfected with non-targeting siRNA control (NTC). As expected, NFκB remained predominantly localized in the cytoplasm after TNFα stimulation when the cells were transfected with siRNA targeting TNFRSF1A.

In this experiment, we used TNFRSF1A Precision LentiORF to rescue the NFκB translocation phenotype of siRNAs targeting the 3' UTR region of TNFRSF1A. This rescue clearly demonstrates that the observed phenotypes associated with the siRNA-mediated knockdown of TNFRSF1A are the result of target specific silencing of this receptor, thus providing additional support for the well established role of TNFRSF1A in NFκB signaling. Moreover, this study demonstrates the practical workflow and the utility of Precision LentiORF expression constructs in a robust rescue strategy for RNAi experiments.

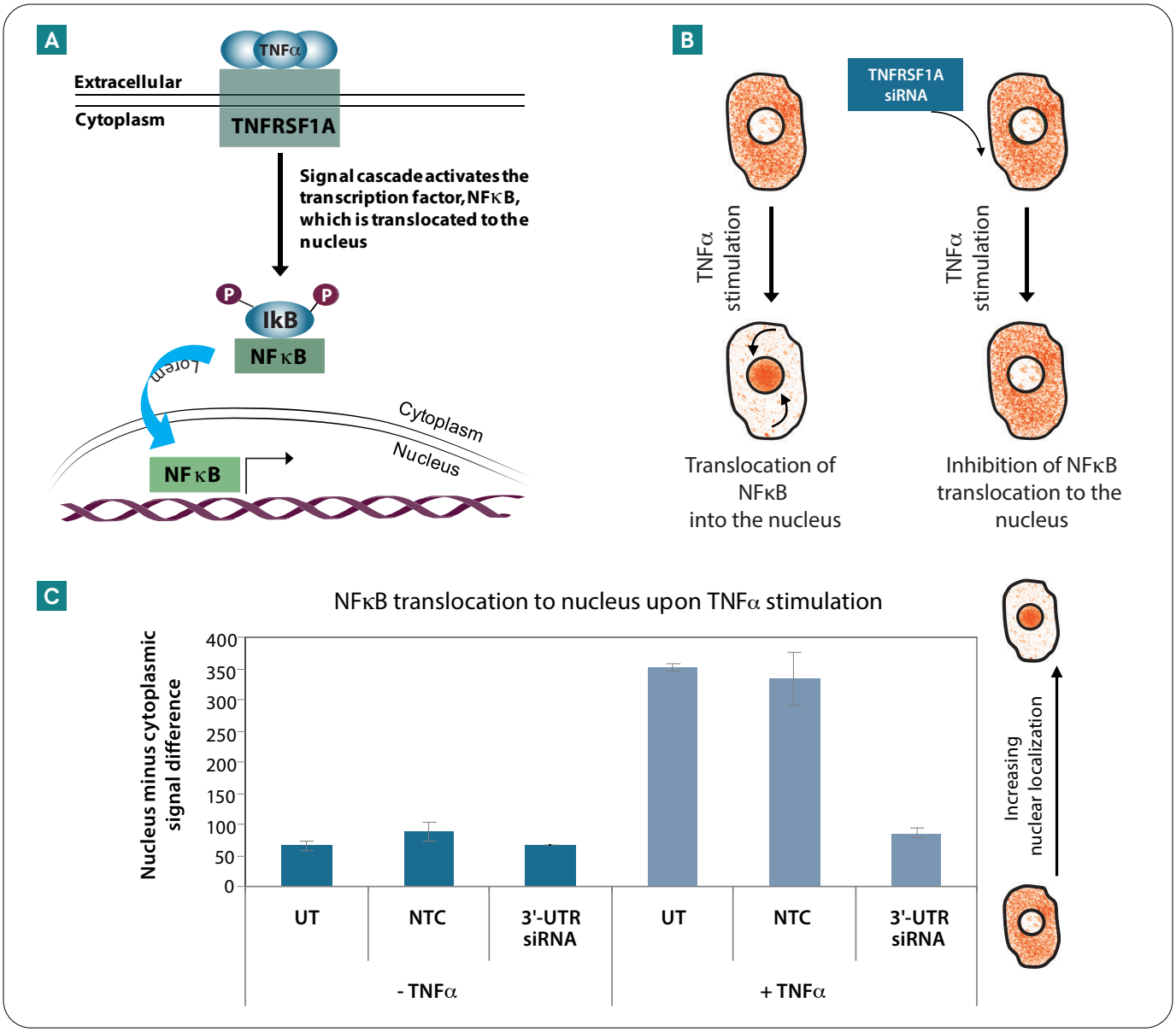


Figure 4: Silencing TNFRSF1A by siRNA causes inhibition of the NFκB translocation response to TNFα stimulation. **(A.)** TNFRSF1A is a receptor for the TNFα cytokine. One of the signaling events induced by TNFα is NFκB activation. NFκB is a transcription factor that is normally sequestered in the cytoplasm by binding to an inhibitory protein, IκBα. Upon TNFα binding to TNFRSF1A, the receptor transmits a signal through a cascade of events, resulting in phosphorylation and subsequent degradation of IκBα, releasing NFκB, which then translocates to the nucleus and activates the transcription of several genes. **(B.)** NFκB is localized in the cytoplasm but translocates into the nucleus upon TNFα stimulation. siRNA targeting the receptor TNFRSF1A inhibits the cell's ability to respond to TNFα stimulation, resulting in inactive NFκB, which remains localized to the cytoplasm. **(C.)** Empirical high-content analysis data acquired with the Thermo Scientific Cellomics ArrayScan VTI from HeLa cells stained for NFκB according to the NFkB activation HCS kit. NFκB translocation is reported as a difference between the nuclear and cytoplasmic fluorescence intensities from the NFκB stain. TNFα stimulation of untreated cells (UT) or cells transfected with the non-targeting siRNA control (NTC) leads to translocation of the NFκB into the nucleus, but not in cells transfected with an siRNA targeting the 3'-UTR of TNFRSF1A.

