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Biological Robustness and the Role of MicroRNAs: A Network Perspective

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Abstract

Over the past decade, microRNA molecules have emerged as critical regulators in the expression and function of animal genomes. This review discusses the relationship between microRNA-mediated regulation and the robustness of biochemical networks that contain microRNAs. Most biochemical networks are robust; they are relatively insensitive to the precise values of reaction constants and concentrations of molecules acting within the network. MicroRNAs involved in network robustness may appear to be nonessential under favourable uniform conditions used in conventional laboratory experiments. However, the function of these molecules can be revealed under environmental and genetic perturbations. Recent advances have revealed unexpected features of microRNA organization in networks that help explain their promotion of robustness.

Biological activities such as development exhibit a property known as robustness. This term has been used to mean many things, and we define robustness as an event that happens reproducibly and uniformly even in the face of variability that can be induced by the environment, informatic (genetic and epigenetic) variation, local effects, and random chance. A few biological processes are quite variable and hence do not require robustness. However, most biological processes, particularly irreversible ones such as differentiation, are strongly robust to ensure a minimal impact of error. This review discusses the particular role that microRNAs (miRNAs) play in biological robustness.

1. MicroRNAs and Large-Scale Networks

Many studies have looked at miRNAs by first-order relationships: what molecules regulate a miRNA and what mRNA transcripts are regulated by a miRNA. Genome-wide studies have shown that, in plants and mammals, mRNAs targeted by miRNAs are overrepresented by GO terms associated with regulation of development (Rhoades *et al.*, 2002; Shalgi *et al.*, 2007). Clearly, miRNAs are important for development (Carthew, 2006). However, a paradox for many miRNAs is their lack of strong phenotypic consequences on development when individually mutated, and yet they are evolutionarily conserved. Some have argued that this paradox is due to the weak repression of target gene expression elicited by most miRNAs. Weak and tunable repression by miRNAs can generally elicit three distinct effects on their targets. MiRNAs can (i) dampen, (ii) denoise, and (iii) set thresholds to the levels of their targets (Bartel and Chen, 2004; Bushati and Cohen, 2007; Cohen *et al.*, 2006; Inui *et al.*, 2010). In the first type of effect, a miRNA reduces the level of target below an activity threshold acting like a switch; in the second type of effect, a miRNA buffers fluctuations in the target, limiting undesired signal propagation; in the third type of effect, a miRNA raises or changes the level of activation at which the target has to be induced to actively regulate a

process. Each of these effects can potentially be harnessed to provide robustness to target gene regulation.

However, miRNAs also exist in higher-order relationships, and less emphasis has been placed on the biochemical networks that include miRNAs. Yet this is fundamental to understand what roles miRNAs play in robustness. Most biochemical networks are robust; they are relatively insensitive to the precise values of reaction constants and concentrations of molecules acting within the network (Barkai and Leibler, 1997; Eldar *et al.*, 2002). Our point of view is that miRNAs help to generate the robustness of biochemical networks. One means that they might generate network robustness is to dampen, denoise, and set thresholds for direct targets in a network. But as we shall see, miRNAs also generate network robustness because of biases in the kinds of targets that they regulate. Understanding how miRNAs do so will provide insight about how higher-level biological processes such as development are also made robust.

1.1. Network hubs and miRNAs

Molecular regulatory systems can be represented as networks composed of nodes and links (Fig. 9.1A). Nodes can be genes, sequence elements, or molecules such as proteins, metabolites, RNAs, etc. Links are the molecular interactions between the nodes. The degree of a node is the number of links that a node has with other nodes in the network. The collection of degrees for each node in the network is the degree distribution and is frequently represented as a graph of the frequency of each node degree type (Fig. 9.1B). While some links such as protein–protein interactions do not necessarily have an associated direction (undirected link), many links such as between a transcription factor (TF) and its gene target, or a miRNA and its mRNA target, are directed (i.e., a miRNA represses a target mRNA when the two are bound together, and not the other way around). In directed networks where links have a directionality associated to them, the degree of a node can be further subdivided into links going in or out of the node (in and out degrees, respectively) (Fig. 9.1C).

