The interplay between transcription factors and microRNAs in genome-scale regulatory networks

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Metazoan genomes contain thousands of protein-coding and non-coding RNA genes, most of which are differentially expressed, *i.e.*, at different locations, at different times during development, or in response to environmental signals. Differential gene expression is achieved through complex regulatory networks that are controlled in part by two types of *trans*-regulators: transcription factors (TFs) and microRNAs (miRNAs). TFs bind to *cis*-regulatory DNA elements that are often located in or near their target genes, while miRNAs hybridize to *cis*-regulatory RNA elements mostly located in the 3′ untranslated region of their target mRNAs. Here, we describe how these *trans*-regulators interact with each other in the context of gene regulatory networks to coordinate gene expression at the genome-scale level, and discuss future challenges of integrating these networks with other types of functional networks.

Keywords: microRNA; network motif; regulatory network; transcription factor

Introduction

Over the last decade, the sequencing of a vast number of genomes revealed that an increase in organismal complexity is not merely explained by a dramatic increase in the number of protein-coding genes. Indeed, highly complex organisms frequently contain roughly the same number of protein-coding genes as organisms with less intricate morphology and behaviors. For example, the nematode *Caenorhabditis elegans* has ~20,000 predicted protein-coding genes with a relatively simple body plan.[1] The fruit fly *Drosophila melanogaster* and humans have a much more complex anatomy and physiology than worms, yet their genomes encode only ~14,000 and ~25,000 predicted protein-coding genes, respectively.[2,3]

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It has been proposed that organismal complexity developed from a gradual increase in protein diversity, mainly due to alternative mRNA splicing, combined with a gradual increase in the extent and intricacy of gene regulation.[4–6] For instance, the human genome is 3.2 Gb in length, whereas *C. elegans* has a genome of only 100 Mb. Since, exon and open reading frame length does not increase with animal complexity, this means that the non-coding part of the human genome can be up to 30 times larger than that of *C. elegans*. In addition to an increase in regulatory genomic space, there is also an increase in the number of *trans*-regulators. First, the proportion of proteins that encode transcription factors (TFs) increases with organismal complexity; around 5% of the protein-coding genes code for TFs in flies and nematodes, compared to almost 10% for mouse and human[7–9] (Table 1). Second, the number of microRNAs (miRNAs) encoded by a genome appears to correlate with organismal complexity as well.[10] For example, 154, 337, and 695 miRNAs have been annotated to date in the *C. elegans*, zebrafish *Danio rerio*, and human genomes, respectively (miRBase).[11]

Both TFs and miRNAs can exert a widespread impact on gene expression. Most, if not all, genes in the genome are controlled by TFs, which either up- or down-regulate transcription. Overall, miRNAs are predicted to target approximately 10–30% of animal protein-coding genes, with each miRNA repressing on average 200 transcripts.[12–15]

Hierarchically, miRNAs function downstream of TFs since miRNAs can repress an mRNA only after it has been transcribed. However, recent observations suggest that transcriptional regulation by TFs and post-transcriptional regulation by miRNAs are often highly coordinated. To gain an understanding of the coordinated effects of TFs and miRNAs, it is critical to delineate and characterize the genome-scale regulatory networks in which these regulators operate. Such networks combine the plethora of regulatory circuits for a tissue, organism, or process of interest, usually into a single model. Analyses of these models provide insights into the mechanisms that control gene expression at a systems level, rather than at the level of (an) individual gene(s). Here, we first briefly describe the main principles of TF- and miRNA-mediated gene regulation, and then discuss our current

Table 1. Overview of TF- and miRNA-mediated gene regulation

	TFs	miRNAs
Abundancy in the genome	~5–10%	~1–5%
De novo identification	Computational	Computational, experimental (high-throughput sequencing)
Classification into families	Yes–based on DNA binding domain	Yes–based on seed sequence
Mode of action	Activation and repression	Mostly repression
Target region	Promoters, *cis*-regulatory modules	Mostly 3′UTRs, coding regions?
Search space for targets	Tens or hundreds of kilobases	One or a few kilobases
Mode of binding	Highly plastic–sites can be highly variant	Stringent, dictated by basepairing of seed sequence
Additional variants	Extensive–created by splicing, PTM, dimerization	Not identified–possibly by editing?
Biological contributions	Often critical in development and physiology	Mostly not critical in development and physiology (fine tuning)

PTM: post-translational modifications.

knowledge of how these factors interact with each other in the context of genome-scale regulatory networks, concentrating primarily on animal systems. We also provide a brief discussion of the need to incorporate spatiotemporal gene expression information, and the long-term incorporation of other functional networks, such as those involving signaling or RNA-binding proteins (RBPs), into dynamic ''meta network models'' that describe gene expression in animal development, homeostasis, behavior, and pathology at high resolution and precision.