Results

To demonstrate the utility of Precision LentiORF reagents for RNAi rescue, we transduced HeLa cells with TNFRSF1A Precision LentiORF viral particles and analyzed the ability of the exogenously expressed ORF to rescue the phenotype caused by siRNA targeting the 3′-UTR region of endogenous TNFRSF1A mRNA. As an experimental control, we used HeLa cells that were transduced with a Precision LentiORF expressing GAPDH, a gene with function unrelated to the TNFRSF1A signaling pathway.

Initial optimization of transduction conditions is necessary to identify an optimal MOI that results in exogenous ORF expression comparable to the level of endogenous mRNA expression, since target over-expression may result in other distinct phenotypes. Figure 5A schematically describes the experimental workflow used in this rescue study. HeLa cells were transduced at MOI 1.5 (Day 1) and selected with Blasticidin S three days post-transduction (Day 4). The selected populations of cells stably expressing GAPDH or TNFRSF1A-ORFs were seeded in 96-well plates (Day 10) for transfection with siRNAs on the next day. Three days after transfection, cells were stimulated with TNFα before fixing and staining NFκB for high-content analysis. A parallel experimental plate was analyzed for TNFRSF1A expression levels on Day 12 using the TNFRSF1A Dharmacon™ Solaris™ qPCR Gene Expression Assay. An illustration of anticipated phenotype in cells expressing TNFRSF1A-ORF or control cells upon siRNA transfections is depicted in Figure 5B. NFκB should be localized to the nucleus in both control and target ORF cells when transfected with NTC siRNA. NFκB should remain in the cytoplasm in both control and target ORF cells when transfected with TNFRSF1A-ORF siRNA (capable of targeting both endogenous mRNA and exogenous ORF transcript). To demonstrate rescue, when cells are transfected with 3′-UTR siRNA (specific for the endogenous mRNA transcript) NFκB should remain in the cytoplasm for control cells, but translocate to the nucleus in the TNFRSF1A-ORF cells.

