

Common logic of transcription factor and microRNA action

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Over the past few years, microRNAs (miRNAs) have emerged as abundant regulators of gene expression. Like many transcription factors (TFs), miRNAs are important determinants of cellular fate specification. Here I provide a conceptual framework for miRNA action in the context of creating cellular diversity in a developing organism, and emphasize the conceptual similarity of TF- and miRNA-mediated control of gene expression. Both TFs and miRNAs are *trans*-acting factors that exert their activity through composite *cis*-regulatory elements that are 'hard-wired' into DNA or RNA. TFs and miRNAs act in a largely combinatorial manner – that is, many different TFs or miRNAs control one gene – and they act cooperatively on their targets – that is, there are several *cis*-regulatory elements for a single TF or miRNA species in a target gene. Just as the set of TFs in a given cell type has been proposed to constitute a 'code' that specifies cellular differentiation, so 'miRNA codes' are likely to have conceptually similar roles in the specification of cell types.

Individual cell types in a multicellular organism are defined by a combinatorial code of coexpressed terminal differentiation genes known as a 'gene battery' (Box 1). Individual genes are linked into a gene battery by their sharing of common *cis*-regulatory elements. Genes usually contain numerous *cis*-regulatory elements and are activated or repressed in a specific type of cell by a set of regulatory factors binding to a subset of these elements. By contrast, in another type of cell a different set of *trans*-acting factors activates or represses the gene by binding to an alternative subset of *cis*-regulatory elements (Box 1 and Figure 1). The information content of *cis*-regulatory elements that are hard-wired into non-protein-coding regions of the genome and the availability of *trans*-acting factors that control these *cis*-regulatory elements are thus crucial determinants of cellular diversity and organismal complexity.

Transcription factors that translate the hard-wired *cis*-regulatory code into specific gene regulatory events are well-studied types of *trans*-acting factor [1]. Over the past few years, miRNAs have emerged as another prominent class of gene regulatory factors [2]. miRNAs, which are single-stranded RNA molecules of about 20–22 nucleotides, were first discovered in *Caenorhabditis elegans*

more than ten years ago [3], but their abundance in animal genomes has become apparent only through recent extensive genome searching and cloning efforts. By means of specific search criteria, about 100–300 miRNA-encoding genes have been discovered in each animal genome examined, corresponding to roughly 1% of the total genes identified in each respective genome [4–8].

In animals, miRNAs are thought to bind to defined complementary sites in target 3' untranslated regions (UTRs) to create imperfectly paired RNA heteroduplexes that inhibit translation of the target RNA by unknown mechanisms. Stimulatory effects of miRNAs have not been described but, in analogy to the action of small RNAs (sRNAs) in bacteria [9] (see below), one cannot exclude their existence. Similar to TF proteins that bind to defined *cis*-regulatory DNA sequences, miRNAs function as '*trans*-acting' factors that bind to *cis*-regulatory elements located in mRNA (Figure 1). As previously pointed out in a report on miRNA target gene identification [10], however, the similarity between TF- and miRNA-regulated control of gene expression goes far beyond this semantic similarity, as I describe in more detail below.

TFs and miRNAs as cell-specific gene regulatory switches

Although the effect of TFs on gene expression is rarely an all-or-nothing event, the ultimate effect of TF-mediated gene regulation in a developmental context can be usually classified as a switch that determines the precise composition of a gene battery and therefore defines the execution of a cellular differentiation program (Box 1). TFs can work as either 'on' or 'off' switches. One of the many previously described examples of an 'on' switch [1,11] is the TTX-3/CEH-10 homeodomain heterodimer, which acts through a single *cis*-regulatory element to turn on the expression of a gene battery that is characteristic of a single type of interneuron [12]. Members of this interneuron-specific gene battery have complex gene regulatory regions with multiple *cis*-regulatory elements, which respond to distinct sets of cell-type-specific TFs.

Negative regulatory switches are also common. Against a 'ground state' of general activation, sequentially acting 'off' switches (i.e. transcriptional repressor proteins) result in the derepression of specific terminal differentiation features and can therefore determine the composition of gene batteries in specific types of cell (Box 2).

