Insights to transcriptional networks by using high throughput RNAi strategies

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Abstract

RNA interference (RNAi) is a powerful method to unravel the role of a given gene in eukaryotic cells. The development of high throughput assay platforms such as fluorescence plate readers and high throughput microscopy has allowed the design of genome wide RNAi screens to systemically discern members of regulatory networks around various cellular processes. Here we summarize the different strategies employed in RNAi screens to reveal regulators of transcriptional networks. We focus our discussion in experimental approaches designed to uncover regulatory interactions modulating transcription factor activity.

Keywords: RNAi screen; transcription factor; reporter; localization; post-translational modification

INTRODUCTION

Cells need to react to changes in the environment by adjusting their behavior and physiological responses to meet the prevailing conditions. In order to integrate information from their surroundings, cells have evolved signaling mechanisms, depending on protein–protein interactions and other signal transmitters such as lipid and nucleotide derivatives [1, 2]. These signaling pathways collect information about the extracellular conditions important for survival, growth and differentiation. This information is integrated in ways that allow modulating the cellular response accordingly by regulating its signaling network activity and ultimately its transcriptional profile. These signaling pathways consist of cell surface or intracellular receptors, intracellular signaling pathway components, transcription factors and other cofactors. Together, these components form intricately refined networks of interactions that regulate the cellular transcriptional profile. Common features in these signal transduction networks are specific protein–protein interactions which transmit unidirectional signals, signal amplification, signal diversification and/or integration and refined mechanisms controlling the amplitude and duration of the signal.

Recent development in sequencing projects, high throughput technology (e.g. fluorescence plate readers, fluorescence activated cell sorters and high throughput microscopes), data analysis software and data storage has revolutionized the way we understand and address questions in biological processes. These technological advances have brought us in transition from classical single gene approaches into an era of systems biology, providing more profound understanding of the composition and function of cellular networks of signal transduction. One of the important experimental strategies in this transformation has been RNAi knockdown screenings, an unbiased systemic approach to discover novel genes and signaling pathways involved in a given biological process. These screens have greatly improved our understanding on the complexity of cellular signaling pathways [3].

RNA interference (RNAi), originally discovered in plants [4, 5], is a powerful method to produce loss-of-function phenotypes in eukaryotic cells. After discovering that RNAi, by introducing dsRNA to cells by various means in vivo (soaking, injecting, feeding) and in vitro (soaking, transfecting), can be successfully used in animal cells [6–8] it rapidly became a popular alternative method to...
traditional genetic screens, as an unbiased experimental strategy for the identification of genes regulating various cellular processes. The first RNAi screens were performed in Caenorhabditis elegans [9, 10]. Since then more than 150 screens have been published and the number is rapidly increasing. The methodology has been applied to various biological processes such as development, cell division, cell size and morphology, innate immunity response, signaling pathway regulation and transcription factor activity.

For comprehensive reviews of the high throughput screening methodology in various platforms and cell types, data collection and data analysis, the reader is referred to several excellent reviews [11–16]. For a discussion on the prevalence of off-target effects in RNAi screens see the following references [17–19]. In addition, RNAi methodology itself is reviewed elsewhere [20]. In this review we will focus on how RNAi screens have been applied to reveal transcriptional network components and, specifically, to identify proteins that regulate transcription factor activity. We will also discuss the different methodological approaches used and the technical issues associated with them.