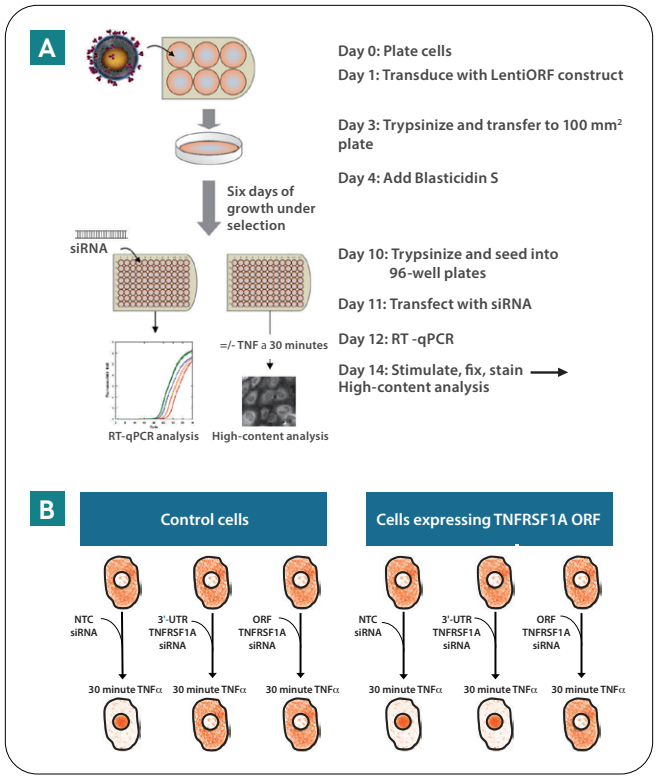


Figure 5: Rescue of the TNFRSF1A siRNA phenotype by Precision LentiORF: Experimental workflow and anticipated phenotypes. **A.** Schematic representation of the workflow used for validation of siRNA target specificity using Precision LentiORFs in a phenotype rescue strategy. HeLa cells were transduced with TNFRSF1A Precision LentiORF and GAPDH Precision LentiORF (as negative control) viral particles. Cells stably expressing the exogenous ORFs were selected by Blasticidin S and seeded in 96-well plates for siRNA transfection. RT-qPCR was employed to confirm expression levels at 24 hours post-transfection and high-content analysis for NFkB activation was performed at 72 hours post-transfection on cells stimulated with TNFα. **B.** Expected results of NFκB cellular localization upon TNFα stimulation of control cells and experimental cells exogenously expressing the TNFRSF1A-ORF after siRNA transfection. Predictions are made in both cell types transfected with non-targeting siRNA (NTC) or siRNAs targeting different regions of the TNFRSF1A gene: 3′-UTR (specific for the endogenous mRNA transcript) or ORF (capable of targeting both endogenous mRNA and exogenous ORF transcript).

Prior to examination of ORF expression on RNAi phenotype rescue, it is extremely important to assess the impact of the ORF expression on overall target mRNA expression levels and gene silencing. To this end we used Solaris qPCR Gene Expression Assays to monitor mRNA levels of TNFRSF1A and GAPDH. Since TNFRSF1A and GAPDH Solaris Assays are ORF-specific, they measure the combined expression level from endogenous mRNA as well as exogenous ORF expression. TNFRSF1A-ORF-expressing cells transduced at MOI 1.5 showed a combined expression almost 2-fold higher than untransduced (MOI 0) HeLa cells (Figure 6). Significant increases in GAPDH transcript level were not observed in the GAPDH-ORF cells. This statement would be greatly bolstered by a reference showing this to be true. siRNA reagents targeting the ORF region of TNFRSF1A (ORF siRNA 1 and 2) led to down-regulation of TNFRSF1A expression levels by greater than 90% in untransduced HeLa cells as well as in TNFRSF1A-ORF and GAPDH-ORF-expressing cells. siRNA reagents targeting the 3′-UTR of TNFRSF1A lead to down-regulation of the TNFRSF1A expression level by greater than 90% in untransduced Hela cells and GAPDH-ORF expressing cells. However, in the TNFRSF1A-ORF-expressing cells, the 3′-UTR-targeting siRNA reagents (selectively targeting endogenous TNFRSF1A mRNA) decreased TNFRSF1A expression to a level similar to untransduced HeLa cells. This indicates that the exogenous TNFRSF1A-ORF transcript is resistant to this siRNA and is expressed at a similar level to that of endogenous TNFRSF1A mRNA.