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Box 1. Creating cellular complexity by differential gene expression

Individual cell types in a multicellular organism are defined by the expression of 'gene batteries' [45]. Gene batteries are sets of terminal differentiation genes that are linked by common sets of *cis*-regulatory elements [1]. An important aspect of the gene battery concept is that the uniqueness of a cell type is not defined by a uniquely expressed gene, but by the unique coexpression of more broadly expressed genes (Figure I). A recent analysis of the gene battery of a single interneuron type in *Caenorhabditis elegans* has made this point particularly clear [12]. This form of combinatorial coding endows an organism with n genes to create, in theory, 2^n different cell-specific gene batteries, as shown in Figure I. This rationalization provides an easy explanation for the fact that the absolute number of genes in a genome does not correlate with

organismal complexity, which instead might result from the amount and usage of hard-wired *cis*-regulatory information.

As has been discussed extensively in the context of transcription factor action [1], and as summarized in Figure I, information concerning when and where a gene is expressed is hard-wired into *cis*-regulatory elements; in other words, genes contain several *cis*-regulatory elements that are occupied by *trans*-acting factors in a cell-type-specific manner. Another essential feature of transcriptional regulatory control is that a *cis*-regulatory element that determines expression in one given cell type is usually occupied by a combinatorially acting set of *trans*-acting factors. These factors often come together in what has been termed an 'enhanceosome' [46].