Overview of the principles of TF- and miRNA-mediated gene regulation

A summary of the differences and similarities between TFs and miRNA-mediated gene regulation is provided in Table 1.

Transcription factors

TFs are modular proteins that often contain separate domains that participate in DNA binding, protein–protein interactions, and transcriptional activation or repression. TFs work largely by interacting with the basal transcriptional machinery and/or chromatin modifying proteins, thereby altering the rate of gene transcription (reviewed in[16,17]). TFs physically interact with genomic *cis*-regulatory DNA elements, referred to as TF-binding sites (TFBSs), through a specific DNA-binding domain. Numerous types of DNA-binding domains have been identified in eukaryotes and known TFs that contain similar DNA-binding domains are grouped into families. By compiling the protein sequence information of known members of a TF family, additional members can be computationally predicted based on protein sequence similarities.[7,8] Since members of TF families by definition have similar DNA-binding domains, their TFBSs can often be highly similar as well. For instance, the 84 homeodomain TFs of *D. melanogaster* can be divided into 11 groups based on their DNA binding specificity, with all the members of a group binding to highly similar DNA sequences.[18] However, members of other families, notably C_2H_2 zinc fingers, can bind a large variety of DNA sequences.[19] An important question in the field of systems biology is how members of TF families attain functional specificity in evolution. It is also important to note that members of a TF family have been reported to bind overlapping sets of target genes and have (partially) redundant functions in biological processes such as development. For instance, a study in human T cell lines showed that three members of the ETS family of TFs frequently bind target genes in a redundant manner.[20] Similarly, multiple members of the FLYWCH family of *C. elegans* TFs bind to and repress overlapping sets of embryonic miRNAs.[21]

For most organisms, only a handful of TFs have been studied in detail. For instance, even in large efforts such as the ENCODE project, only a limited number of TFs have been analyzed by chromatin immunoprecipitation (ChIP) and other methods, and these assays have been conducted only in a small number of human cell lines.[22] Similarly, only ~200 *C. elegans* promoters have so far been used as DNA baits in yeast one-hybrid (Y1H) assays. Although, this already led to the identification of targets for ~25% of all predicted *C. elegans* TFs, these studies are far from complete.[23–25] Extending both types of studies to the genome- and proteome-scale level will uncover more targets for most TFs. There is also accumulating evidence that not all DNA-binding domains, and therefore all TFs, have yet been identified. For instance, Snyder and colleagues have found that the yeast enzyme Arg5,6 specifically binds DNA.[26] Similarly, using Y1H assays, we have retrieved more than 20 *C. elegans* proteins that robustly interact with gene promoters but do not possess a recognizable DNA-binding domain.[23–25] Further computational and experimental studies are needed to obtain more comprehensive predictions of TFs.

MicroRNAs

MiRNAs are short non-coding RNAs that are typically transcribed by RNA polymerase II and further processed in a step-wise manner by a common biogenic pathway (reviewed in[27]). MiRNAs have been identified in numerous organisms by a combination of experimental and computational strategies.[28–33] For most genomes, the repertoire of miRNAs is only partially known, although efforts such as high-throughput sequencing are currently underway to identify all miRNAs in the organism of choice.[10,34–36]

MiRNAs hybridize to complementary *cis*-regulatory elements usually located in the 3′UTR of target mRNAs (reviewed in[37]). In animal systems, this interaction leads to translational repression and/or mRNA destabilization. MiRNAs bind mRNAs mainly through an mRNA-binding motif, often referred to as the ''seed'' (see below). MiRNAs share many characteristics with TFs (Table 1). For instance, they can also be classified into families on the basis of the mRNA-binding motif they possess and, to date, hundreds of miRNA families have already been identified.[11,38] Examples of highly conserved miRNA families include the *let-7* and *mir-1* families that are found in many organisms, including humans. Like TFs, miRNAs from the same family are predicted to share targets, and it has been shown that family members can function redundantly to regulate gene expression.[39,40] For instance, the three related miRNAs *mir-48*, *mir-84*, and *mir-241* function redundantly in the control of developmental timing in *C. elegans*.[39]

Cis-regulatory elements

TFBSs are short DNA sequences, between 5 and 15 bp long, that can be located proximal to the transcription start site in gene promoters, or can reside in *cis*-regulatory modules, such as enhancers, at more distant genomic locations. TFs do not bind just a single unique DNA sequence; rather they are capable of binding multiple closely related sequences, albeit with different affinities. For these reasons, the identification of functional TFBSs in complex genomes has been extremely challenging. So far the complete spectrum of binding specificities is known only for a handful of TFs. Databases such as TRANSFAC and JASPAR, which collect TFBS information, only contain limited number of TFBSs and only for a fraction of all TFs in any organism.[41,42] Two recent efforts experimentally determined the binding specificities of most mouse and *D. melanogaster* homeodomains by protein-binding microarrays and bacterial one-hybrid assays.[18,43] The extension of such studies to other types of TFs will be important to attain comprehensive TFBS information. It will then be crucial to compare how such *in vitro* binding specificities and affinities relate to *in vivo* binding of full-length TFs to their target genes and in the context of chromatinized DNA.