Once the expected effect on expression levels was confirmed, we examined if the phenotype induced by the 3′ UTR siRNA was rescued by TNFRSF1A-ORF expression. For this purpose, we stimulated cells with TNFα and stained NFκB for high-content analysis to assess NFκB activation. Figure 7 shows the quantitative analysis of NFκB translocation reported as the difference between nuclear and cytoplasmic fluorescence intensities. TNFα stimulation caused translocation of NFκB to the nucleus in cells that were either treated with the NTC siRNA or not treated with siRNA regardless of the ORF expressed. NFκB translocation upon TNFα stimulation was inhibited in all cells treated with siRNA targeting the ORF of TNFRSF1A.

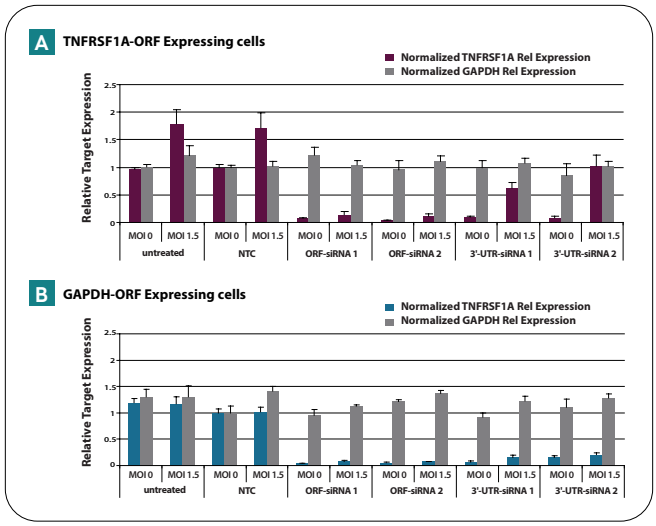


Figure 6: Expression analysis following siRNA transfection of cells expressing TNFRSF1A-ORF **(A.)** or GAPDH-ORF **(B.)** Over-expression due to transduction and down-regulation due to transfection was confirmed with RT-qPCR analysis using TNFRSF1A and GAPDH Solaris qPCR Gene Expression Assays. Expression analysis was performed on untreated HeLa cells (MOI = 0) and either TNFRSF1A-ORF-expressing cells **(A.)** or GAPDH-ORF-expressing cells **(B.)** (MOI = 1.5) upon transfection with siRNA reagents targeting the 3′-UTR or ORF region of TNFRSF1A. siRNA reagents targeting the ORF region of TNFRSF1A lead to down-regulation of the TNFRSF1A expression level in all studied cells. In contrast, siRNA reagents targeting the 3′-UTR of TNFRSF1A lead to down-regulation of the TNFRSF1A expression level in the control cells, but not in TNFRSF1A-ORF-expressing cells.

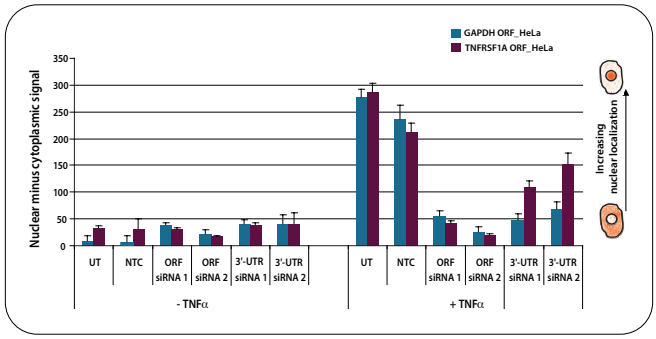


Figure 7: TNFRSF1A Precision Lenti ORF rescues the NFκB translocation phenotype from the siRNAs targeting the 3′-UTR of TNFRSF1A. Cells exogenously expressing GAPDH-ORF (blue) or TNFRSF1A-ORF (purple) were transfected with siRNA reagents targeting the 3′-UTR or ORF region of TNFRSF1A, then fixed and stained for NFκB. High-content analysis was performed with the Cellomics ArrayScan on cells stimulated or not stimulated with TNFα. NFκB translocation is reported as a difference between the nuclear and cytoplasmic fluorescence intensities of the NFκB stain.