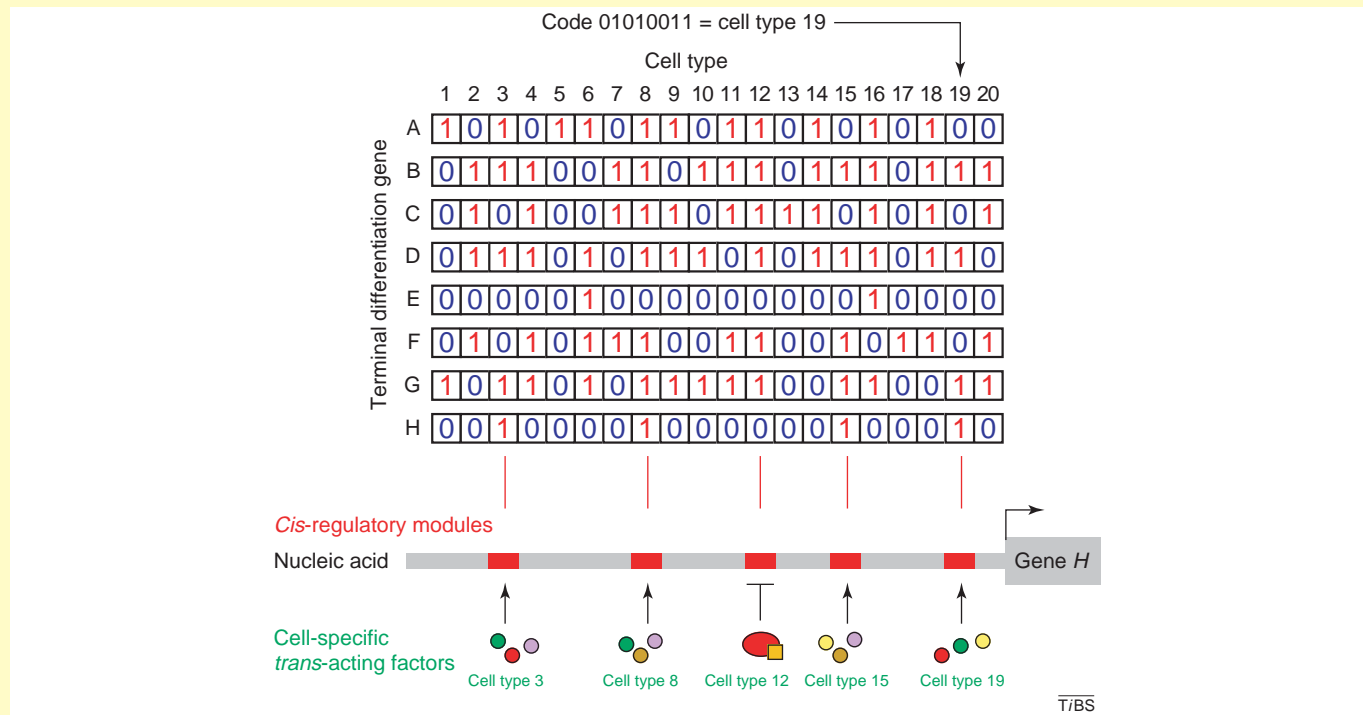


Figure I. Terminal differentiation genes encode proteins that determine the terminal phenotype of a cell, such as the set of neurotransmitter receptors in a neuron, contractile proteins in a muscle cell and metabolite transporters in a kidney cell, among others. Whether such a gene is expressed or not is indicated by '1' or '0', respectively. Of course, this binary behavior of gene expression is an oversimplification because intermediary levels of gene expression can be instructive, but it is still a useful concept if one simply takes '1' to mean that the level of gene expression is sufficient to confer a specific property to a cell and '0' to mean that there are insufficient levels of gene expression. From the perspective of a hypothetical gene *H*, whether *H* is expressed or not is determined by the occupancy of *cis*-regulatory elements located in proximity to the regulated gene. Conceptually, the *cis*-regulatory elements shown here can be binding sites for either TFs or miRNAs (in the latter case, the *cis*-regulatory elements would be located 3' to the gene, which is not indicated here).

miRNAs might function similarly as cell-type-specific switches that determine spatial domains of gene expression. For example, loss-of-function and misexpression approaches have shown that the *lsy-6* miRNA constitutes both a necessary and a sufficient switch to determine a specific program of chemosensory cell differentiation [13] (Figure 2). Hematopoietic lineage differentiation can be also switched by the action of a miRNA [14].

An implicit assumption in the comparison with TFs is that miRNAs, like TFs, are expressed in a spatially restricted manner in vertebrates and invertebrates. Indeed, miRNAs are not only expressed in a tissue-restricted manner but also restricted to individual cell types within specific types of tissue [14–19]. For example, the *lsy-6* miRNA seems to be expressed in only

nine neuronal cells in the adult nervous system of *C. elegans* [13].

TFs and miRNA are tightly linked in gene regulatory pathways

Developmental programs often involve multilayered TF networks, cascades and regulatory loops [1]. miRNAs seem to be tightly linked to TFs in complex regulatory networks; in other words, miRNA expression patterns are determined by complex transcriptional regulatory inputs and miRNAs themselves show a propensity to regulate other TFs. This latter point has been made by genome-wide target predictions of miRNAs [10,20], and also by genetic studies that have provided sound evidence supporting the integration of miRNAs in transcriptional regulatory networks.