MiRNAs bind to two types of targets. The first contains sequences with perfect complementarity to the miRNA seed, a stretch of six to eight nucleotides located in the 5′ end of the miRNA. The second type possesses sequences with imperfect seed complementarity that is compensated by complementarity at the 3′ end of the miRNA.[12] So far, it appears that most detectable target genes belong to the first type (reviewed in[44]). Efforts to study the interaction of miRNAs with their *cis*-regulatory elements have been restricted to the 3′UTR of target mRNAs. The length of 3′UTRs increases with organismal complexity, although even in humans the average 3′UTR length is less than 1 kb.[45,46] Interestingly, it has been shown experimentally that miRNAs can associate with artificial sites located in any position on a target mRNA.[47] In addition, it has been recently demonstrated that functional *cis*-regulatory elements can be located in the coding sequences of target mRNAs.[48,49] Whether this is a general mode of miRNA action or an exception still needs to be determined. However, if it is a general function of miRNAs, the spectrum of putative targets will expand greatly.

Modulation of TF and miRNA activity

TFs and miRNAs are often differentially expressed during the development, differentiation, and homeostasis of cells and tissues.[50–53] Differential spatiotemporal expression of TFs is in part regulated by other TFs at the transcriptional level, and by miRNAs, RBPs, and alternative splicing at the post-transcriptional level.[21,24,50,54–56] At the post-translational level, TF activity can be modulated by selective dimerization with other TFs, interactions with ligands and co-factors, and by modifications such as phosphorylation, acetylation, and ubiquitination (reviewed in[57]).

The spatiotemporal expression of miRNAs is in part regulated by TFs that bind miRNA promoters.[24,51,58] Recent studies have begun to dissect how miRNA activity may be regulated at the post-transcriptional level by miRNA processing and/or (de)stabilization mechanisms.[54,55,59,60] For instance, Viswanathan and colleagues have shown that LIN-28, an RBP, can selectively block processing of the primary *let-7* transcript in mouse embryonic stem cells,[55] while Kawahara *et al*. have shown that miRNAs are subject to extensive RNA editing that converts adenosine to inosine (A to I). Although the mechanism and significance of miRNA editing is unknown, it could potentially alter base pairing specificity (changing the set of target genes) or miRNA stability.[61] Further studies will be needed to determine the extent and variety of post-transcriptional miRNA regulation.

Another way of modulating TF and miRNA activity is to regulate the access to their target sequences. Indeed, the mere presence of a TFBS or a miRNA target sequence is a poor predictor of binding and regulation. For TFs, it is widely accepted that histone modifications and resulting changes in

chromatin structure can alter the accessibility to TFBSs. Similarly, the accessibility of miRNAs to their binding sequences can be altered by the presence of RBPs. For example, the expression of Dead end (Dnd1) impairs the function of several miRNAs by blocking the accessibility to target mRNAs in zebrafish primordial germ cells.[56]

Impact on animal development and physiology

Genome-wide genetic analyses in many organisms have demonstrated a myriad of critical roles that TFs play in controlling gene expression during development and in homeostasis and disease. For instance, more than 30% of *C. elegans* TFs confer a detectable phenotype when knocked down by RNA interference (RNAi) (WormBase WS180).[62] In addition, genome-wide RNAi analysis of growth and viability in *D. melanogaster* cells found that proteins with a predicted DNA-binding domain comprised the largest category of genes that confer the most severe phenotypes.[63] These observations are in agreement with important roles that TFs can play as master regulators of development. For instance, *C. elegans* PHA-4 is necessary and sufficient for development of the pharynx and foregut during embryonic development.[64] Although, mutations in specific miRNAs can produce noticeable phenotypes in several organisms, very few miRNAs have been found in genetic screens. With the exceptions of *lin-4*,[32] *let-7*,[65] *lsy-6*,[66] and *mir-1*,[67] a single null mutation does not result in an easily detectable phenotype for most *C. elegans* miRNAs.[40] Furthermore, with only one exception,[68] genetic inactivation of Dicer (the enzyme required for mature miRNA processing) only has relatively modest effects on organism morphology and patterning.[69–74] Together, these observations indicate that miRNAs make auxiliary, rather than critical contributions to organismal morphology, physiology, and behavior, likely by fine-tuning rather than establishing gene expression programs.[75]