NFκB translocation upon TNFα stimulation was also inhibited by the 3′-UTR specific TNFRSF1A siRNA in the control cells (GAPDH-ORF expressing). However, NFκB nuclear translocation was evident in TNFRSF1A-ORF-expressing cells transfected with 3′-UTR specific TNFRSF1A siRNA. The NFκB translocation was at 50-70% of the level measured in the TNFRSF1A-ORF cells transfected with NTC siRNA, demonstrating that TNFRSF1A-ORF rescues the phenotype induced by the 3′-UTR specific siRNAs. Representative images of NFκB-stained cells upon treatment with NTC siRNA and siRNA reagents targeting the ORF or 3′-UTR regions of TNFRSF1A are shown in Figure 8.

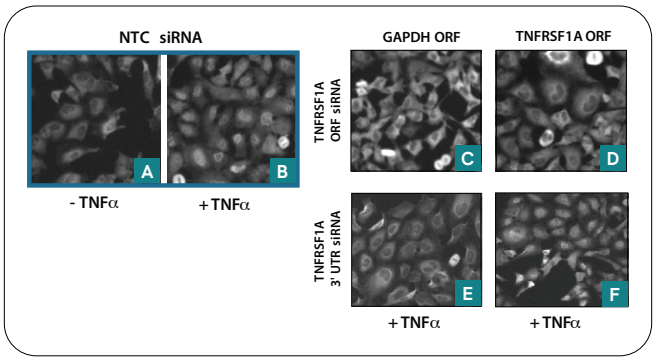


Figure 8: Visualization of the NFκB translocation rescue with high-content microscopy. Representative cell images collected with the Cellomics - ArrayScan during high-content analysis of the experiment outlined in Figure 7. NFκB is **(A.)** predominantly localized to the cytoplasm in HeLa cells or **(B.)** translocated to nuclei after TNFα stimulation. NFκB remains localized to the cytoplasm upon TNFα stimulation in **(C.)** GAPDH-ORF and **D** TNFRSF1A-ORF-expressing cells transfected with an siRNA targeting the ORF region of TNFRSF1A. When transfected with siRNA targeting the 3′-UTR of TNFRSF1A, NFκB remains localized to the cytoplasm **E** in GAPDH-ORF-expressing cells, but is translocated to the nuclei **F** of most of the TNFRSF1A-ORF-expressing cells.

Upon TNFα stimulation, NFκB clearly remains localized to the cytoplasm when cells are transfected with an siRNA targeting the ORF region of TNFRSF1A in the cells exogenously expressing GAPDH-ORF (Figure 8C) and in cells exogenously expressing TNFRSF1A-ORF (Figure 8D). However, when transfected with siRNA targeting the 3′ UTR of TNFRSF1A, NFκB is only translocated to the nuclei in TNFRSF1A-ORF expressing cells (Figure 8F) and not GAPDH-ORF-expressing cells (Figure 8E). This clearly demonstrates that the effect on NFκB translocation observed upon the siRNA-mediated knockdown of TNFRSF1A is the result of target specific silencing of the receptor.

Conclusions

siRNA-mediated gene silencing represents a powerful tool for functional gene analysis. However, siRNA off-target activity can confound the interpretation of phenotypic data and lead to inaccurate assignments of gene function. The gold standard for validating the outcome of a gene knockout or knockdown experiment is often to demonstrate functional restoration by reintroducing the target gene expression. Implementing this strategy for RNAi-based studies on a large scale can be challenging where a researcher must first silence a gene of interest with a siRNA and then attempt rescue with an expression construct that is resistant to siRNA activity. The Precision LentiORF collection of expression-ready lentiviral ORFs simplifies this approach significantly by providing a comprehensive set of ready-to-use clones that permit rescue specifically in studies that utilize 3′-UTR siRNA reagents. In this application note, we demonstrate utility of phenotypic validation using a TNFRSF1A Precision LentiORF expression construct to rescue the NFκB nuclear translocation phenotype induced by a 3′ UTR siRNA targeting TNFRSF1A. With the availability of these lentiviral expressed ORFs, RNAi rescue can become a routine strategy in validating putative gene-function relationships uncovered by RNAi-based screening experiments.