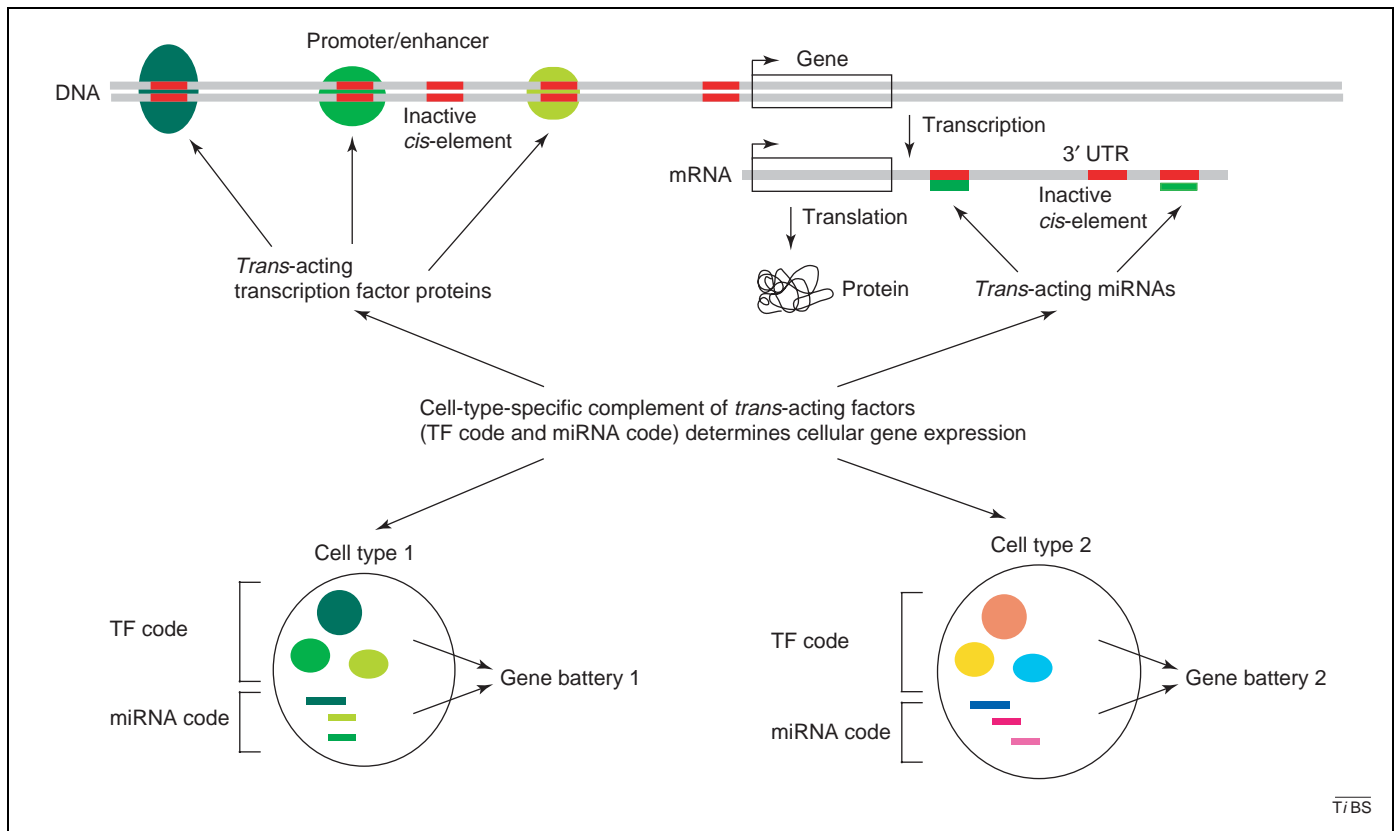


Figure 1. *Cis*-regulatory control by transcription factors (TFs) and microRNAs (miRNAs). Both TFs and miRNAs are *trans*-acting factors that act in a combinatorial manner, such that a distinct combination of TFs (green, numbered 1–3) is bound to the complement of *cis*-regulatory elements (red) present in either DNA or mRNA. Cell-type-specific combinations ('codes') of TFs and miRNAs determine which cell-specific terminal differentiation genes ('gene batteries') are expressed (Box 1). 'Inactive *cis*-element' refers to elements that are not occupied in a specific cellular context. Note that the cell-type-specific expression of combinations of TFs and miRNAs is similarly regulated by sets of *trans*-acting factors. Thus, TF- and miRNA codes are likely to dictate cellular differentiation in a hierarchical manner, with one code setting up the next 'downstream' code. For the sake of conceptual simplicity, other types of 3' untranslated region (UTR)-mediated translation control mechanisms, which function via protein recognition of 3' UTR elements, are not discussed here but have been covered in a recent review [44].

The two most prominent and genetically best-studied examples are miRNAs involved in the timing of cell division events in *C. elegans* and miRNAs involved in neuronal fate determination in *C. elegans*. The first pathway has been extensively reviewed elsewhere [21,22], and the second pathway is shown in Figure 2. Here, a cascade of several miRNAs and TFs regulate each other's activity with the ultimate effect of inducing a different spectrum of putative chemoreceptors in the two main taste receptor neurons, ASEL and ASER, in *C. elegans* [13,23,24] (Figure 2).

Combinatorial action

If the analogy between TF- and miRNA-mediated gene control is truly relevant to complex events in cell type specification, then *cis*-regulatory elements in 3' UTRs, similar to *cis*-regulatory elements in promoters or enhancers, should be also composite in nature to enable them to sample miRNAs in different cell types. Recent genome-wide searches for miRNA targets are consistent with such a hypothesis. Many putative miRNA target genes often contain complementary sites for several different miRNAs [10,19,20,25,26].

The existence of multiple *cis*-regulatory elements for *trans*-acting factors can be viewed from two different angles. First, multiple *cis*-regulatory elements can be an indication of different *trans*-acting factors occupying

different *cis*-regulatory elements in different types of cell (Box 1). Such a scheme is clearly operating in transcriptional control [1]. In light of the tissue-specific expression profiles of miRNAs and composite miRNA-binding sites, this scheme is also likely to apply to miRNA action.

Second, in addition to being an indication of differential occupancy in specific cell types, *cis*-regulatory elements can be an indication of the complex nature of an on or off switch, being composed of several coexpressed, combinatorially acting components. In other words, for a gene to be turned on or off within one given type of cell, a combination of *trans*-acting factors specific for that cell type binds to several *cis*-regulatory elements in proximity to the gene [1] (Box 1).

