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

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**Research Article ((5178 words))****High-throughput RNAi screening to dissect cellular pathways: a how-to guide**

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**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

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3 small interfering RNAs (siRNAs). Such siRNAs can also be chemically  
4 synthesized and artificially introduced, for example by transient liposome-based  
5 transfections. These RNAs are then incorporated into the RNA-induced silencing  
6 complex (RISC) where they serve as templates to recognize and pair to their  
7 complementary mRNAs [9]. Mature miRNAs preferentially bind to the 3' UTR  
8 whereas most synthetic siRNAs target the open reading frame. In case of a  
9 perfect match, the mRNA is cleaved and degraded rapidly whereas imperfect  
10 matching results in translational repression and destabilization of the respective  
11 mRNA (see Figure 1). The decrease in gene expression as a consequence of  
12 reduced mRNA level is referred to as a "knockdown". If synthetic siRNAs are  
13 designed to target the 3'UTR, the rescue construct can be designed without the  
14 3'UTR, becoming "immune" to the siRNAs.  
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19 In recent years systematic screens employing RNAi have become a powerful  
20 technique for studying gene function in various model organisms. The availability  
21 of RNAi-reagent libraries covering full genomes allows high-throughput analysis  
22 of single gene functions as well as characterization of functional gene clusters  
23 and dissection of cellular signaling networks.  
24

25 Model organisms, such as *Caenorhabditis elegans* and *Drosophila melanogaster*  
26 have proved successful for the application of large-scale RNAi screens *in vivo*  
27 and in cultured cell lines [11-14]. In *C. elegans*, RNAi can be performed by  
28 injecting dsRNA [1], by soaking worms in a solution of dsRNA [15], or by feeding  
29 *Escherichia coli* (*E.coli*) that express target-gene dsRNA [16]. In *Drosophila*,  
30 RNAi can be triggered by long dsRNAs, which are taken up very efficiently by  
31 cultured cells from the cell culture medium after a short starvation period [17]. In  
32 addition, *in vivo* fly libraries are available that express RNAi reagents under  
33 tissue or time-specific promoters and allow for the analysis of the RNAi effect in  
34 the whole animal [18, 19].  
35

36 In mammalian cells, the application of long dsRNA induces a strong interferon  
37 response [20, 21]. Therefore, other strategies for RNA-induced gene silencing  
38 have to be applied. To date, genome-wide libraries targeting the human genome  
39 with short dsRNA molecules can be obtained from different sources and in  
40 various formats. These libraries usually contain pools of three to four  
41 independent siRNAs or several independent single sequences targeting one  
42 mRNA to increase the likelihood of a successful knockdown. In addition,  
43 endoribonucleolytically digested small overlapping dsRNA fragments (esiRNAs)  
44 and small hairpin RNA libraries (shRNAs) encoded on vector backbones can be  
45 used for systematic knockdown analysis [10, 22]. Chemically synthesized  
46 siRNAs and esiRNAs are introduced into the cells by liposomal transfection,  
47 electroporation or by linking RNAs to guiding peptides [9, 23-26]. These siRNAs  
48 can be further chemically modified to increase stability and transfection efficiency  
49 [27-29]. Furthermore, lenti-, retro- or adenoviral particles carrying DNA vectors  
50 that encode shRNAs can be used to transduce mammalian cells that are difficult  
51 to transfect with other methods [10, 30, 31]. Viral RNAi constructs can be used to  
52 easily generate cell lines stably expressing the knockdown construct. Thus,  
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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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## 8 **2 High-throughput RNAi screening**

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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:  
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24 Cellular viability: Several assay types based on the detection of fluorescence,  
25 luminescence as well as absorption, are available. Cellular viability is often  
26 correlated by quantifying ATP levels (e.g. CellTiterGlo, Promega), esterase  
27 activity and membrane integrity (e.g. Calcein-AM, Sigma) or cellular redox  
28 potential (e.g. WST-1/MTT, Sigma).  
29

30 Cell morphology: (Immuno)fluorescent staining of cellular features such as  
31 cytoskeleton, DNA and different organelles allows the parallel and automated  
32 analysis of various cellular phenotypes with image analysis software. The  
33 clustering of high-content phenotypic data can be used to generate so called  
34 'phenoprints' that characterize the individual knockdowns.  
35

36 Reporter assay: Fluorescence- or chemoluminescence-based assays in which  
37 the activity of a signaling pathway or biological network can be investigated.  
38 Usually, a transcription-factor binding site for a pathway-specific transcription-  
39 factor is coupled to a reporter gene driven by a minimal promoter. These assays  
40 can be either conducted using transient transfections or cell lines stably  
41 expressing the reporter constructs.  
42

43 Functional assays: A wide variety of functional assays can be used to assess  
44 different cellular events and can be based on different read-outs (e.g. caspase  
45 activity, Ca<sup>+</sup> signaling).  
46

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

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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.

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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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3 and negative controls. A stable assay should result in a Z'-factor between 0.5  
4 and 1 to enable the separation of hits from the background noise [45].