GENERAL RNAi SCREENING CONSIDERATIONS

Several general considerations, dictated by screening objectives and financial restrictions, have to be taken into account when designing an RNAi screen. The most important ones are the selection of the model organism and the cell line within, the scope of the RNAi library and the experimental strategy of the assay (for reviews see [12, 21–23]). Each choice presents its advantages and limitations. For example, when screening for components involved in a well conserved cellular pathway, Drosophila melanogaster cells have several advantages over mammalian systems. Primarily, gene knockdown efficiency on average is higher and the cells have the ability to uptake large dsRNA molecules, easily amplified by in vitro transcription in a microgram scale, directly from the culture medium without the need of costly transfection reagents. Secondly, the fact that Drosophila holds roughly half the number of genes and significantly less redundancy compared to human genome further reduces the screening cost and percentage of false negatives, respectively. These properties make Drosophila cells a very attractive screening platform in terms of efficiency, reliably and cost. On the downside, there are only a few Drosophila cell lines suitable for screening although the use of primary cells, e.g. neurons and muscle cells, has been suggested [23]. Regardless of the exact cell line chosen, it is important to note the limitations arising from that choice. A screening assay in one single cell type will be limited in the identification of global regulatory network components. This is due to the fact that signaling networks are sensitive to context dependent regulators like cell surface receptors, intracellular signal transmitters, transcription factors and other transcriptional co-factors, which may not be present in the cell line used for the screening. This may therefore lead to an underestimation of the total number of regulators of that particular process. Another problem arising from the presence of context-specific regulators is the possibility of drawing conclusions specific for a cell type rather than on general properties of the targeted biological process. This phenomenon was nicely demonstrated by DasGupta and collaborators [17] who tested the activation of Wingless (Wg)-specific Luciferase construct by overexpression or knockdown of known Wg pathway components in different Drosophila cell lines. Their results showed clear cell type dependent differences in pathway activation. A strategy to overcome this caveat is to employ several cell lines in parallel or to use a different cell line when confirming the positive hits in a secondary screen [24, 25].

In vitro screens in cell lines are more amenable to high throughput techniques, but in vivo screens in Drosophila or C. elegans, although technically more challenging, have provided very value insights into the biology of regulatory networks (e.g. [26–30], see below). Several reasons make C. elegans a very good model organism for RNAi studies: its complete cell lineage has been determined, all 959 somatic cells of its transparent body are visible with a microscope, its average life span is only 2–3 weeks, and RNAi delivery is straightforward by soaking worms with RNAi-containing solution or feeding them bacteria expressing RNAi expression constructs [31]. Thus, C. elegans has been often used in genomic RNAi screens, although, unlike Drosophila, its use to characterize transcriptional networks has been somehow limited [30].

Another important selection, the use of a whole genome RNAi library versus a genome subset RNAi library (e.g. kinases or transcription factors), necessarily lays restrictions for assay development, data
analysis and inferences. Targeted RNAi screens, due to the relatively low number of genes, may allow liberty in assay design and a more comprehensive target validation. However, whereas targeted screens in general produce more reliable hits, they are less powerful in drawing systemic conclusions on the networks regulating a given biological process. Ultimately, the success of a screen, measured by its ability to discern genes with a significant role in a particular biological process from a large number of non-significant genes, is determined by experimental parameters such as RNAi knockdown efficiency, assay signal to noise ratio, prevalence of off-target events, assay reproducibility and data analysis. In addition, it is clear that the quality of the RNAi library, in terms of gene annotation and molecular integrity, has a major impact. It is pivotal to understand that the limitations discussed above, if not properly addressed, can result in skewed screening results, make the integration of the data sets from different screens problematic, and prevent drawing systemic conclusions of the biological process tested.

Several assay strategies, i.e. reporter protein, localization and post-translational modification assays, have been applied to high throughput RNAi screens in order to identify regulators of transcriptional networks. Below we review the benefits and caveats of these strategies and give examples of studies where these systems have been successfully applied. We apologize to the colleagues whose work has not been cited by lack of space.

REPORTER PROTEIN ASSAYS

The most straightforward strategy to monitor the activity of a given transcription factor is to measure the accumulation of a reporter protein. The most widely used are the firefly luciferase (Luc), variations of the Green Fluorescent Protein (GFP) or the E. coli β-galactosidase. In this strategy, an endogenous or a synthetic promoter region, known to be responsive for a specific transcription factor, signaling pathway or cellular process, is cloned into an expression vector containing the reporter gene open reading frame (ORF). Upon activation of the given cellular process, the binding of a downstream transcription factor(s) to the promoter will initiate the reporter gene transcription and subsequent reporter protein translation. The accumulation of the reporter protein corresponds to the pathway activity. In an approach to define regulators of a transcriptional activity by a reporter protein, candidate RNAi hits are identified based on their ability to alter basal or induced promoter activity. This kind of assay has been utilized to study regulatory networks around individual transcription factors, e.g. NF-κB [24], E2F [32], Sp3 [33] and FoxO [25]. In addition, several transcriptional reporter-based RNAi screens have been conducted addressing the regulation of cellular signaling pathways, including the Wnt pathway [19, 34–36], the Hedgehog pathway [37, 38] and the JAK-STAT pathway [39, 40]. The reporter constructs can also be employed as surrogates to identify regulators of certain biological processes such as innate immune response or differentiation. For example, activation of the reporter protein under the regulation of Oct4 promoter, a well known marker for pluripotency, has been employed to screen regulators of stem cell identity [41, 42]. The results from these screens have greatly enhanced our knowledge on cellular signaling pathways and many of these studies have been reviewed elsewhere [43–45].