The combinatorial requirement for sets of TFs has led to the proposition of 'TF codes' – namely, specific combinations of TFs that together determine cellular identity [27,28]. A similar scheme might apply to miRNAs. The occurrence of numerous distinct putative miRNA-binding sites in the 3' UTRs of putative target genes is consistent with a combinatorial model in which a given type of cell expresses several miRNAs, which together determine the expression state of a specific set of target genes in that cell. In analogy to TF codes, the complement of miRNAs in a cell type might thus also constitute an 'miRNA code' that determines gene expression profiles.

Box 2. The concept of sequential repression

Hierarchies of sequentially acting transcriptional repressor proteins are an emerging theme in cell fate determination. Specific cell fates are induced by a repressor, which inactivates a repressor of a specific cell fate (Figure 1). Such a model assumes an underlying mechanism of 'basal activation'. In an extreme view of this model, there is latent activation of terminal markers in all types of cell, and precise expression profiles are shaped through the patterned expression of upstream repressor proteins. Sequentially acting transcriptional repressors have been observed in many processes, including motor neuron specification in vertebrates [47–49], *Caenorhabditis elegans* [50] and flies [51,52] and in chemosensory fate specification in *C. elegans* [23] as shown in Figure 1. The importance of such sequential 'off' switches might be a pervasive theme in other regulatory contexts [22,53].

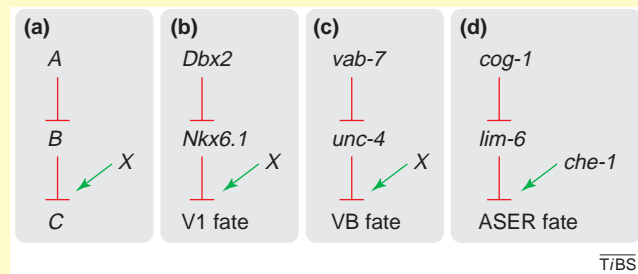


Figure 1. A cascade of sequentially acting negative regulatory factors. (a) The presence of 'A' in a given cell type induces a given cell fate exemplified by gene 'C', through the repression of 'B', which is a repressor of C. Latent activation might be conferred by generally acting transcriptional activators [48]. (b–d) This scheme has been found to apply in, for example, ventral cord (V1) motor neurons in vertebrates [47], VB-type motor neurons in worm [50] and the ASER chemosensory neuron in worm (see also Figure 2) [23]. Note that in VB-type motor neurons, the VAB-7 protein ('X') not only inhibits the *unc-4* gene, but also positively regulates VB fate.

At present, the lack of cellular analyses of miRNA expression patterns in vertebrates hampers the definition of miRNA codes on a cell-type-specific basis, but significant regional overlaps of miRNA expression, for example in the brain, coupled with the coexpression of specific miRNA species in embryonic stem cells [29] and hematopoietic lineages [14], suggests that such 'codes' of miRNAs exist. Further analyses of expression pattern will almost certainly reveal overlaps in the cell-specific expression of miRNAs, thereby substantiating the miRNA code hypothesis. The addition of another combinatorial level of gene expression control to the already very diverse combinatorial expression control that can be achieved through TFs illustrates the vast computational complexity of genomic regulatory information that can be exploited to generate differential patterns of gene expression.

Quantitative aspects of TF and miRNA function: cooperativity

There is a strong quantitative component to the combinatorial action of TFs in a given cell type. The binding affinities of individual TFs to a binding site contained in a complex promoter is often strongly enhanced by the cooperative binding of other, either adjacently or distantly binding, TFs [11]. miRNA-mediated repression of translation has a similar quantitative nature. Synthetic 3' UTRs with binding sites for two distinct miRNAs respond

in a cooperative manner to the presence of both miRNAs [30]. Thus, cell-specific miRNA codes can be translated into precisely tuned levels of gene expression output.

Cooperativity is not observed only with heterotypic *trans*-acting factors (i.e. distinct TFs acting cooperatively on a single promoter); rather, it is common to observe clusters of binding sites for a single TF [11]. Similar homotypic binding of a single miRNA species to several target sites in a given 3' UTR is also commonly observed. For example, the *lin-14*, *lin-41* and *hbl-1* genes each contain several binding sites for their cognate miRNAs *lin-4* and *let-7* [31–35].

Multimerization of homotypic miRNA-binding sites in synthetic 3' UTRs and the ensuing synergistic translational repression response have experimentally confirmed the cooperative nature of miRNA action [36]. Recent *in vivo* work provides further evidence for the cooperative function of miRNAs: the deletion of either of two miRNA target sites in the *die-1* 3' UTR causes a complete disruption of target gene repression in transgenic worms, suggesting that one intact miRNA-binding site is not enough to confer repression [24] (Figure 2). Moreover, genetic evidence suggests that the *lin-28* gene is cooperatively regulated by the *lin-4* miRNA and another unidentified miRNA [37].