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6 The final scale of the RNAi screen should be taken into consideration when  
7 calculating reagent amounts, incubator space, equipment area, handling times  
8 and batch sizes based on the results of the pilot screen. Additional time spent on  
9 optimizations at this stage will pay off in terms of data quality in the subsequent  
10 large-scale screen. Especially, efforts spent on the miniaturization of the assay  
11 system later on save time and money and often improve the stability of the  
12 system and therefore the data quality. Most assay types conducted in human  
13 cultured cell lines can be done in 384-well high-density cell culture plates. For  
14 smaller *Drosophila* cells 1536-well plates are feasible. To avoid variations during  
15 handling of those plates, the screening procedure should be automated in most  
16 steps. The screen itself is done relatively fast even on a genome-wide scale.  
17 Care should be taken to avoid every possible variation between plate and batch  
18 handling, the use of different reagent lots, and incubation periods.  
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### 22 23 **3 Analysis of RNAi screening data**

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26 The analysis of raw data from a screen can be complex and time consuming.  
27 Computational data analysis methods are commonly used to normalize the data  
28 and to correct for e.g. plate position effects and batch to batch variations.  
29 Specific software solutions can be applied for normalization of the screen and for  
30 supplying an initial hit list of the strongest phenotypes (e.g. cellHTS,  
31 Spotfire/Tibco). cellHTS, a software package based on the programming  
32 language R and the opensource platform Bioconductor ([www.bioconductor.org](http://www.bioconductor.org))  
33 was specifically designed to analyze RNAi screens capturing both workflow and  
34 data analysis [46]. In 2009, a web-based version of cellHTS was launched  
35 allowing for online data analysis (<http://web-cellhts2.dkfz.de>).  
36  
37

38 The analysis of genome-wide RNAi screens will result in a gene list with an  
39 associated list of z-scores. These scores describe the difference in standard  
40 deviations between a given value and the median of all values [47]. The range of  
41 z-scores will be large and the values for positive and negative controls should be  
42 nicely separable from each other (see Figure 2). A high z-score reflects a  
43 significant deviation of a phenotype from the median of a screen. Moderate  
44 phenotypes, reflected by lower z-scores, can be caused by an incomplete  
45 knockdown of a gene with a strong effect or by a strong knockdown of a gene  
46 with a weak phenotype.  
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49  
50 For analysis of microscopy-based imaging screens, open source software  
51 packages such as CellProfiler ([www.cellprofiler.org](http://www.cellprofiler.org); [48]), EBImage  
52 ([www.bioconductor.org/packages/bioc/1.9/html/EBImage.html](http://www.bioconductor.org/packages/bioc/1.9/html/EBImage.html)), and DetectIFF  
53 ([www.detectiff.com](http://www.detectiff.com); [49] ) are available. These software tools analyse cell count  
54 and shape phenotypes as well as specific changes in staining levels created by  
55 antibody or dye staining of certain cellular events. Cells are identified and  
56 analysed for a variety of parameters, which are collected and saved as specific  
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3 phenotypic fingerprints of each phenotype. These phenotypic clusters can be  
4 used to identify novel gene functions by similarity analysis. However, storage of  
5 large-scale datasets, especially of microscopy-based imaging screens that can  
6 easily exceed a terabyte per screen remains challenging.  
7

8 Depending on what the actual read-out of the screen is, several artefacts can  
9 occur and need to be corrected if possible. The most common are plate position  
10 effects such as edge, row and column effects. Edge effects usually derive from  
11 evaporation from assay plates. Artefacts can also arise due to chemicals used in  
12 a screen carried over from liquid handling steps or a poor chemical or biological  
13 stability of reagents over time. Most of these effects should be eliminated during  
14 the optimization procedure, others can be minimized by, for example, reserving  
15 an incubator exclusively for a screen, using so-called plate hotels/stackers and  
16 avoiding small volumes of liquid when possible. Plate position and other effects  
17 can be visualised by different software tools (e.g. cellHTS, Spotfire) and can be  
18 corrected by fitting functions that model these effects. Other artefacts can be  
19 harder or impossible to correct, thus leading to the exclusion of individual wells or  
20 plates from the screen (e.g. signaling decay of some luciferases). The final  
21 output of the initial data analyses is a list of the strongest hits in the screen,  
22 which can comprise of up to several dozens potential candidates.  
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#### 28 **4 Retests and secondary assays**

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32 To validate the phenotype of candidate genes found in an RNAi screen a  
33 comprehensive retesting strategy should be applied. This is necessary because  
34 RNAi can cause sequence-dependent and -independent off-target effects,  
35 meaning that not the candidate gene, but another (unknown) gene is responsible  
36 for the observed phenotype. Sequence-dependent off-targets can occur when  
37 the siRNA sense instead of the antisense strand is incorporated into the RISC  
38 and subsequently mediates silencing of non-targeted genes. The siRNA  
39 antisense strand can also partially match to another mRNA sequence  
40 consequently cause silencing of non-targeted genes. Sequence-independent off-  
41 target effects mostly originate from liposomal transfection procedures, from  
42 stimulation of pathways associated with the immune response and from  
43 saturating the endogenous RNAi machinery [50].  
44  
45

