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High-throughput RNAi screening to dissect cellular pathways: a how-to guide

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High-throughput RNAi screening to dissect cellular pathways: a how-to guide

Christina Falschlehner, Sandra Steinbrink, Gerrit Erdmann and Michael Boutros

German Cancer Research Center and University of Heidelberg, Div. Signaling and Functional Genomics, D-69120 Heidelberg, Germany

Abstract

RNA interference (RNAi) has become a powerful tool to dissect cellular pathways and characterize gene functions. The availability of genome-wide RNAi libraries for various model organisms and mammalian cells has enabled high-throughput RNAi screenings. These RNAi screens successfully identified key components that had previously been missed in classical forward genetic screening approaches and allowed the assessment of combined loss-of-function phenotypes. Crucially, the quality of RNAi screening results depends on quantitative assays and the choice of the right biological context. In this review, we provide an overview on the design and application of high-throughput RNAi screens as well as data analysis and candidate validation strategies.

1 Introduction

In the past, reverse genetic techniques have successfully been used to identify and characterize gene functions. One of the most powerful tools in functional genomics emerged from the discovery of RNAi.

RNAi is a sequence-specific, post-transcriptional silencing process that is mediated by double-stranded RNA (dsRNA) molecules [1]. RNAi is a widespread cellular process that can be found in fungi, plants and animals. It is thought that RNAi has evolved as a defence mechanism to eliminate dsRNA from viruses. In recent years, RNAi has also been implicated in the regulation of transposons and in the formation and maintenance of heterochromatic regions in the genome [2-6].

The RNAi mechanism can be induced by endogenous microRNAs (miRNAs) encoded in the genome or by exogenous dsRNAs that have been introduced into the cell by viruses (shRNA) or synthetic experimental interventions [7, 8]. In mammalian cells, the transcription of an endogenously encoded miRNA leads to the formation of a hairpin-shaped RNA molecule, which is processed by the nuclease Drosha. The resulting pre-miRNA is transported from the nucleus into the cytoplasm and further processed by the RNase III-family member DICER into smaller fragments, typically between 21 and 23 nucleotides (nts) long, named

small interfering RNAs (siRNAs). Such siRNAs can also be chemically synthesized and artificially introduced, for example by transient liposome-based transfections. These RNAs are then incorporated into the RNA-induced silencing complex (RISC) where they serve as templates to recognize and pair to their complementary mRNAs [9]. Mature miRNAs preferentially bind to the 3' UTR whereas most synthetic siRNAs target the open reading frame. In case of a perfect match, the mRNA is cleaved and degraded rapidly whereas imperfect matching results in translational repression and destabilization of the respective mRNA (see Figure 1). The decrease in gene expression as a consequence of reduced mRNA level is referred to as a "knockdown". If synthetic siRNAs are designed to target the 3'UTR, the rescue construct can be designed without the 3'UTR, becoming "immune" to the siRNAs.

In recent years systematic screens employing RNAi have become a powerful technique for studying gene function in various model organisms. The availability of RNAi-reagent libraries covering full genomes allows high-throughput analysis of single gene functions as well as characterization of functional gene clusters and dissection of cellular signaling networks.

Model organisms, such as *Caenorhabditis elegans* and *Drosophila melanogaster* have proved successful for the application of large-scale RNAi screens *in vivo* and in cultured cell lines [11-14]. In *C. elegans*, RNAi can be performed by injecting dsRNA [1], by soaking worms in a solution of dsRNA [15], or by feeding *Escherichia coli* (*E.coli*) that express target-gene dsRNA [16]. In *Drosophila*, RNAi can be triggered by long dsRNAs, which are taken up very efficiently by cultured cells from the cell culture medium after a short starvation period [17]. In addition, *in vivo* fly libraries are available that express RNAi reagents under tissue or time-specific promoters and allow for the analysis of the RNAi effect in the whole animal [18, 19].