Integrating TFs and miRNAs into genome-scale gene regulatory networks

Computational and experimental interaction data can be combined into functional network models to elucidate the systems-level mechanisms of gene regulation mediated by TFs and miRNAs. Such models can then be examined for their architecture and organization. Compared to regulatory networks involving protein-coding genes, until recently, little was known about genome-scale networks that involve miRNAs. Two types of regulatory networks involving miRNAs and TFs can be distinguished: transcription regulatory networks that consist of protein-DNA interactions between TFs and the *cis*-regulatory DNA regions of their target genes (TF → target) (Fig. 1A), and post-transcription regulatory networks that consist of RNA–RNA interactions between miRNAs and the 3′UTR of their target mRNAs (miRNA → target) (Fig. 1B). Both types of networks can be combined into gene regulatory networks (Fig. 1C). While several efforts have identified post-transcriptional miRNA → target networks, the transcriptional networks that control miRNA expression (TF → miRNA) had received little

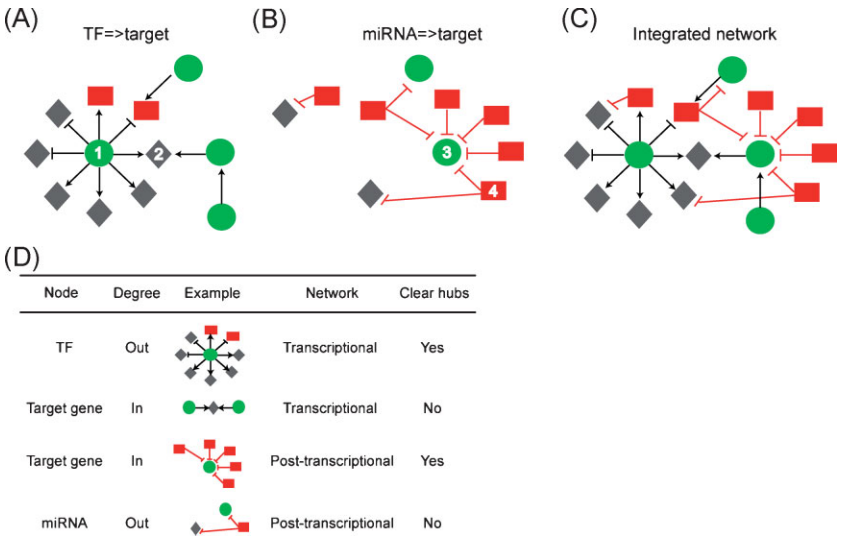

Figure 1. TF and miRNA-containing regulatory networks. (A) Transcriptional network (TF → target). (**B**) Post-transcriptional network (miRNA → target). (**C**) Integrated gene regulatory network. Nodes: green circles, TFs; red rectangles, miRNAs; gray diamonds, other protein-coding genes. Edges: black arrows, transcriptional activation; black blunted arrows, transcriptional repression; red blunted arrows, post-transcriptional repression. Examples of in- and out-degrees: 1, out-degree of TF is 8; 2, in-degree of target gene is 2; 3, in-degree of target gene (in this case a TF) is 5; 4, out-degree of miRNA is 2. (**D**) Summary of presence of hub nodes in transcriptional and post-transcriptional networks.

attention. We have recently mapped the first genome-scale TF → miRNA transcription regulatory network in *C. elegans* and integrated this network with a computationally predicted miRNA → TF post-transcriptional network.[24] We investigated the topology and properties of this network to understand how TFs and miRNAs interact to regulate gene expression. Our recent findings, as well as findings by other groups, are discussed below.

Gene regulatory networks

Regulatory network models are composed of nodes (*e.g.*, TFs, miRNAs, and target genes) and edges that describe the relationships between nodes (*e.g.*, binding, activation, repression). The visualization of biological interactions as network graphs allows the investigation of network topology and its correlation to network function using graph-theoretical concepts or network parameters. One example of a network parameter is ''node degree'', defined as the number of interacting partners or number of edges per node. The degree distribution of most biological networks characterized to date is scale free, which means that the majority of the nodes in the network exhibit relatively low connectivity, whereas a small number of nodes, referred to as network hubs, are extremely highly connected.[76] The biological significance of this observation became apparent when it was found that hubs in protein–protein interaction networks are more often essential for survival or development of the organism than other nodes.[77] Transcriptional and post-transcriptional networks are bipartite and directed; they each contain two types of nodes, and the edges between these types of nodes are unidirectional (*i.e.*, a TF binds its target gene and not the other way around). Because of this, the regulatory networks have two types of degree: in- and out-degree, which reflect, for example, the number of TFs that bind a gene and the number of genes bound by a TF, respectively (Fig. 1). Whereas the out-degree distribution of TF nodes in transcriptional networks is scale free, the in-degree distribution of gene nodes is not scale free.[23,25,78] TF hubs tend to be essential for viability, whereas target genes bound by many TFs do not have a tendency to be essential for viability. Thus, network analysis provides insights into biological systems that cannot be obtained in single gene studies.