Materials and methods

Precision LentiORF transduction

HeLa cells (ATCC, Cat #CCL-2) were cultured under the ATCC recommended medium conditions. On day zero, 250,000 cells were plated into each well of a six-well tissue culture plate with growth medium. The following day (day 1), medium was removed and replaced with either GAPDH or TNFRSF1A Precision LentiORF viral particles (Dharmacon, Cat #OHS5900-100999005 and OHS5899-101003901, respectively) with an MOI of 1.5 in 1 mL serum-free medium. Titer was determined by fluorescent colony count in HEK293T cells transduced with serial dilutions of virus and was used to calculate the number of viral particles needed for a desired MOI. Six hours post-transduction, 4 mL of full-serum medium was added to each well. On day three, 48 hours post-transduction, cells were examined microscopically for

TurboGFP expression then trypsinized and passaged to p100 tissue culture plates. On day four, Blasticidin S was added at a concentration of 5 µg/mL. Cells were maintained under selection for six days, replacing medium or passaging every 2-3 days as needed. The selected populations of cells stably expressing GAPDH or TNFRSF1A-ORFs were used for siRNA transfection experiments. Untreated HeLa cells maintained alongside transduced cells and passaged at the same time as transduced cells served as a control population.

siRNA transfections

The selected populations of cells stably expressing GAPDH-ORF or TNFRSF1A-ORF and the control HeLa cells were plated in 96-well plates at a density of 2,000 cells per well in full serum medium and allowed to grow overnight. Biological triplicate wells were transfected with 25 nM final concentration siRNA targeting either the TNFRSF1A-ORF or 3′ UTR. Similarly, biological triplicate wells were transfected with NTC siRNA (Cat #D-001810-02) to serve as a treatment control. Finally, biological triplicate wells were spared transfection to serve as an additional control (UT). All siRNAs were transfected with a cell-density optimized amount of transfection reagent, 0.1 µL/well DharmaFECT 1 (Cat #T-2001). Parallel plates were transfected and analyzed by RT-qPCR at 24 hours post-transfection or by High-Content Microscopy at 72 hours post-transfection. The siRNAs targeting the ORF of TNFRSF1A (geneID 7132) used for transfection included: Cat #J-005197-08 and Cat #A-005197-16. The siRNAs targeting the 3′ UTR of TNFRSF1A included Cat #A-005197-15 and an siRNA that was custom designed using the siDESIGN Center (sense strand sequence 5′-GGUU CCCUGAGCCUUUUU), a free online siRNA design tool.

RT-qPCR

RNA was isolated using Promega™ SV 96 Total™ RNA Isolation System (Promega, Cat #Z3505), cDNA synthesis was performed using Thermo Scientific™ Verso™ cDNA Synthesis Kit (Cat #AB-1453) with 3:1 (volume:volume) random hexamer primers to oligo dT and gene expression analysis was performed using Solaris qPCR Gene Expression Assays and Master Mix (Cat #AB-4350), according to manufacturer's protocols. qPCR was executed with a Roche™ *Light*Cycler™ 480 in 384-well white plates (Roche, Cat #04729749001). Samples were assayed for TNFRSF1A (Cat #AX-005197-00), GAPDH (Cat #AX-004253-00) and PPIB (Cat #AX-004606-00). Relative expression was calculated using a ΔΔCq method[16], normalizing to PPIB expression and then to NTC siRNA control samples and is reported as a percentage of the NTC expression level.

High-content analysis for NFκB activation

Cells from each transfection treatment group as described above were incubated for 72 hours. Triplicate wells were either untreated or treated with 10 ng/µL TNFα (Thermo Scientific, Cat #RTNFAI) for 30 minutes at 37 °C to stimulate NFκB translocation to nucleus prior to fixing for high-content analysis. All cells were stained with Cellomics HCS Reagent Kit for NFκB (Thermo Scientific, Cat #8400401) to assay translocation of NFκB from the cytoplasm to nucleus. Cellomics™ ArrayScan™ VTI (Cat #N010002) was employed for high-content analysis of three replicate wells for each siRNA transfection or treatment. Cells were imaged using the 10x objective and the MEAN_CircRingAvgIntenDiffCh2 metric from the Molecular Translocation BioApplication software (Thermo Scientific, Cat #S507030V2) was utilized to quantify NFκB translocation. A minimum threshold of 600 cells was analyzed in each well. The averages and standard deviations from triplicate wells are reported for each treatment.

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