The degree distribution is an important property of large-scale network organization, and it measures how the connectivity is distributed overall in the network. Most molecular networks studied to date such as protein–protein, signaling, or TF-gene networks have a degree distribution that deviates from random (i.e., a normal distribution) and follows instead an exponential distribution or a power law (Barabási and Oltvai, 2004; Martinez and Walhout, 2009). In such networks, the majority of nodes have a low to medium number of links, while a few nodes, called hubs, are highly connected to other nodes (Fig. 9.1B). Hubs mediate interactions among numerous and less connected nodes, allowing rapid coordination between different parts of the network. This type of system is robust to random loss of the less connected nodes but is sensitive to deletion of the hubs (Albert *et al.*, 2000). For example, in protein–protein interaction networks, there is a lethality–centrality relationship where highly connected components induce lethality when lost (Jeong *et al.*, 2001; Zotenko *et al.*, 2008).

Do miRNAs exhibit this lethality–centrality relationship? Systematic mutagenesis of many individual miRNA genes (Miska *et al.*, 2007) and paralogous families of miRNA genes (Alvarez-Saavedra and Horvitz, 2010) were performed in the nematode *Caenorhabditis elegans*. Most miRNAs either individually or in collective families are not essential for viability or development. Only miRNAs like let-7, one of the most highly connected miRNAs in the animal (Martinez *et al.*, 2008), elicit observable lethal phenotypes when knocked out (Reinhart *et al.*, 2000). The degree distribution of *C. elegans* miRNA-TF networks could explain why most miRNAs trigger subtle or nondetectable mutant phenotypes. If a miRNA node has only one or a few links to target within a given network, then loss of this node would generally have a small impact on network behavior. In *C.*

elegans, there is a significant difference between nodes composed of TFs and nodes composed of miRNAs. TFs bind promoters in a scale-free manner, that is, the TF-out degree distribution follows a power law, and there are clear TF hubs binding many promoters. In contrast, the miRNA-out degree distribution follows an exponential distribution, that is, there are no clear miRNA hubs even though some miRNAs are more connected than others (Martinez and Walhout, 2009; Martinez *et al.*, 2008).

Although miRNAs do not exhibit hub-like properties, frequently the direct targets of miRNAs behave as network hubs. These target hubs often contain many in-links from different miRNAs (Fig. 9.2). In the *C. elegans* and human miRNA–mRNA target networks, the target in-links follow a power law distribution (Martinez *et al.*, 2008; Mookherjee *et al.*, 2009a). In such networks, target hubs exist that are linked by 15 or more miRNAs. Strikingly, these target hubs are enriched for TFs (Martinez *et al.*, 2008) and factors involved in regulation of development (Shalgi *et al.*, 2007). Target hubs also tend to be more connected by protein–protein interaction links than lowly connected targets. A positive correlation has been seen between the number of miRNA-binding sites in the 3^0UTR of a gene and the connectivity of its protein product to other proteins (Liang and Li, 2007). This propensity is not a side result of longer or evolutionarily conserved 3′UTRs (Liang and Li, 2007; Shalgi *et al.*, 2007). In the case of *C. elegans*, experimental evidence indicates such hubs are important for miRNA function. Combined knockout of individual miRNA genes and TF hubs led to synthetic phenotypes that were otherwise undetectable when either miRNA or hub was knocked out alone (Brenner *et al.*, 2010).

Target hubs can be more connected in other ways as well. miRNAs were found to preferentially target genes encoding enzymes that are metabolic hubs or cut point enzymes (Tibiche and Wang, 2008). These are capable of regulating metabolic mass flow at global and local scales, respectively (Tibiche and Wang, 2008). Highly connected scaffold proteins in signaling networks are also preferentially linked to miRNAs (Cui *et al.*, 2007). Scaffold proteins are important components of signaling pathways that lack enzyme activity but physically interact with upstream and downstream components of the pathways, often simultaneously. It has been found that miRNAs more frequently target highly connected scaffold proteins than less connected nodes of the same pathways (Cui *et al.*, 2007). Altogether these results suggest that miRNAs preferentially regulate highly connected nodes in various types of networks.

1.2. Signal flow and miRNAs

Signaling networks are crucial to establish expression patterns that lead to cell decisions. In such systems, signal transduction has a directional flow; it often begins with membrane-bound receptors that bind ligands to intracellular transduction proteins interacting with other proteins, to translocation of effectors to the nucleus resulting in altered gene expression. Cui *et al.* (2007) found that miRNAs target signaling proteins of a human network more frequently than what would be expected by chance. Importantly, the distribution of miRNA targets in the signaling network is correlated with signal flow and the position of the factors within the signaling network. The propensity for a factor to be regulated by miRNAs increases in the direction of signal flow, from ligands (9.1%) to cell surface receptors (18.8%), to intracellular transducers (31.2%), and to nuclear proteins (50%) (Cui *et al.*, 2007).