In mammalian cells, the application of long dsRNA induces a strong interferon response [20, 21]. Therefore, other strategies for RNA-induced gene silencing have to be applied. To date, genome-wide libraries targeting the human genome with short dsRNA molecules can be obtained from different sources and in various formats. These libraries usually contain pools of three to four independent siRNAs or several independent single sequences targeting one mRNA to increase the likelihood of a successful knockdown. In addition, endoribonucleolytically digested small overlapping dsRNA fragments (esiRNAs) and small hairpin RNA libraries (shRNAs) encoded on vector backbones can be used for systematic knockdown analysis [10, 22]. Chemically synthesized siRNAs and esiRNAs are introduced into the cells by liposomal transfection, electroporation or by linking RNAs to guiding peptides [9, 23-26]. These siRNAs can be further chemically modified to increase stability and transfection efficiency [27-29]. Furthermore, lenti-, retro- or adenoviral particles carrying DNA vectors that encode shRNAs can be used to transduce mammalian cells that are difficult to transfect with other methods [10, 30, 31]. Viral RNAi constructs can be used to easily generate cell lines stably expressing the knockdown construct. Thus,

1
2
3 persistent loss of a gene product enables monitoring of the knockdown over
4 longer time periods allowing the analysis of many relevant biological questions.
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6

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8 **2 High-throughput RNAi screening**
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10
11 Systematic RNAi screenings can be used to elucidate a biologically relevant
12 question that is reflected in the assay system of interest. For a precise and
13 meaningful result, this question needs to be well-defined, supplemented with a
14 carefully chosen read-out system, followed by an accurate and optimized
15 technical screening procedure. Special care should be taken when choosing the
16 read-out system. The system needs to be technically stable and robust to avoid
17 misleading background noise, but sensitive enough to detect subtle changes in
18 the analysed setting. In general, assays that are straightforward are easier to
19 handle and to control. The more complex an assay system is, the more controls
20 are needed to check for stability and correctness. Common read-out options to
21 analyse a variety of biological questions are the following:
22

23 Cellular viability: Several assay types based on the detection of fluorescence,
24 luminescence as well as absorption, are available. Cellular viability is often
25 correlated by quantifying ATP levels (e.g. CellTiterGlo, Promega), esterase
26 activity and membrane integrity (e.g. Calcein-AM, Sigma) or cellular redox
27 potential (e.g. WST-1/MTT, Sigma).
28

29 Cell morphology: (Immuno)fluorescent staining of cellular features such as
30 cytoskeleton, DNA and different organelles allows the parallel and automated
31 analysis of various cellular phenotypes with image analysis software. The
32 clustering of high-content phenotypic data can be used to generate so called
33 'phenoprints' that characterize the individual knockdowns.
34

35 Reporter assay: Fluorescence- or chemoluminescence-based assays in which
36 the activity of a signaling pathway or biological network can be investigated.
37 Usually, a transcription-factor binding site for a pathway-specific transcription-
38 factor is coupled to a reporter gene driven by a minimal promoter. These assays
39 can be either conducted using transient transfections or cell lines stably
40 expressing the reporter constructs.
41

42 Functional assays: A wide variety of functional assays can be used to assess
43 different cellular events and can be based on different read-outs (e.g. caspase
44 activity, Ca⁺ signaling).
45

46 In addition, many of the above mentioned assays can be combined or modified to
47 answer different questions or to draw further conclusions (combinatorial RNAi-
48 drug treatment, viability assessment of morphology screens by DNA content
49 quantification, and combinatorial viability and reporter screens).
50
51

52
53 A further decision has to be made on the model organism to use for the screen.
54 Human cultured cell lines offer the possibility to dissect the full human genome
55 for various processes e.g. those that are disturbed in different diseases. In
56 certain cases it might be advantageous to make use of model organisms such as
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58
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60

1
2
3 *C. elegans* or *Drosophila*. The RNAi procedure in worms and cultured fly cells is
4 relatively cheap and easy and the dissection of conserved signaling pathways or
5 biological processes is often more successful in identifying novel pathway
6 mediators due to a low genetic redundancy in these organisms. Furthermore,
7 when analysing complex biological questions such as behaviour or
8 developmental processes, the use of whole animal systems for screening is
9 indispensable. Nevertheless, full organism screens, especially in *Drosophila* flies,
10 are laborious and time consuming and the use of cultured cells is preferable to
11 facilitate the throughput and handling complexity.

12
13
14 When using human cells, the selection of the cell line has to be considered
15 carefully. Classical cultured cell lines, such as human embryonic kidney
16 (HEK293T) or cervix carcinoma (HeLa) cells are often used for genome-wide
17 RNAi screens as they are easy to grow and to transfect with siRNAs. However,
18 the use of these cell lines is probably not the right choice to analyse specific
19 biological procedures such as T- or B-cell receptor signaling. The right cell line
20 should always be defined by the biological question to answer. Such initial
21 considerations are crucial as they may predetermine the significance and
22 relevance of potential screening hits.