46 The initial analysis of genome-wide RNAi screening data leads to a long list of  
47 candidate genes that are ranked according to their z-scores reflecting the  
48 strength of the phenotype. To validate these phenotypes as a consequence of  
49 candidate gene silencing and not silencing of an unintended gene, multiple  
50 independent siRNA sequences should be used, which target the same gene at  
51 different locations in the mRNA sequence [51]. When a library of pooled siRNAs  
52 was used during the screening procedure, the candidate pools should be  
53 deconvoluted into single sequences. In addition, the gene phenotype can be  
54 confirmed with RNAi reagents from different vendors that target the respective  
55 mRNA at different sites. Furthermore, the knockdown efficiency of single siRNAs  
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3 should be validated using qRT-PCR or immunoblotting and correlated with the  
4 observed phenotype (see Figure 3A and B). Candidate gene phenotypes should  
5 at least be reproducible with two independent RNAi reagents.  
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8 The most reliable way of excluding off-target effects is by substituting the  
9 silenced gene with an RNAi-insensitive expression construct, reverting the RNAi  
10 phenotype. In the case of a long candidate gene list, this approach is rather  
11 laborious and time-consuming as the respective expression constructs have to  
12 be cloned first. Nevertheless, the phenotype of previously evaluated top-ranked  
13 candidates should finally be confirmed using this method.  
14

15 Thus, several different strategies are commonly used to reduce initial hit lists  
16 depending on the screen type. Usually, the initial hits are retested with the same  
17 assay as used in the large-scale screen to eliminate false positives.  
18

19 Secondary small-scale screens are often conducted using a different assay that  
20 elucidates the same biological question to eliminate false positives that are  
21 related to the specificity of the primary assay and to provide a hit list of highly  
22 probable candidates for further studies. Therefore a number of secondary assays  
23 can be performed that validate the phenotype with methods independent to those  
24 used in the screen. These assays should be specific for the question addressed  
25 by the screen thus eliminating unspecific hits. There is a wide variety of  
26 secondary assays that can be performed, for example microscopy-based  
27 translocation assays or immunoblot analysis (see Figure 3C and D).  
28

29 After retesting and phenotype validation the candidate screen list will be  
30 shortened and improved. Finally, the remaining candidates can be investigated  
31 further to find their exact mechanism of action in the respective signaling  
32 pathway(s). A summary of the high-throughput RNAi procedure, including all  
33 essential screening steps described in the review, is given in Figure 4.  
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## 36 **5 Outlook**

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

- [1] Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., *et al.*, Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* 1998, 391, 806-811.
- [2] Aliyari, R., Ding, S. W., RNA-based viral immunity initiated by the Dicer family of host immune receptors. *Immunol Rev* 2009, 227, 176-188.
- [3] Pal-Bhadra, M., Bhadra, U., Birchler, J. A., RNAi related mechanisms affect both transcriptional and posttranscriptional transgene silencing in *Drosophila*. *Mol Cell* 2002, 9, 315-327.
- [4] Tabara, H., Sarkissian, M., Kelly, W. G., Fleenor, J., *et al.*, The *rde-1* gene, RNA interference, and transposon silencing in *C. elegans*. *Cell* 1999, 99, 123-132.
- [5] van Rij, R. P., Berezikov, E., Small RNAs and the control of transposons and viruses in *Drosophila*. *Trends Microbiol* 2009, 17, 163-171.
- [6] Volpe, T., Schramke, V., Hamilton, G. L., White, S. A., *et al.*, RNA interference is required for normal centromere function in fission yeast. *Chromosome Res* 2003, 11, 137-146.
- [7] Fedorov, Y., King, A., Anderson, E., Karpilow, J., *et al.*, Different delivery methods-different expression profiles. *Nat Methods* 2005, 2, 241.
- [8] Whitehead, K. A., Langer, R., Anderson, D. G., Knocking down barriers: advances in siRNA delivery. *Nat Rev Drug Discov* 2009, 8, 129-138.
- [9] Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., *et al.*, Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* 2001, 411, 494-498.
- [10] Brummelkamp, T. R., Bernards, R., Agami, R., Stable suppression of tumorigenicity by virus-mediated RNA interference. *Cancer Cell* 2002, 2, 243-247.
- [11] Bartscherer, K., Pelte, N., Ingelfinger, D., Boutros, M., Secretion of Wnt ligands requires Evi, a conserved transmembrane protein. *Cell* 2006, 125, 523-533.
- [12] Boutros, M., Kiger, A. A., Armknecht, S., Kerr, K., *et al.*, Genome-wide RNAi analysis of growth and viability in *Drosophila* cells. *Science* 2004, 303, 832-835.
- [13] Fraser, A. G., Kamath, R. S., Zipperlen, P., Martinez-Campos, M., *et al.*, Functional genomic analysis of *C. elegans* chromosome I by systematic RNA interference. *Nature* 2000, 408, 325-330.
- [14] Gonczy, P., Echeverri, C., Oegema, K., Coulson, A., *et al.*, Functional genomic analysis of cell division in *C. elegans* using RNAi of genes on chromosome III. *Nature* 2000, 408, 331-336.