1.3. Network modules and miRNAs

Biochemical networks are frequently organized into a set of distinct subnetworks called modules. A module exists as a group of nodes that are more highly connected to each other than to the rest of the network. Modules are interconnected, typically through nodes called

bottlenecks (Fig. 9.3). Bottlenecks can in some cases be hubs. Since bottlenecks connect modules, they have a high betweenness centrality, that is, are nodes with many “shortest paths” going through them. They are analogous to major bridges and tunnels with multiple parallel links between two nodes that themselves are part of two distinct modules. Given their central position in networks, bottlenecks are often essential proteins (Yu *et al.*, 2007). Modularity can impart robustness to networks, as it provides the ability for certain functions to be carried out in a semiautonomous manner through the coordinated interactions of relatively small subsets of molecules more densely connected to themselves than to other network components (Hartwell *et al.*, 1999). Modularity can make systems more evolvable and in some cases more tolerant to the random loss or modification of a module's component. Modules have been mapped for developmental processes such as early embryogenesis of *C. elegans* (Gunsalus *et al.*, 2005) and have been in some cases shown to operate as robust entities despite variations in input signals and kinetic constants that govern their behavior (von Dassow *et al.*, 2000).

MicroRNAs frequently regulate module bottlenecks in networks analyzed on the genome scale (Hsu *et al.*, 2008) (Fig. 9.4A). More detailed studies have found corroboration for this bias. One study focused on a protein–protein network regulated by miR-204, a miRNA that was shown to function as a tumor suppressor (Lee *et al.*, 2010). Within the network, two distinct modules (cell adhesion and cell cycle) are found, and each module is connected to the other via bottlenecks. Within each module, miR-204 preferentially targets mRNAs encoding hub and bottleneck proteins (Lee *et al.*, 2010). Interestingly, miR-204 suppression significantly augments cell cycle and extracellular matrix remodeling *in vitro* and *in vivo* (Lee *et al.*, 2010). Another study showed that several miRNAs are predicted as regulators for various modules of tightly coexpressed genes (Bonnet *et al.*, 2010). miR-200a is the top regulator of a small module of nine genes that is part of a larger network, such that this miRNA regulates the module via the TF ZEB1. Interestingly, this module is most likely involved in epithelial homeostasis, and its dysregulation could contribute to the malignant process in cancer cells (Bonnet *et al.*, 2010).

A second modularity-related property of miRNAs is that they cotarget molecules belonging to the same module (Fig. 9.4B). Cotargeting occurs by subsets of either unrelated miRNAs or a particular miRNA family. For example, there is pervasive regulation of several related transcription repressors that function in the Notch signaling network by three different classes of miRNAs that each recognizes a similar seed sequence in their targets (Lai *et al.*, 2005). Further support for this property has come from computational studies. Basu *et al.* (2011) found that coregulated targets tend to be organized within network modules. More than half of modules with prevalent coregulated targets are not simply explained by seed similarity (Mookherjee *et al.*, 2009b). Two other computational analyses showed that cotargeted genes and their interacting neighbors jointly show significantly higher modularity, and clustered miRNAs jointly regulate proteins in close proximity within a protein–protein interaction network (Hsu *et al.*, 2008; Yuan *et al.*, 2009). These analyses support the notion that coregulation of targets within modules is a prevalent phenomenon.

As factors belonging to a module are corepressed, modular regulation increases the redundancy of the cotarget network, making it robust to the individual loss or rewiring of some of the miRNAs. Consistent with this feature, miRNAs are individually not essential (Miska *et al.*, 2007). The concept of miRNA regulative modularity is also in accordance with observations that relate specific miRNAs to cancer development. Up- or downregulation of miRNAs repressing particular modules is associated with loss of robustness that is linked with cancer progression (Bandyopadhyay *et al.*, 2010). For example, five miRNAs (miR-19b, miR-20a, miR-26a, miR-92, and miR-223), which are capable of promoting T-cell acute lymphoblastic leukemia (T-ALL) in a mouse model,

account for the majority of miRNA expression in human T-ALL. This small set of miRNAs is responsible for the cooperative suppression of several tumor suppressor factors, achieved through an overlapping and cooperative regulation of these miRNAs (Mavrakis *et al.*, 2011).