23
24 The biological question also determines the choice of the RNAi library. The
25 options include distinct subset libraries, for instance kinase or phosphatase
26 libraries, libraries covering the “druggable” genome or genome-wide libraries. A
27 collection of different available RNAi libraries is given in Table 1.

28
29
30 ((Table 1))

31
32
33 The use of siRNA pools, usually consisting of 3-4 single siRNA sequences,
34 versus the use of individual siRNAs is another choice to make [44]. On the one
35 hand, siRNA pools are likely to increase the knockdown efficiency and reduce
36 off-target effects due to the relatively lower concentration of the individual siRNAs
37 in the respective pool. On the other hand, the use of individual siRNA sequences
38 in a screen might facilitate hit evaluation as it enables target selection on the
39 condition that at least two or more individual siRNAs directed against the
40 respective gene show the same phenotype. The latter approach, however,
41 multiplies the consumption of materials and the time necessary to conduct a
42 screen. Thus, the final decision of which system to use also involves cost and
43 time considerations.

44
45 Before conducting a genome-wide screen, the screening approach has to be
46 evaluated on a small scale. For the pilot screen, a subset of randomly selected
47 genes that represent the respective library as well as positive and negative
48 controls have to be defined. These controls are an absolute requirement for the
49 reliance of the screen. The results of the pilot screen will allow conclusions to be
50 drawn on assay stability, robustness of controls and the overall dynamic range.
51 Performing a pilot screen will also help identify bottlenecks in the screening
52 procedure that may have to be optimized for a genome-wide screening protocol.
53 A common quality criterion for high-throughput RNAi screens is the Z'-factor that
54 describes the variability and the dynamic range of the assay between the positive
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and negative controls. A stable assay should result in a Z'-factor between 0.5 and 1 to enable the separation of hits from the background noise [45]. The final scale of the RNAi screen should be taken into consideration when calculating reagent amounts, incubator space, equipment area, handling times and batch sizes based on the results of the pilot screen. Additional time spent on optimizations at this stage will pay off in terms of data quality in the subsequent large-scale screen. Especially, efforts spent on the miniaturization of the assay system later on save time and money and often improve the stability of the system and therefore the data quality. Most assay types conducted in human cultured cell lines can be done in 384-well high-density cell culture plates. For smaller *Drosophila* cells 1536-well plates are feasible. To avoid variations during handling of those plates, the screening procedure should be automated in most steps. The screen itself is done relatively fast even on a genome-wide scale. Care should be taken to avoid every possible variation between plate and batch handling, the use of different reagent lots, and incubation periods.

3 Analysis of RNAi screening data

The analysis of raw data from a screen can be complex and time consuming. Computational data analysis methods are commonly used to normalize the data and to correct for e.g. plate position effects and batch to batch variations. Specific software solutions can be applied for normalization of the screen and for supplying an initial hit list of the strongest phenotypes (e.g. cellHTS, Spotfire/Tibco). cellHTS, a software package based on the programming language R and the opensource platform Bioconductor (www.bioconductor.org) was specifically designed to analyze RNAi screens capturing both workflow and data analysis [46]. In 2009, a web-based version of cellHTS was launched allowing for online data analysis (<http://web-cellhts2.dkfz.de>).

The analysis of genome-wide RNAi screens will result in a gene list with an associated list of z-scores. These scores describe the difference in standard deviations between a given value and the median of all values [47]. The range of z-scores will be large and the values for positive and negative controls should be nicely separable from each other (see Figure 2). A high z-score reflects a significant deviation of a phenotype from the median of a screen. Moderate phenotypes, reflected by lower z-scores, can be caused by an incomplete knockdown of a gene with a strong effect or by a strong knockdown of a gene with a weak phenotype.

For analysis of microscopy-based imaging screens, open source software packages such as CellProfiler (www.cellprofiler.org; [48]), EBImage (www.bioconductor.org/packages/bioc/1.9/html/EBImage.html), and DetecTIFF (www.detectiff.com; [49]) are available. These software tools analyse cell count and shape phenotypes as well as specific changes in staining levels created by antibody or dye staining of certain cellular events. Cells are identified and analysed for a variety of parameters, which are collected and saved as specific

phenotypic fingerprints of each phenotype. These phenotypic clusters can be used to identify novel gene functions by similarity analysis. However, storage of large-scale datasets, especially of microscopy-based imaging screens that can easily exceed a terabyte per screen remains challenging.