The utilization of the reporter assay in high throughput screening has several advantages: relatively easy assay adaptation to the high throughput format, rapid data collection by automated high throughput screening platforms, ability to distinguish negative and positive regulators and sensitivity to identify subtle target regulators. These characteristics have made the reporter assay a very popular screening tool. However, in spite of its popularity, it holds several important limitations, which have to be considered when drawing conclusions from the obtained screening data. Primarily, the utilization of one promoter-reporter construct often oversimplifies the concept of signaling pathways assuming that all inputs from a pathway will converge to that particular downstream event. Intracellular signal transduction employs alternative signaling routes and transcription factors in different cells and upon different stimulus. Additionally, the activity of a reporter expression can reflect the integration of several, unknown pathways (Figure 1). This limitation is especially evident when employing endogenous promoter regions whose activation is often context dependent, reflecting only a subset of the targets activated by a given signaling pathway or transcription factor. In principle, the use of a synthetic promoter construct could overcome this problem. However, synthetic promoters often do not reach the same level of transcription efficiency as
endogenous ones, probably because they lack some important sequence for transcription factor co-regulation or because the transcription factor binding sites are not situated at the optimal distance in regard to the core promoter. It is also important to note that endogenous promoter constructs, lacking their normal epigenetic regulation and detached from their original chromosomal location, might differ from their normal activation pattern. These limitations will result in accumulation of false negative hits (true regulators that are missed in the screen), which can also result from inefficient knockdown of a true positive hit gene. In general, the occurrence of false negatives cannot be avoided, but it can be estimated roughly if the screen fails to identify known pathway regulators.

An additional layer of complexity comes from the fact that the accumulation of a reporter protein is determined by cellular processes independent from the transcription factor activity. These include, for example, general transcriptional and translational regulation as well as cell proliferation. Hence, suitable normalization procedures are needed to exclude false positives caused by interference of general cellular processes. Normally, another reporter protein such as Renilla Luciferase under a constitutively active promoter is employed. Proteins used for normalization should have similar properties in terms of mRNA processing and decay, as well as protein translation and half life. It is also important to note that ‘constitutive’ promoters can also be hit by RNAi. For example, Klein and collaborators [46] found that ~2% of their dsRNA treatments affected specifically their actin-promoter reporter construct used for normalization. In addition, it is even possible that the promoter used for normalization is targeted by the pathway components under investigation. DasGupta and collaborators [17] demonstrated that an actin-promoter-Renilla construct was sensitive for Wingless-pathway activation. Therefore an

Figure 1: The use of one promoter-reporter construct does not faithfully represent the activity of a given signaling pathway. (A) Transcription from a reporter promoter can reflect the integration of signals from several unknown signaling pathways. Hence, a proportion of the total reporter protein concentration is derived from unknown signal activities, which may lead to the identification of positive hits that are not related to the pathway/transcription factor under investigation. (B) A signaling pathway may employ several different effectors, i.e. transcription factors, with different target promoters (genes X, Y and reporter). Hence, the reporter protein might only partially reflect the signal activity, which may result in the accumulation of false negative hits.
alternative normalization method, such as total protein content or cell viability, is necessary. The hits likely to be unspecific, i.e. general regulators of transcription, translation or cell viability can also be identified by comparing the results of several RNAi-based screens which can help to exclude the hits that appear consistently in different assay strategies (see http://www.flyrnai.org/). In addition, computational analyses based on protein interactions have been developed in order to enhance the identification of true positive hits [47].