The cooperativity of TF action depends on the precise spacing of *cis*-regulatory sites [11]. Similarly, the correct relative spacing of miRNA-binding sites might be crucial for miRNA function [35]. TF cooperativity is usually based on a direct interaction between the TF proteins [11]; by contrast, such a direct interaction of miRNAs is difficult to envisage. However, protein factors recruited by duplexes comprising miRNA and its target mRNA might confer such cooperativity. Alternatively, miRNA binding to 3' UTRs could have a cooperative effect through the potential necessity to 'open' suprastructural elements of the 3' UTR.

The clustering of several *cis*-regulatory elements is often an imperative for the efficient binding of *trans*-acting factors that show a low affinity *in vitro* for an individual *cis*-regulatory element. Low binding affinity is another common feature of miRNAs and many TF families. For example, the core consensus binding motif for an individual homeodomain TF is less than ten bp and often contains only a conserved TAAT sequence in its center [38]. Similarly, miRNAs seem to recognize their cognate targets largely through sequences of less than ten bp located at the 5' end of the miRNA [26,30]. For both TFs and miRNAs, these short binding sites are not long enough to target specifically binding motifs located within the large genomic sequence space, and for both factors phenomena such as cooperativity and combinatorial action present one way out of this apparent conundrum. An alternative way out is cofactor action, as discussed below.

Cofactors for TFs and miRNAs

The conceptual similarities between TFs and miRNAs invites speculation about other potential commonalities. For example, many DNA-binding TFs were identified long before it was recognized that they function with numerous cofactors to affect gene expression. Cofactors also bind