Global properties of gene regulatory networks involving miRNAs and TFs

We have recently used Y1H assays to map a genome-scale miRNA transcriptional network (TF → miRNA) by experimentally identifying the TFs that can interact with *C. elegans* miRNA promoters.[24] We found that the out-degree of TFs in the miRNA transcriptional network follows a scale-free distribution (Fig. 1D). This indicates that the topology of miRNA transcription regulatory networks is similar to that of protein-coding gene networks, and that, therefore, miRNA expression overall is regulated in a similar manner to protein-coding genes.[24]

Shalgi and colleagues have analyzed the global degree of a mammalian post-transcriptional miRNA → target network. This network consisted of computationally predicted interactions between miRNAs and their target genes, which were evolutionarily conserved in four species: human, mouse, rat, and dog. By plotting the in-degree distribution of target genes, they showed the existence of hundreds of genes that are subject to extensive miRNA regulation, referred to as ''target hubs''. Noticeably, many TFs are among these target hubs, suggesting that ''regulating the regulators'' is of particularly great importance (Fig. 1D). The out-degree distribution of miRNA nodes is not scale-free and, although multiple miRNAs appear to target many genes, no clear ''miRNA hubs'' could be identified.[79] Similarly, we have shown that in the predicted *C. elegans* post-transcriptional miRNA → TF network, the out-degree distribution of miRNA nodes is not scale free, indicating the absence of clearly identifiable miRNA hubs (Fig. 1D).[24] Interestingly, *C. elegans* can tolerate removal of most individual miRNAs without obvious developmental defects.[40] The lack of clear miRNA hubs and the fact that most of them are not essential for viability both agree with the hypothesis that miRNAs do not function as master regulators, but rather predominantly function to fine tune gene expression instead of establishing crucial developmental gene expression programs.[75,80] Taken together, transcriptional and post-transcriptional regulatory networks exhibit different in- and out-degree distributions that correlate with the overall biological roles for TFs and miRNAs.

Network motifs

Network models can be analyzed globally but can also provide more local information through the characterization of smaller sub-graphs such as network modules and motifs (reviewed in[81]). Network motifs were first identified in transcriptional networks of bacteria and yeast[82,83] and can provide insights into the mechanisms of differential gene expression at a systems level. Network motifs are defined as small gene regulatory circuits that occur more often in real networks than expected by chance, *i.e.*, compared to randomized networks in which the edges have been reassigned computationally. Examples of network motifs include feedback, feed-forward, bi-fans, single and multi-input motifs. Gene regulatory networks of different organisms contain the same types of network motifs, suggesting that they not only constitute successful mechanisms of gene expression in one particular organism, but throughout evolution as well. Although feedback mechanisms are

prominent throughout biology, Alon and colleagues found that feedback loops are not overrepresented in pure transcriptional networks.[83] Based on this observation, Margalit and colleagues proposed that when protein–protein interactions are combined with transcriptional interactions, feedback motifs could be identified.[84] Several examples of feedback loops involving transcriptional interactions and miRNA-mediated post-transcriptional interactions have been either postulated,[85] or demonstrated (see below).[86–89] Based on these observations, we hypothesized that miRNAs may, together with TFs, be involved in general mechanisms of feedback regulation in metazoan regulatory networks. Indeed, by combining transcriptional and post-transcriptional interactions, we have recently shown that miRNAs play a role in feedback motifs in *C. elegans* regulatory networks.[24]

MiRNA- and TF-containing circuits in regulatory networks

Although examples of feedback and feed-forward loops have been found experimentally in several organisms (see below), their function and implications on gene expression have been mainly studied by mathematical models or by creating small synthetic networks with loops in *Escherichia coli*.[90–92] The functions of these circuits are much more difficult to assess in metazoan networks, mostly because these circuits are part of highly interconnected sub-graphs and their disruption may not result in detectable effects. The generation and study of synthetic networks in metazoan systems will be essential to further understand how network motifs perform during multicellular development and physiology.

Feedback loops can be classified into coherent and incoherent loops (Fig. 2). In coherent loops the regulatory paths have the same overall effect (either activation or repression of the target), while in incoherent loops the regulatory paths have opposite effects (Fig. 2).[93] It has been proposed that coherent feedback loops can generate mutually exclusive or bi-stable expression of both the miRNA and TF involved,[87] and that a transient signal can cause the loop to be locked into an irreversible state, even after the signal is gone. For example, in human hematopoietic cells, *mir-233* and NFI-A function in a coherent feedback loop to control granulocytic differentiation. In undifferentiated cells, *mir-233* levels are low and NFI-A levels are high; however, upon retinoic acid signaling, *mir-233* levels increase (due to activation by the TF C/EBPα) and NFI-A is repressed, facilitating differentiation to the myeloid lineage[86] (Fig. 2). This feedback loop ensures mutually exclusive expression of *mir-233* and NFI-A, thereby generating a bi-stable system (undifferentiated *versus* differentiated hematopoietic cells).