Many reporter assays are based on overexpression of transcription factors or their upstream regulators in cell culture. Protein overexpression has the advantage of significantly increasing the signal to noise ratio, which helps to distinguish the reporter activity caused by the protein under investigation from other activators causing background signal. However, protein concentrations far above the endogenous level might produce aberrant protein–protein interactions resulting into false hit identification. Finally, as opposed to qualitative phenotypic screens, reporter protein screens yield quantitative data. This has important implications in terms of hit identification: the number of hits identified by a quantitative measurement will be based on arbitrary thresholds set by the researcher, significantly affected by the signal to noise ratio, the specificity of the particular assay, and the experimental conditions. Most importantly, the thresholds should depend on the error of assay measurement (determined by several repetitions of the same RNAi treatment) which can roughly estimate the number of false positives after a given cut off [48]. Thus, if the thresholds are not set correctly there is a risk to identify a very variable number of regulators (for discussion, see [3]).

Taken together, reporter-based screens have the ability to rapidly identify pathway regulators but also the tendency to identify a significant amount of noise as false positives. In fact, some reports suggest that most of the hits in RNAi screens are false positives [48]. In addition, the results of a reporter assay screen always represent a context dependent set of hits understimating the true number of regulators. Therefore, rigorous downstream target validation and caution in drawing systemic conclusions are necessary. In general, integration of results coming from different screens has been problematic, even when the same RNAi library was used by different groups. The lack of appropriate controls, variability in RNAi knockdown efficiency, cell-type specific differences, or discrepancies in data processing and analysis are some factors that may have contributed to this problem. Thus, confirmation of primary hits in secondary screens or by other means is necessary in order to draw systemic conclusions.

A good example of a successful reporter-based genome wide RNAi screen of transcriptional regulators is the work of Lu and collaborators [32] who developed an RNAi screening for regulators of E2F, a member of a transcription factor family well known for its role in cell proliferation, apoptosis and development [49, 50]. E2F transcription factors are best known for their role in regulating the cell cycle progression from G1 to S phase through the induction of Cyclin E1 expression. Their aberrant function, due to the inactivation of the Retinoblastoma (Rb) proteins, seems to be a common feature in cancer progression [51]. The authors employed a synthetic promoter comprising of four E2F-binding elements upstream of an Hsp70 minimal promoter and a Luciferase ORF. By using this reporter construct they were able to detect the endogenous E2F protein activity in Drosophila cell culture with a signal to noise ratio of ~2, as estimated from their E2F control knockdown experiments. Interestingly, only 18 dsRNAs up-regulated the reporter construct more than 50%, an arbitrary cut off introduced by the authors, suggesting that the screen was stringent enough to avoid false positives. Among the hits, three putative members of the fly SWR1-like chromatin remodeling complex (Domino, CG14514 and CG4621) and two Polycomb Group-like MBT proteins, seem to be a common feature in cancer progression [49, 50]. E2F transcription factors are best known for its role in cell proliferation, apoptosis and development [49, 50].

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of the versatile usage of the RNAi in combination of a reporter assay.

The use of reporter assay system is not solely restricted to cell lines. In fact, the development of a Drosophila RNAi library for in vivo studies (Vienna Drosophila stock center) has made possible to utilize this model organism as an in vivo platform for RNAi screens in search for pathway/transcription factor regulators. In an elegant study by Mummery-Widmer and collaborators [29] a LacZ reporter for Suppressor of hairless (Su(H)) expression in the wing disc was utilized in a secondary screen for Notch pathway regulators. In another study by Kambris and coworkers [28] a drosomycin-LacZ reporter was employed in search for Serine proteases involved in infection-induced Toll pathway activation. Although the false positive ratio in in vivo screens is expected to be low, their application to high throughput platforms is time consuming and laborious. Hence, in vivo reporter screens are better suitable for targeted small scale primary screenings or for target validation in secondary screens.