- 1  
2  
3 [15] Tabara, H., Grishok, A., Mello, C. C., RNAi in *C. elegans*: soaking in the  
4 genome sequence. *Science* 1998, *282*, 430-431.
- 5 [16] Timmons, L., Fire, A., Specific interference by ingested dsRNA. *Nature*  
6 1998, *395*, 854.
- 7 [17] Clemens, J. C., Worby, C. A., Simonson-Leff, N., Muda, M., *et al.*, Use of  
8 double-stranded RNA interference in *Drosophila* cell lines to dissect signal  
9 transduction pathways. *Proc Natl Acad Sci U S A* 2000, *97*, 6499-6503.
- 10 [18] Kambris, Z., Brun, S., Jang, I. H., Nam, H. J., *et al.*, *Drosophila* immunity: a  
11 large-scale in vivo RNAi screen identifies five serine proteases required for Toll  
12 activation. *Curr Biol* 2006, *16*, 808-813.
- 13 [19] Dietzl, G., Chen, D., Schnorrer, F., Su, K. C., *et al.*, A genome-wide  
14 transgenic RNAi library for conditional gene inactivation in *Drosophila*. *Nature*  
15 2007, *448*, 151-156.
- 16 [20] Manche, L., Green, S. R., Schmedt, C., Mathews, M. B., Interactions  
17 between double-stranded RNA regulators and the protein kinase DAI. *Mol Cell*  
18 *Biol* 1992, *12*, 5238-5248.
- 19 [21] Stark, G. R., Kerr, I. M., Williams, B. R., Silverman, R. H., Schreiber, R. D.,  
20 How cells respond to interferons. *Annu Rev Biochem* 1998, *67*, 227-264.
- 21 [22] Kittler, R., Buchholz, F., Functional genomic analysis of cell division by  
22 endoribonuclease-prepared siRNAs. *Cell Cycle* 2005, *4*, 564-567.
- 23 [23] Gresch, O., Engel, F. B., Nestic, D., Tran, T. T., *et al.*, New non-viral method  
24 for gene transfer into primary cells. *Methods* 2004, *33*, 151-163.
- 25 [24] Muratovska, A., Eccles, M. R., Conjugate for efficient delivery of short  
26 interfering RNA (siRNA) into mammalian cells. *FEBS Lett* 2004, *558*, 63-68.
- 27 [25] Brazas, R. M., Hagstrom, J. E., Delivery of small interfering RNA to  
28 mammalian cells in culture by using cationic lipid/polymer-based transfection  
29 reagents. *Methods Enzymol* 2005, *392*, 112-124.
- 30 [26] Kittler, R., Putz, G., Pelletier, L., Poser, I., *et al.*, An endoribonuclease-  
31 prepared siRNA screen in human cells identifies genes essential for cell division.  
32 *Nature* 2004, *432*, 1036-1040.
- 33 [27] Birmingham, A., Anderson, E., Sullivan, K., Reynolds, A., *et al.*, A protocol  
34 for designing siRNAs with high functionality and specificity. *Nat Protoc* 2007, *2*,  
35 2068-2078.
- 36 [28] Deleavey, G. F., Watts, J. K., Damha, M. J., Chemical modification of siRNA.  
37 *Curr Protoc Nucleic Acid Chem* 2009, *Chapter 16*, Unit 16 13.
- 38 [29] Ge, Q., Dallas, A., Ilves, H., Shorestein, J., *et al.*, Effects of chemical  
39 modification on the potency, serum stability, and immunostimulatory properties of  
40 short shRNAs. *RNA*, *16*, 118-130.
- 41 [30] Rubinson, D. A., Dillon, C. P., Kwiatkowski, A. V., Sievers, C., *et al.*, A  
42 lentivirus-based system to functionally silence genes in primary mammalian cells,  
43 stem cells and transgenic mice by RNA interference. *Nat Genet* 2003, *33*, 401-  
44 406.
- 45 [31] Meacham, C. E., Ho, E. E., Dubrovsky, E., Gertler, F. B., Hemann, M. T., In  
46 vivo RNAi screening identifies regulators of actin dynamics as key determinants  
47 of lymphoma progression. *Nat Genet* 2009, *41*, 1133-1137.
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51  
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2  
3 [32] Kamath, R. S., Ahringer, J., Genome-wide RNAi screening in *Caenorhabditis*  
4 *elegans*. *Methods* 2003, *30*, 313-321.
- 5 [33] Kamath, R. S., Fraser, A. G., Dong, Y., Poulin, G., *et al.*, Systematic  
6 functional analysis of the *Caenorhabditis elegans* genome using RNAi. *Nature*  
7 2003, *421*, 231-237.
- 8 [34] Sonnichsen, B., Koski, L. B., Walsh, A., Marschall, P., *et al.*, Full-genome  
9 RNAi profiling of early embryogenesis in *Caenorhabditis elegans*. *Nature* 2005,  
10 *434*, 462-469.
- 11 [35] Hild, M., Beckmann, B., Haas, S. A., Koch, B., *et al.*, An integrated gene  
12 annotation and transcriptional profiling approach towards the full gene content of  
13 the *Drosophila* genome. *Genome Biol* 2003, *5*, R3.
- 14 [36] Gildsford, M., Horn, T., Arziman, Z., Pelz, O., *et al.*, GenomeRNAi: a database  
15 for cell-based RNAi phenotypes. 2009 update. *Nucleic Acids Res*, *38*, D448-452.
- 16 [37] Foley, E., O'Farrell, P. H., Functional dissection of an innate immune  
17 response by a genome-wide RNAi screen. *PLoS Biol* 2004, *2*, E203.
- 18 [38] Berns, K., Hijmans, E. M., Mullenders, J., Brummelkamp, T. R., *et al.*, A  
19 large-scale RNAi screen in human cells identifies new components of the p53  
20 pathway. *Nature* 2004, *428*, 431-437.
- 21 [39] Paddison, P. J., Silva, J. M., Conklin, D. S., Schlabach, M., *et al.*, A resource  
22 for large-scale RNA-interference-based screens in mammals. *Nature* 2004, *428*,  
23 427-431.
- 24 [40] Reynolds, A., Leake, D., Boese, Q., Scaringe, S., *et al.*, Rational siRNA  
25 design for RNA interference. *Nat Biotechnol* 2004, *22*, 326-330.
- 26 [41] Silva, J. M., Li, M. Z., Chang, K., Ge, W., *et al.*, Second-generation shRNA  