Depending on what the actual read-out of the screen is, several artefacts can occur and need to be corrected if possible. The most common are plate position effects such as edge, row and column effects. Edge effects usually derive from evaporation from assay plates. Artefacts can also arise due to chemicals used in a screen carried over from liquid handling steps or a poor chemical or biological stability of reagents over time. Most of these effects should be eliminated during the optimization procedure, others can be minimized by, for example, reserving an incubator exclusively for a screen, using so-called plate hotels/stackers and avoiding small volumes of liquid when possible. Plate position and other effects can be visualised by different software tools (e.g. cellHTS, Spotfire) and can be corrected by fitting functions that model these effects. Other artefacts can be harder or impossible to correct, thus leading to the exclusion of individual wells or plates from the screen (e.g. signaling decay of some luciferases). The final output of the initial data analyses is a list of the strongest hits in the screen, which can comprise of up to several dozens potential candidates.

4 Retests and secondary assays

To validate the phenotype of candidate genes found in an RNAi screen a comprehensive retesting strategy should be applied. This is necessary because RNAi can cause sequence-dependent and -independent off-target effects, meaning that not the candidate gene, but another (unknown) gene is responsible for the observed phenotype. Sequence-dependent off-targets can occur when the siRNA sense instead of the antisense strand is incorporated into the RISC and subsequently mediates silencing of non-targeted genes. The siRNA antisense strand can also partially match to another mRNA sequence consequently cause silencing of non-targeted genes. Sequence-independent off-target effects mostly originate from liposomal transfection procedures, from stimulation of pathways associated with the immune response and from saturating the endogenous RNAi machinery [50].

The initial analysis of genome-wide RNAi screening data leads to a long list of candidate genes that are ranked according to their z-scores reflecting the strength of the phenotype. To validate these phenotypes as a consequence of candidate gene silencing and not silencing of an unintended gene, multiple independent siRNA sequences should be used, which target the same gene at different locations in the mRNA sequence [51]. When a library of pooled siRNAs was used during the screening procedure, the candidate pools should be deconvoluted into single sequences. In addition, the gene phenotype can be confirmed with RNAi reagents from different vendors that target the respective mRNA at different sites. Furthermore, the knockdown efficiency of single siRNAs

should be validated using qRT-PCR or immunoblotting and correlated with the observed phenotype (see Figure 3A and B). Candidate gene phenotypes should at least be reproducible with two independent RNAi reagents.

The most reliable way of excluding off-target effects is by substituting the silenced gene with an RNAi-insensitive expression construct, reverting the RNAi phenotype. In the case of a long candidate gene list, this approach is rather laborious and time-consuming as the respective expression constructs have to be cloned first. Nevertheless, the phenotype of previously evaluated top-ranked candidates should finally be confirmed using this method.

Thus, several different strategies are commonly used to reduce initial hit lists depending on the screen type. Usually, the initial hits are retested with the same assay as used in the large-scale screen to eliminate false positives.

Secondary small-scale screens are often conducted using a different assay that elucidates the same biological question to eliminate false positives that are related to the specificity of the primary assay and to provide a hit list of highly probable candidates for further studies. Therefore a number of secondary assays can be performed that validate the phenotype with methods independent to those used in the screen. These assays should be specific for the question addressed by the screen thus eliminating unspecific hits. There is a wide variety of secondary assays that can be performed, for example microscopy-based translocation assays or immunoblot analysis (see Figure 3C and D).

After retesting and phenotype validation the candidate screen list will be shortened and improved. Finally, the remaining candidates can be investigated further to find their exact mechanism of action in the respective signaling pathway(s). A summary of the high-throughput RNAi procedure, including all essential screening steps described in the review, is given in Figure 4.

5 Outlook

During recent years, the identification of numerous novel gene functions have shown that genome-wide RNAi screens are a valuable tool for the analysis of various biological processes and the dissection of signaling pathways. Data sets resulting from these experiments are now being collected and compared in databases, enabling a systems biology approach which gives insight into the regulation of complex cellular signaling networks (<http://www.genomernai.org>). The collection of phenotypic parameters enables the clustering of genes according to the similarity of their phenotypes and the subsequent interpretation of putative related functions. Used in combination with drugs, systematic RNAi approaches can help unravel the mode of action of drugs, identify enhancers of the therapeutic mechanism or shed light on putative markers of resistance of a certain treatment [52-54]. Thus, RNAi screens might help in the near future to improve the treatment of highly resistant and aggressive tumours by identifying targets for efficient cotreatments [55-58].