1.4. How do these properties affect network robustness?

Nonuniform perturbation in network activity is most keenly "felt" by critical nodes such as hubs and bottlenecks since their extensive in-links act to amplify any perturbation. This amplification must be dampened in order to maintain these nodes working in synchrony with less connected nodes that do not experience such a degree of amplification. miRNAs weakly repress protein expression and thus are well suited to dampen hubs or bottlenecks in times of perturbation. Another reason critical nodes are targeted is one of impact. If a hub or bottleneck is perturbed, it has the most impact on the network's stability. Therefore, miRNAs that dampen such perturbation will contribute more greatly to network stability than miRNAs that target other types of nodes. A flip side of the impact theme is that perturbation of one section of a network is transduced most strongly to the rest of the network by hubs and bottlenecks. Dampening the transduced perturbation at these central nodes has both the broadest and swiftest effect on stabilizing the entire network and achieving synchronization. It also has the advantage of being able to respond to a wider variety of perturbations.

The modularity of miRNA targeting is also well suited to providing robustness. As a module is semiautonomous with distinctive biochemical properties, it specifically responds to perturbation relative to the rest of the network. A perturbation can thus be contained within a module by exact and coordinated regulation of the handful of nodes acting within a module or by regulation of bottlenecks. Clearly, miRNAs with tunable and parallel (redundant) regulatory capabilities are well suited to dampen perturbations within a module. This activity then would help prevent destabilization of the remainder of the network.

The pattern of miRNA regulation in signaling networks further provides robustness. Preferential regulation of the most downstream nodes in signal flow facilitates rapid responses with minimal lag when upstream nodes are perturbed. This pattern of regulation also makes signaling networks less prone to respond to noise resulting from signal propagation, since amplification of upstream noise would be dampened downstream.

2. MicroRNAs and Circuits

In this section, we review how small-scale circuits can provide robustness to biological processes, associating miRNA function with particular features of networks.

2.1. Circuits are recurrent patterns in large-scale networks

Large-scale networks can be deconstructed into circuits composed of smaller groups of nodes. Analysis of prokaryotic TF-target gene networks led to the discovery that certain types of circuits occur inside networks at frequencies much higher than in randomized control networks (Shen-Orr *et al.*, 2002). Such overrepresented circuits are called network motifs, and they constitute "building blocks" of larger networks that preserve their functions independent of the network environment in which they are embedded (Alon, 2007; Milo *et al.*, 2002).

One class of network motif is a circuit called a feedforward loop (FFL). FFLs have two paths or arms of regulation, one direct (short arm) and one indirect (long arm). The upstream node in the loop regulates the downstream node directly and indirectly through an intermediate node (Fig. 9.5). Eight different FFL configurations exist, and four variables determine which combination of dynamic behaviors emerges in a particular FFL (Box 9.1).

Computational modeling and experimental studies of prokaryotic FFLs show that such loops have specific information processing properties that differ from direct circuits (Goentoro *et al.*, 2009; Kaplan *et al.*, 2008; Mangan and Alon, 2003; Mangan *et al.*, 2003; Shen-Orr *et al.*, 2002). Such properties include acceleration or delay of a response, generation of signal pulses, and the ability to buffer the downstream node against fluctuations in the upstream node such that only persistent changes in the upstream node are transduced through the loop. FFLs provide robustness against stochastic fluctuations in the upstream node of the circuit.

Another type of circuit is the feedback loop (FBL). Positive and negative FBLs are known to be of central importance in biological processes (Fig. 9.5). Positive FBLs can amplify signals, create ultrasensitivity, and enable irreversible states of gene expression to occur (Brandman and Meyer, 2008; Chang *et al.*, 2010; Ferrell, 2002). Positive FBLs can give rise to bistable switches, that is, two alternative stable states without stable intermediates in between them (Ferrell, 2002). Double-negative FBLs can also stabilize gene expression in one state, though simulations have shown that double-negative FBLs are not sufficient to create bistable switches. Other features such as nonlinear positive feedback or balanced link strength are needed for a double-negative FBL to generate bistable behavior (Ferrell, 2002; Graham *et al.*, 2010). Single-negative FBLs are associated with homeostasis and desensitization (Ferrell, 2002). Thus, in different ways, positive and negative FBLs provide robustness of a circuit against fluctuation or perturbation.