Incoherent feedback loops potentially function to fine-tune gene expression and to maintain precise steady state levels of both components of the loop.[85] Additionally, incoherent

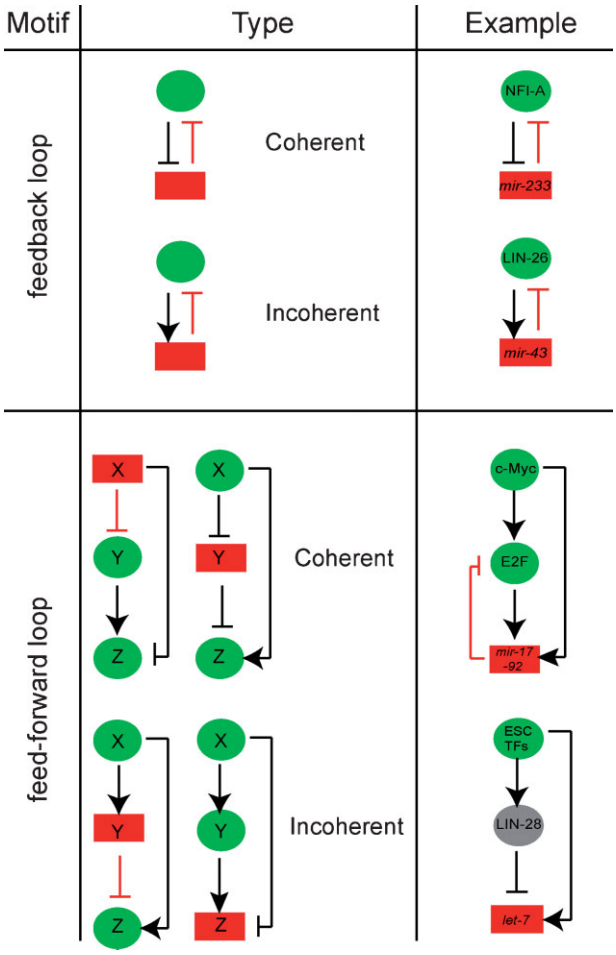

Figure 2. Cartoon depicting coherent and incoherent feedback and feed-forward motifs. Note that for feed-forward loops, other arrangements between regulators and targets are possible. Green circles,TFs; red rectangles, miRNAs; gray diamonds, other protein-coding genes; black arrows, activation; black blunted arrows, repression. LIN-28 is an RNA-binding protein. ESC TFs: embryonic stem cell TFs Nanog, Oct4, Tcf3 and Sox2.

loops can result in oscillatory expression of both components, which depends on additional input signals.[94] We have shown that *C. elegans mir-43* and LIN-26 function together in an incoherent feedback loop where both components of the loop are co-expressed (Fig. 2).[24]

In feed-forward loops, a regulator X regulates the expression of a target Z *via* a direct as well as an indirect path, through a regulator Y. Note that not necessarily all targets or regulators are TFs and miRNAs (Fig. 2). Feed-forward loops can also be coherent or incoherent depending on the overall effect of both regulatory paths (Fig. 2). Coherent feed-forward loops involving TFs and miRNAs can function to suppress leaky transcription or as toggle switches where an initial signal can be converted into a long-lasting cellular response.[85,95] The c-Myc/E2F/*miR-17-92* circuit is an

example of coherent feed-forward loop. The TF c-Myc, which promotes cell cycle progression, activates the expression of many genes, including the E2F family of TFs and the *mir-17-92* cluster. In addition, E2F family members can bind the promoter of the *mir-17-92* cluster, activating its transcription. This coherent feed-forward loop is embedded in a more complex circuit, since some of the miRNAs from the *mir-17-92* cluster negatively regulate E2F family members (Fig. 2). This feed-forward loop reveals a mechanism through which c-Myc simultaneously activates E2F transcription and limits its translation, through activation of *mir-17-92* cluster, allowing a tightly controlled proliferative signal.[58,96,97] Incoherent feed-forward loops have been proposed to provide response acceleration upon signal detection.[95] For instance, Marson and colleagues have reported that the core embryonic stem cell (ESC) TFs, Oct4, Nanog, Sox2 and Tcf3, promote the transcription of the miRNA *let-7* and the RBP LIN-28. Mature *let-7* is scarce in ESCs but abundant in differentiated cells. Upon signal detection, LIN-28-mediated inhibition is released and mature *let-7* starts to accumulate. This circuit poises ESCs for rapid and efficient cellular differentiation (Fig. 2).[98]