27 libraries covering the mouse and human genomes. *Nat Genet* 2005, *37*, 1281-  
28 1288.
- 29 [42] Huesken, D., Lange, J., Mickanin, C., Weiler, J., *et al.*, Design of a genome-  
30 wide siRNA library using an artificial neural network. *Nat Biotechnol* 2005, *23*,  
31 995-1001.
- 32 [43] Giuliano, K. A., Haskins, J. R., Taylor, D. L., Advances in high content  
33 screening for drug discovery. *Assay Drug Dev Technol* 2003, *1*, 565-577.
- 34 [44] Parsons, B. D., Schindler, A., Evans, D. H., Foley, E., A direct phenotypic  
35 comparison of siRNA pools and multiple individual duplexes in a functional  
36 assay. *PLoS One* 2009, *4*, e8471.
- 37 [45] Zhang, J. H., Wu, X., Sills, M. A., Probing the primary screening efficiency by  
38 multiple replicate testing: a quantitative analysis of hit confirmation and false  
39 screening results of a biochemical assay. *J Biomol Screen* 2005, *10*, 695-704.
- 40 [46] Boutros, M., Bras, L. P., Huber, W., Analysis of cell-based RNAi screens.  
41 *Genome Biol* 2006, *7*, R66.
- 42 [47] Larsen, R. J., Marx, M. L., An Introduction to Mathematical Statistics and Its  
43 Application. *Third Edition*. ISBN 0139223037. 2000, 282.
- 44 [48] Carpenter, A. E., Jones, T. R., Lamprecht, M. R., Clarke, C., *et al.*,  
45 CellProfiler: image analysis software for identifying and quantifying cell  
46 phenotypes. *Genome Biol* 2006, *7*, R100.
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3 [49] Gilbert, D. F., Meinhof, T., Pepperkok, R., Runz, H., DetecTiff: a novel image  
4 analysis routine for high-content screening microscopy. *J Biomol Screen* 2009,  
5 14, 944-955.  
6  
7 [50] Martin, S. E., Caplen, N. J., Applications of RNA interference in mammalian  
8 systems. *Annu Rev Genomics Hum Genet* 2007, 8, 81-108.  
9 [51] Echeverri, C. J., Beachy, P. A., Baum, B., Boutros, M., *et al.*, Minimizing the  
10 risk of reporting false positives in large-scale RNAi screens. *Nat Methods* 2006,  
11 3, 777-779.  
12 [52] Barbie, D. A., Tamayo, P., Boehm, J. S., Kim, S. Y., *et al.*, Systematic RNA  
13 interference reveals that oncogenic KRAS-driven cancers require TBK1. *Nature*  
14 2009, 462, 108-112.  
15 [53] Whitehurst, A. W., Bodemann, B. O., Cardenas, J., Ferguson, D., *et al.*,  
16 Synthetic lethal screen identification of chemosensitizer loci in cancer cells.  
17 *Nature* 2007, 446, 815-819.  
18 [54] Aza-Blanc, P., Cooper, C. L., Wagner, K., Batalov, S., *et al.*, Identification of  
19 modulators of TRAIL-induced apoptosis via RNAi-based phenotypic screening.  
20 *Mol Cell* 2003, 12, 627-637.  
21 [55] Lopez-Fraga, M., Martinez, T., Jimenez, A., RNA interference technologies  
22 and therapeutics: from basic research to products. *BioDrugs* 2009, 23, 305-332.  
23 [56] Dykxhoorn, D. M., RNA interference as an anticancer therapy: a patent  
24 perspective. *Expert Opin Ther Pat* 2009, 19, 475-491.  
25 [57] Kassner, P. D., Discovery of novel targets with high throughput RNA  
26 interference screening. *Comb Chem High Throughput Screen* 2008, 11, 175-184.  
27 [58] Lee, A. J., Kolesnick, R., Swanton, C., RNAi-mediated functional analysis of  
28 pathways influencing cancer cell drug resistance. *Expert Rev Mol Med* 2009, 11,  
29 e15.  
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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]	C. elegans	<a href="http://www.gurdon.cam.ac.uk/~ahrin/gerlab/pages/rnai.html">www.gurdon.cam.ac.uk/~ahrin/gerlab/pages/rnai.html</a>
RNAi library for injection	[14]; [34]	C. elegans	<a href="http://www.worm.mpi-cbg.de/phenobank2">www.worm.mpi-cbg.de/phenobank2</a>
Long dsRNA library (21,306 PCR templates)	[35]	Drosophila	<a href="http://www.flyrnai.org">www.flyrnai.org</a>
Genome-wide dsRNA library (DRSC)	[12]	Drosophila	<a href="http://www.flyrnai.org">www.flyrnai.org</a>
Genome-wide dsRNA library	[36]	Drosophila	<a href="http://rnai.dkfz.de">http://rnai.dkfz.de</a>
Long dsRNA library	[37]	Drosophila	<a href="http://www.openbiosystem.com">http://www.openbiosystem.com</a>
Non-mammalian subset libraries	Distributed by Open Biosystems	Drosophila, C.elegans, Arabidopsis thaliana, Zebrafish morpholinos	<a href="http://www.openbiosystems.com">www.openbiosystems.com</a>
esiRNA library	[26]	Human	<a href="http://www.mpi-cbg.de/esiRNA/index.html">www.mpi-cbg.de/esiRNA/index.html</a>
shRNA library (TRC1/2)	Distributed by Sigma and Open Biosystems	Human	<a href="http://www.broadinstitute.org/rnai/trc">www.broadinstitute.org/rnai/trc</a>
NKI shRNA library (targeting 8000 genes, retroviral)	[38]	Human	<a href="http://www.screeninc.nl">http://www.screeninc.nl</a>
Library of retroviral vectors	[39]	Human	<a href="http://codex.cshl.edu">http://codex.cshl.edu</a>
Genome-wide siRNA library, subset libraries e.g. kinases, phosphatases	Dharmacon; [40]	Human, Mouse	<a href="http://www.dharmacon.com">www.dharmacon.com</a>
Genome-wide shRNA library (GeneNet™)	System Biosciences	Human, Mouse	<a href="http://www.systembio.com">www.systembio.com</a>
shRNA-mir library (retroviral)	[41]	Human, Mouse	
Genome-wide siRNA library, subset libraries	Qiagen; [42]	Human, Mouse, Rat	<a href="http://www.qiagen.com">www.qiagen.com</a>