We review the role of circuits that contain or are regulated by miRNAs, focusing on their possible roles in providing robustness. We refer the reader to four reviews on the topic that relate these circuits to developmental canalization (Hornstein and Shomron, 2006; Wu *et al.*, 2009), noise (Herranz and Cohen, 2010), and signal transduction (Inui *et al.*, 2010).

2.2. Feedback loops and miRNAs

Genome-wide studies have shown that FBLs containing miRNAs are network motifs in *C. elegans* and mammals (Martinez *et al.*, 2008; Tsang *et al.*, 2007). FBLs containing miRNAs can be double-negative, where the miRNA represses a repressor of the miRNA (Fig. 9.5). The double-negative FBLs containing miRNAs can be associated with bistable dynamics that give rise to mutually exclusive expression of the miRNAs and their targets. miRNAs can also be found in single-negative FBLs, where the miRNA represses an activator of the miRNA. A limitation of these genome-wide studies has been their ability to experimentally verify the existence of computationally predicted links. For example, Martinez *et al.* (2008) derived their network from a combination of computational predictions of miRNA targetsand Y1Hexperiments that established which TFs bound a library of DNA elements containing predicted promoters for miRNA genes.

Several experimentally verified examples of miRNA-containing FBLs have been described. Some of these are listed in Table 9.1 and have been reviewed extensively. Instead, we focus on two examples where a role for miRNA-mediated robustness has been shown. In *Drosophila melanogaster*, the transcription repressor YAN binds and represses the transcriptional enhancer of the *miR-7* gene. In turn, miR-7 binds and represses the protein expression of *YAN*. YAN and miR-7 are part of a network that regulates the transition from multipotent retinal progenitor cells to differentiated photoreceptors (Li and Carthew, 2005; Li *et al.*, 2009). The YAN network is a bistable system that transitions from a high YAN/low miR-7 to a high miR-7/low YAN stable state (Graham *et al.*, 2010). These two states are stabilized through double-negative FBLs between YAN and its repressors,and dictate whether a cell remains multipotent or differentiated (Graham *et al.*, 2010). EGF receptor signaling induces the phosphorylation of YAN and a switching from the multipotent to the differentiated state. Nevertheless, the FBL between YAN and miR-7 is essential neither for the switch nor for the stable maintenance of the cells' states. Instead, the role of this FBL is

probably to generate robustness to the network. When development is perturbed in a *miR-7* mutant by oscillating temperature, the switch occurs less robustly and errors in cell fate are observed (Li *et al.*, 2009). These errors are undetectable under uniform temperature conditions. Thus, this FBL can make differentiation robust to environmental perturbation.

A different mechanism is found in the differentiation of the *Drosophila* sensory organ precursors (SOPs; Li *et al.*, 2006). In this case, the miRNA is not itself part of a FBL but rather it regulates the responsiveness of the FBL. *miR-9a* represses expression of *Senseless*, which encodes a TF that induces SOP differentiation. The SOP cell fate choice is made by coupling Notch signaling to a positive FBL between various TFs, including Senseless. This choice does not fundamentally depend on *miR-9a* repression of *Senseless*, as *miR-9a* mutants are still capable of specifying their SOPs. Nevertheless, up to 40% of mutant animals make extra sensory organs (Li *et al.*, 2006). *miR-9a* thresholds *Senseless* expression such that, unless a threshold of *Senseless* expression is achieved, the TF FBL is not engaged and SOPs are not specified (Cohen *et al.*, 2006). Thus, *miR-9a* buffers SOP differentiation against fluctuations of Senseless.

2.3. Feedforward loops and miRNAs

Genome-wide studies have found FFLs that contain miRNAs are network motifs (Re *et al.*, 2009; Shalgi *et al.*, 2007; Tsang *et al.*, 2007). Thus, miRNAs could provide robustness by participating in FFLs (Fig. 9.5C). Nevertheless, the existence of a FFL topology is no guarantee that the processing property predicted for a FFL containing a miRNA follows the principles established for other kinds of FFLs. This is because the properties of such loops not only depend on the pattern of interactions but also on the molecular stoichiometry of the nodes and relative kinetics of the links. Finally, it is worth noting that not every functional FFL containing a miRNA might necessarily satisfy the computational assumptions made by Mangan and Alon (2003) for protein-based FFLs.