LOCALIZATION-BASED ASSAYS

As seen above, reporter transcription strategy has been used to detect regulators of the transcriptional activity of a given transcription factor or signaling pathway. However, regulation of transcription factors can be detected by means other than direct transcriptional measurement. For example, many transcription factors are known to shuttle between the nucleus and the cytoplasm in a regulated manner (Figure 2). Transcription factor subcellular localization often correlates with its dynamic post-translational modification status, usually phosphorylation, either priming the protein to chaperone binding and nuclear exclusion, or by driving its nuclear import by unmasking the nuclear localizing signal (NLS). For example, Akt dependent phosphorylation of FoxO transcription factors results in the binding of the 14-3-3 chaperone proteins leading to subsequent cytoplasmic retention and transcriptional inactivation [53]. This property gives an opportunity for an unambiguous screening assay lacking many of the caveats associated with reporter strategies, especially the problem of normalization against unspecific transcriptional and translational regulation. In general, the use of cellular localization assays to detect transcription factor regulators minimizes the identification of false positives and it is a sensible approach when combined with downstream assays monitoring transcriptional activity. In addition, the subcellular localization of transcription factors is nowadays amenable for fully automated detection by high throughput fluorescence microscopy, giving the possibility to identify subtle differences in protein localization [25]. However, localization assays are less powerful in detecting positive, i.e. activating, regulatory interactions since nuclear localization is not necessarily sufficient for transcriptional activation. Hence, additional assays are necessary to correlate localization to activity limiting the scope of the screen and its applications to elucidate global regulatory networks. Nevertheless, the rapid development of high throughput microscopy technology, by reducing the labor and increasing the efficiency, reliability, and sensitivity, has made localization assays very attractive.

To date, transcription factor localization assays have not yet been applied to many RNAi screens. Gwack and coworkers [54] used this regulatory feature to search for kinases that phosphorylate Nuclear factor of activated T cells (NFAT) transcription factor, thereby regulating its localization and activity. NFAT is a vertebrate transcription factor initially implicated in T-cell maturation but recently also associated with carcinogenesis [55, 56]. NFAT transcription factor subcellular localization is highly responsive to the Ca\(^{2+}\)/Calcineurin signaling, providing an efficient screening strategy. Upon stimulation with Ca\(^{2+}\), NFAT becomes dephosphorylated by Calcineurin and shuttles to the nucleus [54]. The authors performed a genome wide RNAi screen utilizing GFP-tagged human NFAT in order to identify aberrant subcellular localization of NFAT in Drosophila S2R+ cells, finding 662 positive hits. Since positive hits were scored by eye, this could negatively impact the results of their screen by increasing the number of false positives. However, their hit list included several regulators of the Ca\(^{2+}\) signaling, which validated their experimental approach. This work is interesting and unique in the sense that it uses an invertebrate screening model to search for regulators of a vertebrate transcription factor. However, the chosen experimental setup may prevent from making any systemic conclusions since it is likely that not all the NFAT regulators are expressed in the Drosophila cells used for the screen. A very similar strategy to screen for regulators of transcription factor localization was used by Xu and coworkers [57] who performed a genome
wide screen for genes regulating Mothers against decapentaplegic (Mad) localization. Mad is the Drosophila ortholog of mammalian Smad proteins, a transcription factor downstream of TGF-β signaling. Xu and co-workers used cellular conditions where Mad was nuclear, i.e. upon overexpression of the Drosophila TGF-β receptor kinases Punt and Tkv, and spotted diffuse Mad localization upon individual gene knockdown. Hence, the assay only allowed for screening of positive regulators of nuclear localization. The authors identified 346 such regulators including, among others, moleskin (msk) a known regulator of nuclear import. A third study combining localization and RNAi screening was published by Mattila and collaborators [25] who searched for regulators of dFoxO among kinases and phosphatases regulating its subcellular localization together with transcriptional activity. It is notable that in this study several proteins were found to regulate dFoxO transcriptional activity but not its localization. This finding emphasizes the lack of correlation that sometimes exists between these two processes. Finally, a very elegant strategy combining reporter protein and localization assay in the same screen was used by Zanella and collaborators [58] who developed a U2-OS cell line to identify FoxO regulators. By stably transfecting these cells with a FoxO-GFP fusion protein and Daf-16 Binding Element (DBE)-luciferase constructs, the authors were able to simultaneously detect the localization and transactivation of FOXO3 in these cells. The application of a combined localization-transactivation assay would greatly enhance the power of a genome wide RNAi screen to identify transcription factor regulators.