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5 Genome-wide siRNA Ambion; [43] Human, [www.ambion.com](http://www.ambion.com)  
6 library, subset Mouse, Rat,  
7 libraries Drosophila  
8 (subset)  
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5 **Figure 1: Schematic view of RNAi in mammalian cells.** Endogenous microRNAs (miRNA) and  
6 exogenous small hairpin RNAs (shRNA) are transcribed from DNA by RNA polymerase II. The  
7 resulting RNA hairpin is then processed by the nuclease Drosha, resulting in pre-  
8 miRNA/processed shRNA which is transported from the nucleus into the cytoplasm via exportin 5  
9 (Exp5). The RNA is further processed into ~21bp fragments by the ribonuclease DICER.  
10 Synthetic short-interfering RNAs (siRNAs) resemble DICER processed double-stranded RNAs.  
11 These RNAs are incorporated into the RNA-induced silencing complex (RISC) where they are  
12 unwound and bind to complementary mRNA. Mature miRNAs preferentially bind to the 3' UTR  
13 while most siRNAs target the open reading frame. In case of a perfect match, the mRNA is  
14 cleaved and degraded. In contrast, imperfect matching leads to translational repression and  
15 consequent mRNA destabilization [9, 10].  
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18 **Figure 2: Scatter plot of average z-scores from a genome-wide RNAi screen.** Luciferase  
19 reporter activity was measured using Mithras LB940 plate reader (Berthold Technologies) and  
20 TOPflash (TCF reporter plasmid). Cell viability was determined using Calcein-AM. siRNAs  
21 targeting b-catenin that result in reduction of wnt reporter activity were used as positive control.  
22 siRNAs targeting UBC cause loss of cell viability and were used as viability control. Non-targeting  
23 siRNAs were used as negative control.  
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26 **Figure 3: Hit validation with retests and secondary assays.** A) Phenotype scores of control,  
27 single siRNAs and corresponding siRNA pool. B) Matching qRT-PCR validating knockdown of  
28 control, single siRNAs and corresponding siRNA pool. C) Nuclear translocation assay showing  
29 localization of the wnt pathway component  $\beta$ -catenin upon signal cascade activation by RNAi  
30 (scale bars equal 10  $\mu$ m). D) Western blot analysis indicating knockdown of  $\beta$ -catenin on protein  
31 level.  
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35 **Figure 4: Schematic view of the RNAi screening procedure.** After definition of the biological  
36 question and choice of the right cellular system, the read-out assay needs to be established. A  
37 pilot screen is performed to determine the robustness of the screening procedure and to identify  
38 possible optimization steps. After analysis of the pilot screen, genome-wide RNAi screenings can  
39 be performed, leading to a candidate list ranked according to the z-scores reflecting the strength  
40 of the phenotype. The candidates are then validated by retesting and secondary assays.  
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Figure 1