Several experimental examples of miRNA-containing FFLs have been described. One such example involves *Drosophila* miR-7 and its action in the eye differentiation network. Three FFLs are contained within the YAN network (Graham *et al.*, 2010; Li *et al.*, 2009) (Fig. 9.6). The first of these has a topology where miR-7 is in the middle of the long arm of the loop; the TF *Pnt-P1* directly represses *YAN* and indirectly represses *YAN* through miR-7 (Li and Carthew, 2005; Li *et al.*, 2009). This FFL was predicted to buffer variations of *Pnt-P1*, only accepting persistent *Pnt-P1* changes. Interlocked in the opposite direction is another FFL, where YAN directly represses *miR-7* and indirectly represses *miR-7* by preventing *Pnt-P1* transcription. This FFL is predicted to buffer *miR-7* expression from sudden nonpersistent changes in YAN abundance. In a third FFL, *Pnt-P1* directly activates *miR-7* and indirectly activates *miR-7* by repressing *YAN*. This last FFL is predicted to buffer *miR-7* expression from sudden nonstable changes in *Pnt-P1*. Collectively, these three interlocked FFLs could buffer sudden and nonpersistent changes in the abundance of two key regulatory factors, imparting robustness to the network.

The roles of these FFLs in providing robustness were tested in *miR-7* mutant flies (Li *et al.*, 2009). When mutant animals were subjected to temperature fluctuations, *YAN* showed abnormal overexpression and there were errors in differentiation. Under uniform temperature, *YAN* expression was normal. This result suggests that miR-7 acting in FBL and FFLs plays a specific role in buffering the network against environmental perturbation.

FFLs with a TF at the beginning of the loop, a miRNA in the middle of the long path, and a target gene at the end of the loop could provide robustness through several mechanisms. If both TF and miRNA repress the target, they would augment target repression asynchronously since the kinetics of synthesis and action between TFs and miRNAs are

different. This could cause a delay or an acceleration in the expression of a target, thus reducing or increasing the time necessary to trigger a response. Second, compared to simple regulation circuits, such FFLs provide redundancy. Third, they would act as persistence detectors that only transduce stable changes in the activity of the upstream node. If the FFL is structured where the upstream TF activates and the miRNA represses the target, then the miRNA would set a threshold, assuring that unless a given level of TF activity is achieved, the downstream target is not affected.

Theoretical studies support the notion that FFLs containing miRNAs could provide robustness to the expression of miRNA targets (Hornstein and Shomron, 2006). A quantitative comparison of small RNA-based and protein-based regulation showed that small RNA-based mechanisms are better at filtering noise in input signals (Mehta *et al.*, 2008). Osella *et al.* (2011) computationally analyzed the ability of FFLs containing miRNAs to buffer noise, and they found that miRNAs can confer efficient noise control in the face of fluctuations of the upstream nodes. Thus, miRNA FFLs are predicted to buffer noise, as Ghosh *et al.* (2005) found for protein-only FFL models. Interestingly, Osella *et al.* (2011) found that optimal noise filtering does not necessarily require strong repression. Indeed, weak repression is a common feature of most miRNA–target interactions. It is possible that the networks that use miRNA-containing FFLs for buffering might have selected miRNAs for modest repression. This could explain a frequent paradox existing for many miRNAs, that is, an apparent dispensability and lack of strong phenotypic consequences when individually knocked out but a strong evolutionary conservation.

3. Conclusion

Robustness triggered by miRNAs is generally thought to be a consequence of the way in which miRNAs act upon their gene targets. While this principle is no doubt at work, we have attempted to discuss miRNAs in the context of simple and complex networks of regulation. miRNAs regulate circuits that can provide robustness to networks. Within networks, miRNAs favor regulation of central hubs and bottlenecks. Their regulation is frequently module-centric, and targeting propensity increases toward the downstream effectors of signaling networks. These biases in miRNA targeting are, in and of themselves, other means by which miRNAs more effectively generate robustness. These biases would then imply that the acquisition of targets by miRNAs is not necessarily to generate novel gene regulation but to stabilize gene networks. This idea would explain why experimentalists frequently observe few phenotypic changes when highly conserved miRNAs are mutated. It also begs the question as to whether the extraordinary high birth and death rates of animal miRNA genes (Lu *et al.*, 2008) might reflect dynamic buffering of gene expression prior to and subsequent to speciation.