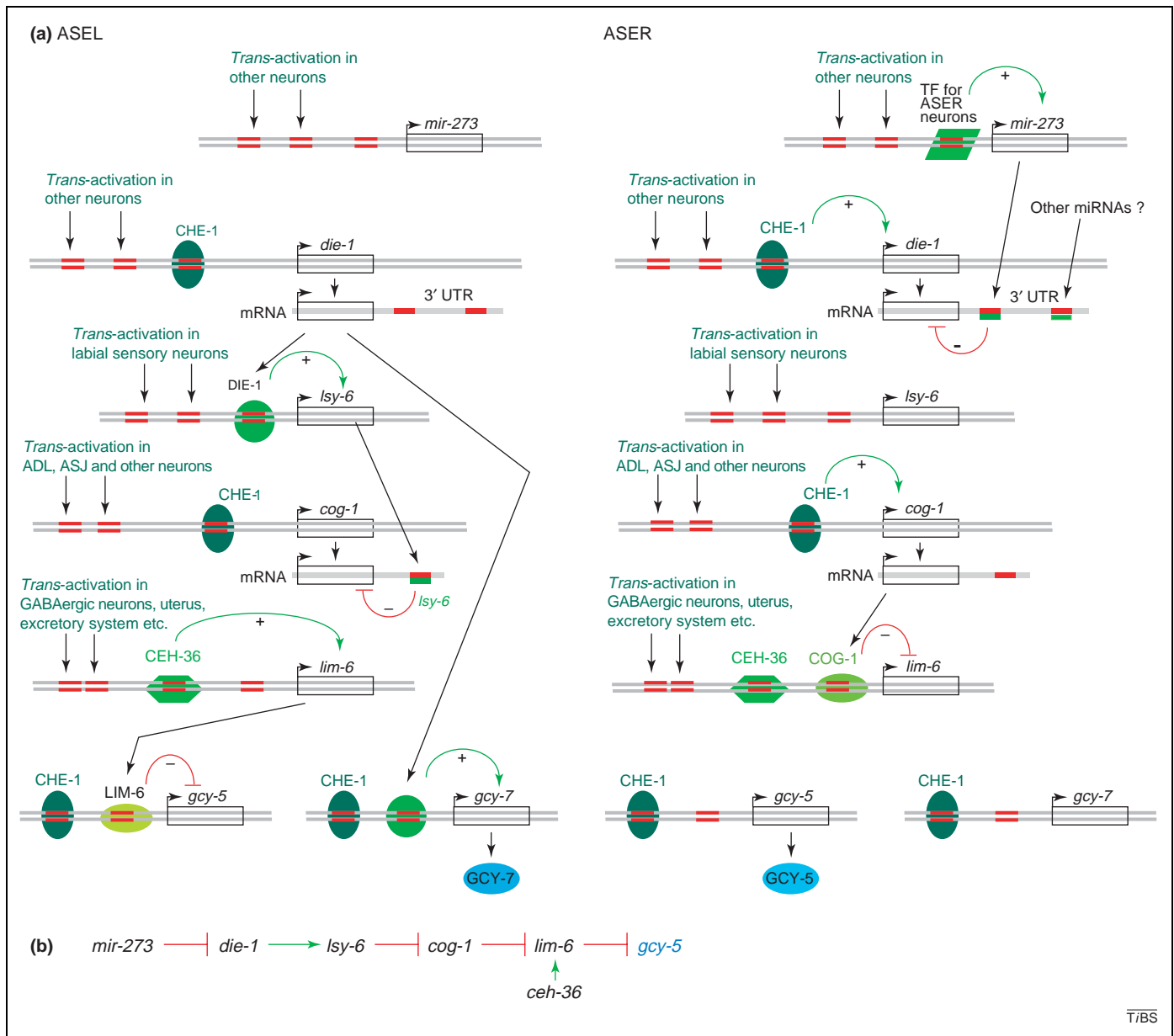


Figure 2. Integration of microRNA (miRNA) and transcription factors (TFs) in gene regulatory networks. The ASE chemosensory neurons of *Caenorhabditis elegans* use a cascade of TFs and miRNAs to control cell fate [13,23,24]. **(a)** There are two ASE neurons, one on the left side of the brain (ASEL) and one on the right (ASER), which each express a different set of GCY chemoreceptors. In ASER, the miRNA *mir-273* (and presumably other miRNAs acting through phylogenetically conserved elements in the *die-1* 3' untranslated region) downregulates expression of the zinc-finger TF gene *die-1*, whose transcription is activated in both neurons by CHE-1, a zinc-finger TF. In ASEL, less *mir-273* is expressed (either because *mir-273* is repressed in ASEL or because it is more strongly activated in ASER, as shown here); thus, *die-1* is not downregulated and it can activate expression of the *Isy-6* miRNA. In turn, this miRNA represses expression of the *cog-1* homeobox gene, a repressor of the *gcy-7* gene and the *lim-6* homeobox gene, which itself represses expression of the *gcy-5* chemoreceptor. CHE-1, DIE-1 and CEH-36 are each required either directly or indirectly for expression of *gcy-7* in ASEL (CEH-36 is not shown). **(b)** Summary of the chain of interactions, as revealed by genetic analysis. Whether interactions between individual components are direct, as shown schematically in (a), remains to be determined. Note the sequential repressor interactions that follow the scheme shown in Box 2. Several more complex aspects of this regulatory pathway, including additional pathway members and feedforward and feedback loops are omitted for the sake of clarity. Other aspects of this cascade, such as the precise mechanisms of CHE-1 action, are also not understood. Finally, it is notable that the regulatory relationship between the genes indicated here exclusively exists in the ASE neurons. There is little to no overlap in expression of these regulatory factors in other cell types and, accordingly, their expression is determined by independent *cis*-regulatory elements and *trans*-acting factors in other cell types, as indicated in (a). This example therefore follows the concept presented in Box 1 and Figure 1.

DNA and have a significant impact on the DNA-binding specificity of the partner (e.g. HOX and Exd complexes) [38]. It would not be surprising if we were to find that similar principles apply to miRNA function.

Specifically, target recognition by miRNAs might be accompanied and further specified by cofactors binding to the double-stranded RNA (dsRNA) heteroduplex and thus contributing to the specificity of miRNA–target duplex formation. It is possible that such factors, if they exist,

might themselves be tissue-specific, thereby increasing the complexity of the system analogous to transcriptional control. Systematic searches for dsRNA-binding proteins that specifically recognize mismatched or bulged miRNA–target duplexes are likely to identify such cofactors.

Antiquity of similarities between TFs and miRNAs

Small regulatory RNAs, or sRNAs, abound in bacteria [9,39] and show intriguing similarities to animal miRNAs.

First, a single sRNA can control the expression of several genes by binding to its respective target mRNA; second, the complementarity of an sRNA to its target is not perfect; and third, the consequence of an sRNA–target interaction is translation regulation. The effects of sRNAs on translation, similar to the effects of animal miRNAs, can be inhibitory, but they can also be stimulatory through the disruption of inhibitory secondary structural elements in the mRNA [9].