PROTEIN POST-TRANSLATIONAL MODIFICATION ASSAYS

Protein post-translational modifications such as phosphorylation, acetylation, ubiquitinylation and methylation are markers of signaling activities and amenable for detection by a variety of means. These include, for example, band shift analysis from SDS-PAGE gels, generic anti-p-Ser/p-Thr/Ac-Lys antibodies and mass spectrometric analysis. The applications of these methods in RNAi screens have not yet been widely explored, mainly because of the lack of available high throughput technology. For example, whereas powerful mass spectrometric analysing methods such as Stable Isotope Labelling by Amino acids in Cell culture (SILAC) have been utilized to
study single gene knockdown effects [59] their use in high throughput strategies is yet to be substantiated. An exception is the use of immunocytochemistry in combination with either fluorescent plate readers or high throughput microscopy.

In the work of Friedman and Perrimon [52], an assay to detect the phosphorylation of *Drosophila* Extracellular Regulated Kinase (ERK) by a phosphoprotein specific antibody was developed. In their primary screen, the authors employed a *Drosophila* cell line stably transfected with YFP-tagged ERK and an Alexa647 conjugated pERK antibody recognizing the dually phosphorylated, active ERK. After dsRNA treatments, a high throughput fluorescence plate reader was utilized to detect antibody binding along with the total YFP tagged ERK levels. After a whole genome RNAi screening, the authors found a staggering 1168 annotated genes which caused deviations in the pERK-YFP ratio. The effect of these regulators was then further categorized under different cell lines and stimulatory conditions and the hits were grouped into context dependent and independent ERK signaling regulators. As a proof of principle, the authors characterized two of the identified genes, a novel Ste20-like kinase and a novel PPM phosphatase as mediators of ERK signaling in vivo.

The advantage of monitoring a post-translational modification event is mainly an increase in specificity. Unlike reporter assays which can be influenced by other activities targeting the same promoter, direct measurement of post-translational modifications as readout of transcription factor activity is not influenced by unrelated signaling pathways. On the other hand, a single post-translational modification event might not faithfully reflect the activation/repression of the protein in question. Hence, more refined assay strategies are needed in parallel in order to detect several post-translational modifications.

**CONCLUDING REMARKS**

RNAi high throughput screens have been very powerful in the identification of novel regulators of a given biological process. The assay strategies employed in RNAi screens in search for transcription factor regulators differ in their sensitivity, specificity, cost and informational output (Table 1). An important challenge remains the complexity of network interactions around a given transcription factor. Since transcription factor activity is regulated in multiple ways, none of the approaches on its own is able to reveal the complete network of proteins that regulate it. In addition, it is important to be aware of the limitations of each of these strategies, the possible sources of false positive and negative hits, and the setting of adequate thresholds to minimize them. Therefore, it is advised to use caution when results from RNAi screens are used to draw general conclusions implying systemic regulation. On the other hand, RNAi screens present a unique tool to study the function of thousands of genes simultaneously in a high throughput manner, in ways that no other approach can. In conclusion, if the experimental conditions and design of the study are well controlled, RNAi screens are a very powerful and efficient method to investigate complex transcriptional networks.

**Table 1**: Comparison of the different assay methods used in RNAi screens for pathway/transcription factor activity detection

<table>
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<tr>
<th>Reporter protein assays</th>
<th>Localization assay</th>
<th>Protein post-translational modification assay</th>
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<tr>
<td>Advantages</td>
<td>Sensitive when used with automated fluorescent detection</td>
<td>Detection specific for direct effects</td>
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<td></td>
<td>No normalization needed</td>
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<td>Disadvantages</td>
<td>Localization does not necessarily correlate to transactivation</td>
<td>Does not necessarily correlate to protein activation</td>
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<td></td>
<td>Requires significant assay optimization and specific instrumentation</td>
<td>Lack of high throughput assay platforms</td>
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[59] Mattila and Puig
Key Points
- RNAi screens allow for an efficient way to interrogate the function of thousands of genes simultaneously, providing more profound understanding of the composition and function of cellular networks.
- In order to identify proteins interacting with transcription factors, three major strategies that directly correlate to transcription factor activity have been used:
  - measure the activity/accumulation of a reporter protein
  - determine the subcellular localization of the transcription factor
  - assess transcription factor post-translational modifications
- RNAi screens have several shortcomings that need to be taken into consideration, for example arbitrary thresholds set to identify positive hits, choice of promoter, normalization, etc, which may prevent from extracting systemic conclusions during data interpretation.
- Overall, when used with appropriate controls and with careful experimental design, RNAi screens present a very powerful and efficient method to investigate complex transcriptional networks.

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