Several lines of evidence suggest that regulatory circuits involving miRNAs and TFs are not isolated instances but are in fact prevalent mechanisms of gene expression at the genome-scale level. First, using information derived from genome-scale gene expression assays, several bioinformatic studies found that the expression of miRNAs and their targets is often highly correlated or anti-correlated.[85,99–101] Tsang *et al*. proposed that correlation or anti-correlation in expression between miRNAs and their targets can be explained by the existence of various types of feed-forward and feedback loops involving miRNAs and TFs.[85] However, they did not identify any actual loops in which the participating components are known. Second, Shalgi and colleagues searched for pairs of human miRNAs and TFs that regulate sets of common target genes by identifying putative miRNA sites and TFBSs that co-occur in individual genes. They observed that such miRNA-TF pairs are predicted to regulate each other more frequently than randomly picked pairs, suggesting the existence of feed-forward and feedback loops. Third, Wang *et al*. identified conserved *cis*-regulatory elements surrounding miRNAs in 12 *Drosophila* species.[102] These elements were enriched in known TFBSs. By integrating their predictions with miRNA target interactions, they identified putative regulatory feedback loops between miRNAs and TFs. Finally, by integrating the experimentally defined *C. elegans* TF → miRNA transcription regulatory network with a predicted miRNA → TF target network, we uncovered more than 20 miRNA ↔ TF feedback loops, where the TF that regulates a miRNA is itself regulated by that same miRNA. We found that such feedback loops occur more frequently than expected by chance (*i.e.*, in computationally randomized networks) and, hence, constitute a network motif. This demonstrates that such loops provide a general mechanism of gene expression.[24] We have also identified several feed-forward motifs by integrating the upstream regulators of TFs; however, further mapping of transcriptional networks of protein-coding genes will be necessary to determine if miRNA-containing feed-forward loops constitute network motifs at a genome scale.

Altogether, these network studies revealed not only the existence of reciprocal regulation between miRNAs and TFs, but also the existence of extensive coordination in the regulation of shared target genes at the genome-scale level. As a result, genes that are regulated both by transcriptional and by post-transcriptional mechanisms may be tuned at a higher level of precision than could be obtained by either mechanism alone. In support of this idea, a recent computational study found that genes with many TFBSs have a higher probability to be controlled by miRNAs than genes with fewer TFBSs, illustrating the coordinated action that TFs and miRNAs exert on gene expression.[103]

By examining the degree of the TFs and miRNAs that participate in feedback loops in the integrated *C. elegans* network, we found that most loop participants exhibit both a high in- and out-degree.[24] In most miRNA ↔ TF feedback loops, the miRNAs regulate many TFs and are regulated by many TFs, and *vice versa*. The fact that both the miRNAs and the TFs involved in feedback loops have many downstream targets implies that the expression of not only the TF and the miRNA, but also of their respective targets is tightly coordinated. Based on these observations, we propose that gene regulatory effects exerted by TFs and miRNAs can "spread" to large sets of genes. This "regulatory spreading" could, in principle, be important in regulating large sets of genes, or gene batteries, *e.g.*, in different tissues or in response to developmental or environmental cues (Fig. 3A). By integrating human protein-protein interactions with miRNA-target interactions, Liang and Li found that proteins regulated by several miRNAs tend to have a higher degree of connectivity in protein-protein interactions networks. The targeting of protein-protein interaction hubs by many miRNAs may consequently affect a large number of interacting proteins as well (Fig. 3B).[104]

Conclusions and future challenges

The genes of an organism are differentially expressed through the activity of gene regulatory circuits that we are just beginning to uncover. The first studies of genome-scale regulatory networks involving miRNAs and TFs have already revealed the existence of reciprocal regulation between these two types of regulators, as well as extensive coordination in the regulation of shared target genes. These studies have also identified common mechanisms by which miRNAs and