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Table 1: Collection of available RNAi libraries

Description	Reference/Supplier	Organisms	Links
RNAi feeding library (87% genome coverage)	[13]; [32, 33]	<i>C. elegans</i>	www.gurdon.cam.ac.uk/~ahrin/gerlab/pages/rnai.html
RNAi library for injection	[14]; [34]	<i>C. elegans</i>	www.worm.mpi-cbg.de/phenobank2
Long dsRNA library (21,306 PCR templates)	[35]	<i>Drosophila</i>	www.flyrnai.org
Genome-wide dsRNA library (DRSC)	[12]	<i>Drosophila</i>	www.flyrnai.org
Genome-wide dsRNA library	[36]	<i>Drosophila</i>	http://rnai.dkfz.de
Long dsRNA library	[37]	<i>Drosophila</i>	http://www.openbiosystem.com
Non-mammalian subset libraries	Distributed by Open Biosystems	<i>Drosophila</i> , <i>C. elegans</i> , <i>Arabidopsis thaliana</i> , Zebrafish morpholinos	www.openbiosystems.com
esiRNA library	[26]	Human	www.mpi-cbg.de/esiRNA/index.html
shRNA library (TRC1/2)	Distributed by Sigma and Open Biosystems	Human	www.broadinstitute.org/rnai/trc
NKI shRNA library (targeting 8000 genes, retroviral)	[38]	Human	http://www.screeninc.nl
Library of retroviral vectors	[39]	Human	http://codex.cshl.edu
Genome-wide siRNA library, subset libraries e.g. kinases, phosphatases	Dharmacon; [40]	Human, Mouse	www.dharmacon.com
Genome-wide shRNA library (GeneNet™)	System Biosciences	Human, Mouse	www.systembio.com
shRNA-mir library (retroviral)	[41]	Human, Mouse	
Genome-wide siRNA library, subset libraries	Qiagen; [42]	Human, Mouse, Rat	www.qiagen.com

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Genome-wide siRNA library, subset libraries	Ambion; [43]	Human, Mouse, Rat, Drosophila (subset)	www.ambion.com
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Figure 1: Schematic view of RNAi in mammalian cells. Endogenous microRNAs (miRNA) and exogenous small hairpin RNAs (shRNA) are transcribed from DNA by RNA polymerase II. The resulting RNA hairpin is then processed by the nuclease Drosha, resulting in pre-miRNA/processed shRNA which is transported from the nucleus into the cytoplasm via exportin 5 (Exp5). The RNA is further processed into ~21bp fragments by the ribonuclease DICER. Synthetic short-interfering RNAs (siRNAs) resemble DICER processed double-stranded RNAs. These RNAs are incorporated into the RNA-induced silencing complex (RISC) where they are unwound and bind to complementary mRNA. Mature miRNAs preferentially bind to the 3' UTR while most siRNAs target the open reading frame. In case of a perfect match, the mRNA is cleaved and degraded. In contrast, imperfect matching leads to translational repression and consequent mRNA destabilization [9, 10].

Figure 2: Scatter plot of average z-scores from a genome-wide RNAi screen. Luciferase reporter activity was measured using Mithras LB940 plate reader (Berthold Technologies) and TOPflash (TCF reporter plasmid). Cell viability was determined using Calcein-AM. siRNAs targeting b-catenin that result in reduction of wnt reporter activity were used as positive control. siRNAs targeting UBC cause loss of cell viability and were used as viability control. Non-targeting siRNAs were used as negative control.

Figure 3: Hit validation with retests and secondary assays. A) Phenotype scores of control, single siRNAs and corresponding siRNA pool. B) Matching qRT-PCR validating knockdown of control, single siRNAs and corresponding siRNA pool. C) Nuclear translocation assay showing localization of the wnt pathway component β -catenin upon signal cascade activation by RNAi (scale bars equal 10 μ m). D) Western blot analysis indicating knockdown of β -catenin on protein level.

Figure 4: Schematic view of the RNAi screening procedure. After definition of the biological question and choice of the right cellular system, the read-out assay needs to be established. A pilot screen is performed to determine the robustness of the screening procedure and to identify possible optimization steps. After analysis of the pilot screen, genome-wide RNAi screenings can be performed, leading to a candidate list ranked according to the z-scores reflecting the strength of the phenotype. The candidates are then validated by retesting and secondary assays.

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