In line with TF-mediated gene expression, there can be numerous sites for a single sRNA in its target mRNA and several different sRNAs can control the same mRNA species. In contrast to miRNA-mediated gene regulation in multicellular organisms, however, the complexity of bacterial sRNA-mediated mRNA target regulation obviously does not lie in different types of cells expressing distinct sRNAs. Rather, the spatial control of miRNA activity in animals is replaced by the control of sRNAs by environmental stimuli in bacteria [9]. In both cases, the regulation of small RNA activity is conferred at the level of transcriptional regulation; in other words, spatially controlled TFs regulate animal miRNA transcription, whereas environmental stimuli induce TFs that regulate bacterial sRNA expression.

The conceptual similarity of animal miRNAs and prokaryotic sRNAs points to the intriguing issue of reversibility. Environmental regulation of gene expression by sRNAs is reversible in bacteria: genes under the control of an sRNA in one environmental condition become independent of that sRNA on changes to that environment. When more targets of miRNAs have been identified and experimentally verified, it will be possible to assess whether miRNA-mediated gene regulation is as readily reversible as bacterial sRNA function. The observation that the fly miRNA *miR-277* potentially targets many enzymes in a specific metabolic pathway whose activity is under the control of environmental conditions [26] might provide a good test case. It will be intriguing to assess whether the plasticity of translational control that exists in some specific cellular contexts, such as local translational control at the synapse [40], uses such a reversibility of miRNA action.

Differences between TF and miRNA action

The conceptual similarities between TF and miRNA action should not obscure our view of the dissonances between these two factors. In contrast to miRNAs, TFs can act as either activators or repressors, their size enables them to interact simultaneously with various distinct, often tissue-specific, protein interaction partners, and their function can be modulated by posttranslational modifications. The last point is exemplified by the fact that the integration of many developmental signaling pathways occurs at the level of TF modifications [41]. Although miRNAs themselves do not seem to have these properties, unidentified proteins that bind to miRNA–target heteroduplexes to control translation might introduce these features – in other words, the resulting protein–heteroduplex complex might be subject to control by signaling pathways. A caveat to this suggestion is that if such mechanisms were really prominent, then unbiased

genetic analysis in worms and flies, which has been very effective in identifying TFs, should have revealed such proteins by now.

At first sight, another difference between TF- and miRNA-mediated gene regulation lies in their distinct cellular localization: namely, one takes place in the nucleus, the other at ribosomes. The latter can be distributed to rather distinct compartments in a cell, which provides miRNA-mediated gene expression with the potential for subcellular spatial control, such that variations in the subcellular localization of miRNAs might dictate local changes in gene expression. This is a particularly relevant issue in the field of neurobiology, where local regulation of translation occurs at specific synaptic sites [40]. It should be kept in mind, however, that the nucleus is also divided into specific subdomains and the differential distribution of TFs might be functionally relevant [42].

Size constraints constitute another relevant difference in the contribution of TFs and miRNAs to gene regulation. *Cis*-regulatory elements for TFs can be dispersed over hundreds of kilobases, or sometimes megabases, whereas *cis*-regulatory elements targeted by miRNAs are constrained in their localization to 3' UTRs, which are, on average, smaller than 1 kilobase in both vertebrates and invertebrates [43]. Although this constraint significantly hampers the evolvability of miRNA-mediated control as compared with TF-mediated control, it does not fundamentally call into question the conceptual similarities of TFs and miRNAs. The limited compositional complexity of 3' UTRs implies only that the means to decipher miRNA codes are more limited than the means to decipher TF codes.

Concluding remarks

The non-gene-coding information content of a genome sequence can create extraordinary regulatory complexity. The *cis*-regulatory elements of genes that are expressed cell-specifically sample the existing set of *trans*-acting factors (TFs and miRNAs) in a given cell type, resulting in the precise spatial and temporal control of gene expression. In so far as every cell type can be described by the terminal differentiation gene battery that it expresses, it can be similarly defined by the set of *trans*-acting factors (TFs and miRNAs) that it expresses. These TF codes and miRNA codes ultimately determine the composition of cell-type-specific gene batteries. The combined complexity of both codes not only is of crucial relevance for creating cellular complexity in a developing organism, but also provides evolution with abundant opportunity for creating novelty by tinkering with *cis*-regulatory information content.

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