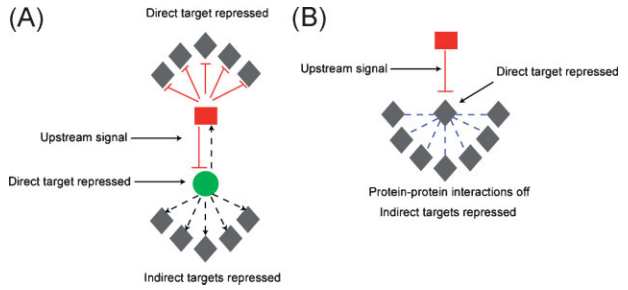

Figure 3. Network circuits allow the spreading of regulatory effects. (**A**) TF and miRNAs that participate in feedback loops are highly connected and not only regulate each other but also each others' targets. In this example, an upstream signal activates the miRNA, which in turns represses all its direct targets, including the TF in the feedback loop. As a result, all downstream targets of the TF are also repressed (indirect targets of the miRNA). Red blunted arrows, post-transcriptional repression; black dashed arrows, inhibition of transcriptional activation; gray diamonds, protein-coding genes; red rectangle, miRNA; green circle, TF. (**B**) MiRNAs tend to target hubs in protein-protein interaction networks, hence spreading its regulatory effects to large set of proteins. In this example, an upstream signal activates the miRNA, which in turn represses the hub (direct target). As a result, all protein-protein interactions between the hub and other nodes (indirect targets) are inhibited. Red blunted arrow, post-transcriptional repression; blue dashed lines, inhibition of protein-protein interactions; red rectangle, miRNA; gray diamonds, protein-coding genes.

TFs function to control gene expression and have suggested an inherited difference in the network properties of both types of regulators.

Complete, dynamic, and integrated networks

Network representations of TF and miRNA interactions are undoubtedly powerful when they incorporate reliable, complete, and unbiased data. To date, the computationally and/or experimentally mapped gene regulatory networks available for most genomes are only a small representation of all the interactions that occur. Thus, continued efforts for the experimental mapping of transcriptional networks using a variety of complementary methods such as ChIP and Y1H assays are essential. Importantly, the scope of regulatory network mapping may turn out to be greater than expected. For instance, miRNA sites that are not conserved or not seed-like, or miRNA sites outside 3′UTRs may be general rather than exceptions, and their interactions need to be considered. Similarly, as the complete spectrum of TFs in an organism of interest expands they need to be incorporated in regulatory networks. Finally, transcriptional networks have so far used binary information, *i.e.*, a TF either regulates a target gene or it doesn't. In the longer term, it will be important to include the full spectrum of binding specificities and affinities of all TFs.

Complex cellular and developmental processes depend in part on the precise spatiotemporal expression of genes, which is acquired by regulatory interactions that specifically occur at particular developmental times and/or in a tissue-specific manner. Most gene regulatory networks mapped to date consist of static depictions of all the possible interactions between TFs or miRNAs and their targets that can possibly occur *in vivo*. However, it will be important to integrate all available miRNA, TF and target expression patterns to limit the network to only those interactions that can occur, and to expand the set of environmental and experimental conditions tested. Together, this information will lead to highly dynamic network models that can help us understand how gene expression relates to development, physiology and, ultimately, disease.

It will also be crucial to integrate TF- and miRNA-containing regulatory networks with other functional data, such as protein-protein interactions on which many efforts have heavily focused, as well as other types of interactions that remain largely uncharacterized, such as those involving RBPs (Fig. 4). Such studies will provide insights into how TFs, miRNAs and RBPs together coordinate control of their targets, thereby affecting differential gene expression in a concerted fashion.

In the longer term, it will also be important to generate ''meta network models'' in which different types of nodes and interactions are combined to reveal how signaling networks, regulatory gene expression networks and protein–protein interaction networks function to regulate biological processes

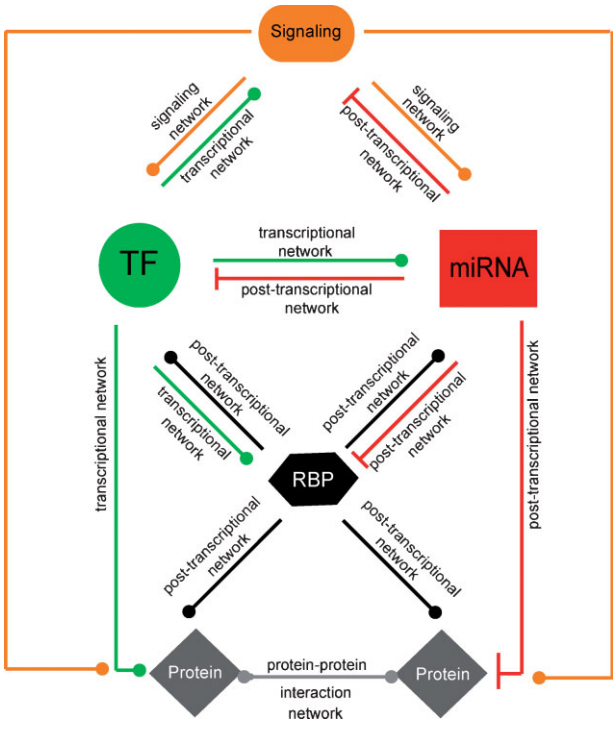

Figure 4. Integration of functional data into "meta network models".

that relate to development and homeostasis and how these networks are perturbed in disease (Fig. 4).

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