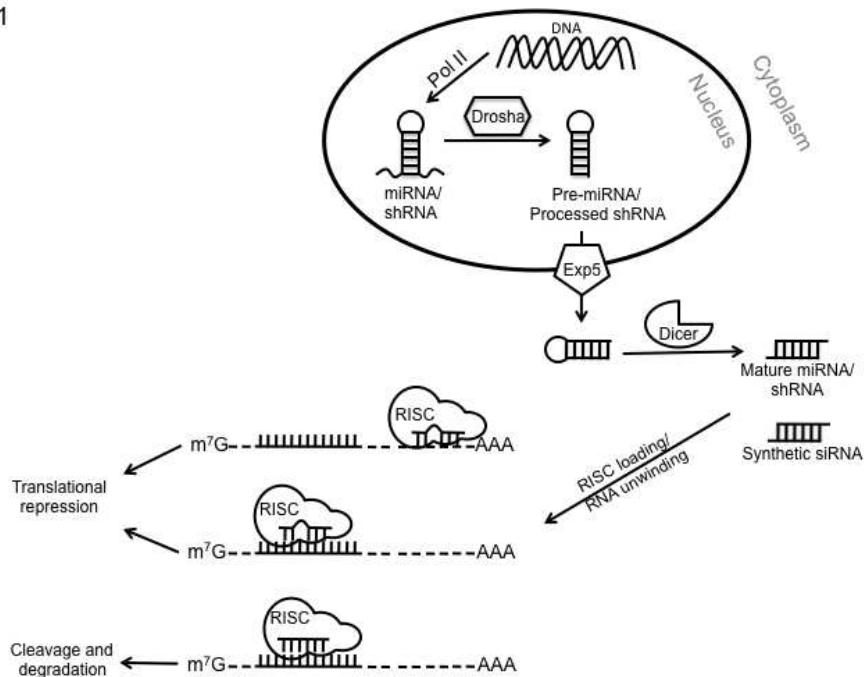


Figure 1: Schematic view of RNAi in mammalian cells. Endogenous microRNAs (miRNA) and exogenous small hairpin RNAs (shRNA) are transcribed from DNA by RNA polymerase II. The resulting RNA hairpin is then processed by the nuclease Drosha, resulting in premiRNA/processed shRNA which is transported from 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]

254x190mm (72 x 72 DPI)

Figure 2

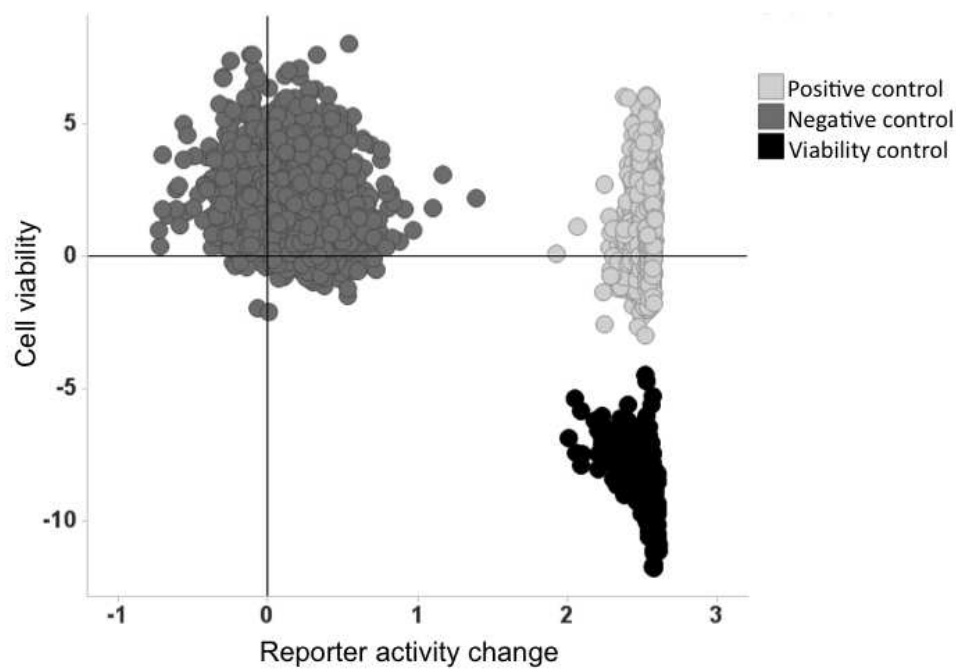


Figure 2: Scatter plot of average z-scores from a genome-wide RNAi screen. Luciferase reporter activity was measured using Mithras LB940 plate reader (Berthold Technologies) and TOPflash (TCF reporter plasmid). Cell viability was determined using Calcein-AM. siRNAs targeting  $\beta$ -catenin that result 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.

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Figure 3

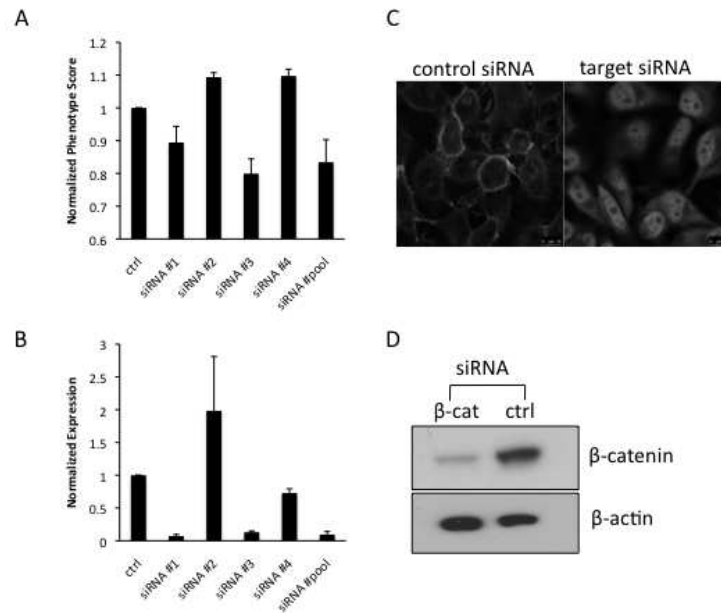


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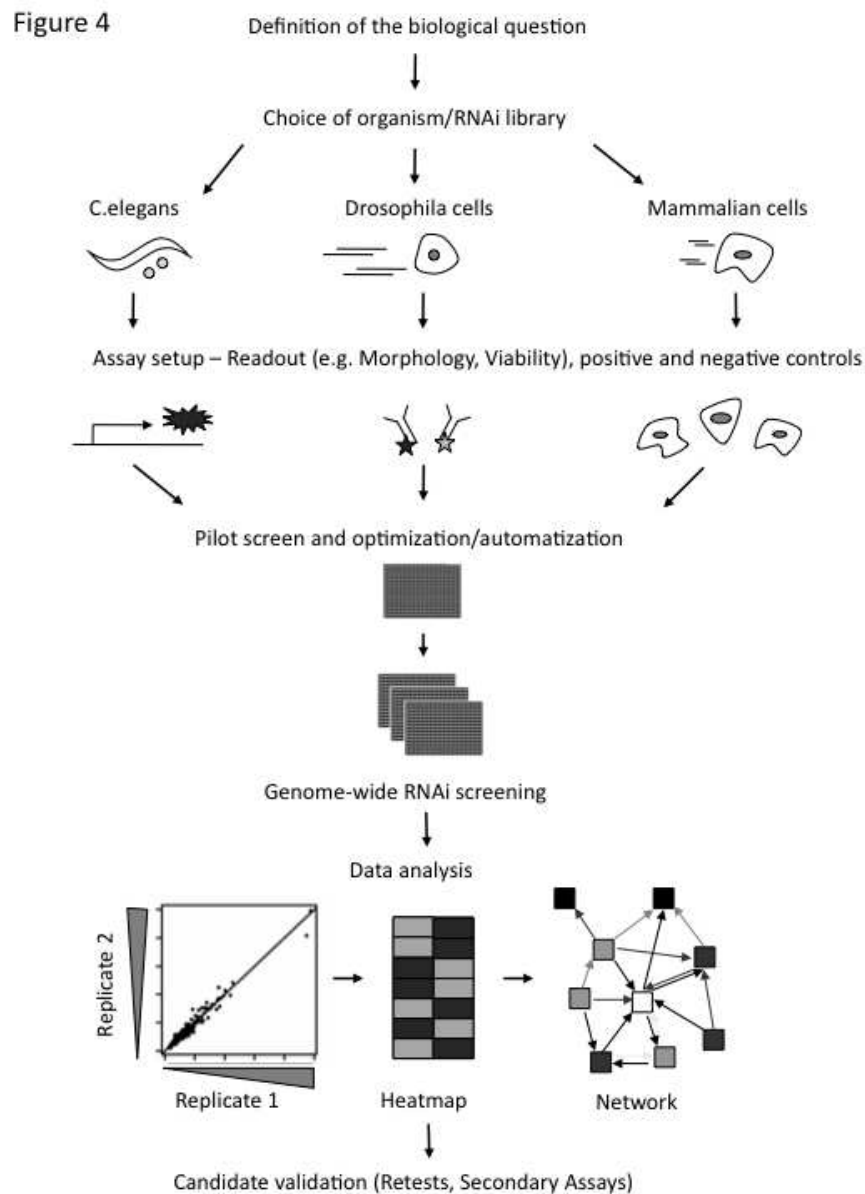


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