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Box 9.1 Feedforward loops are network motifs

In a FFL, the first factor of the loop regulates the last factor directly (short arm) and indirectly (long arm) through an intermediate factor. Four variables determine the dynamic behavior of a FFL. These variables are (i) the overall regulatory effect of the arms. The long arm can be positive or negative. (ii) Whether arms are coherent or incoherent (the same or opposite sign). (iii) The logic gate that integrates the signals from the two upstream nodes. This determines whether the downstream node can be regulated by both arms (AND gate) or by either arm (OR gate). (iv) The type of change in signal that passes through the loop. Does the change in the signal directed from an active to a repressed state (ON-to-OFF), or its inverse? Depending on the combination of these four variables, FFLs can act as pulsers, persistence detectors of change, or accelerators of response time. For example, consider the Type 3 coherent FFL where the upstream node directly and indirectly represses the downstream node through a second inhibitor activated by the first. When this specific architecture operates with an OR Gate, the resulting FFL produces a delayed response to a decrease (ON-to-OFF step) in the upstream node. As result, this type of FFL can filter ON-to-OFF pulses and only respond to persistent changes in the upstream node. This type of architecture exists in the Yan network linking *Pnt-P1*, *miR-7*, and *YAN* and is predicted to buffer YAN expression from stochastic decreases of *Pnt-P1*.

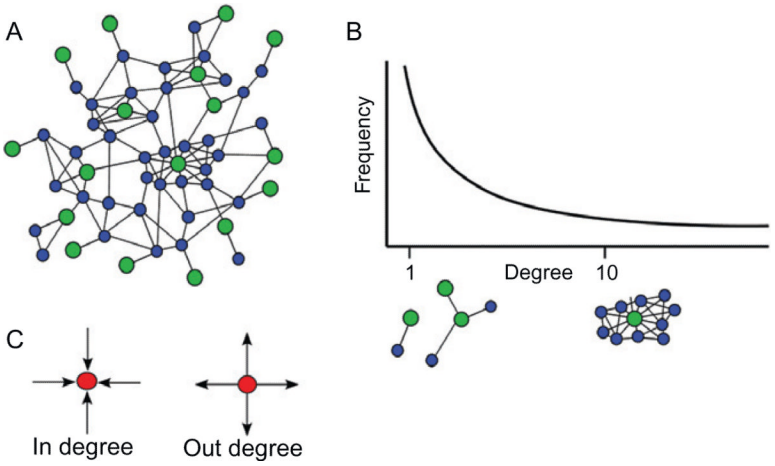

Figure 9.1.

Biochemical network organization. (A) Schematized network of nodes (circles) and links (lines). Different molecular classes of nodes are highlighted in green and blue. (B) A typical degree distribution for a network, illustrating that most nodes have few links and a few nodes have many links. This organization gives rise to a power law distribution that has a long tail. (C) Each link between nodes can be directed in terms of cause–effect relationship. Links directed into a node effect that node, while links directed out from a node effect the other node connected by the link.

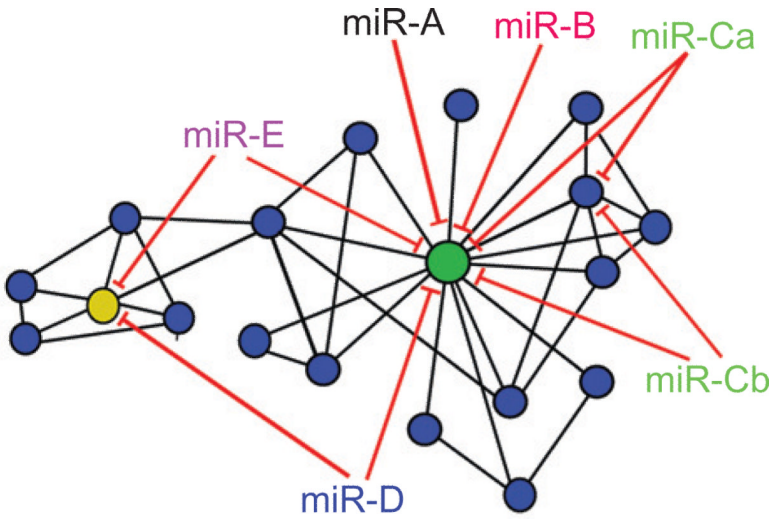

Figure 9.2.
MicroRNAs regulate network hubs. Two highly connected nodes are highlighted in green and yellow, and these have hub-like features. Various miRNAs preferentially regulate the network through these hubs.

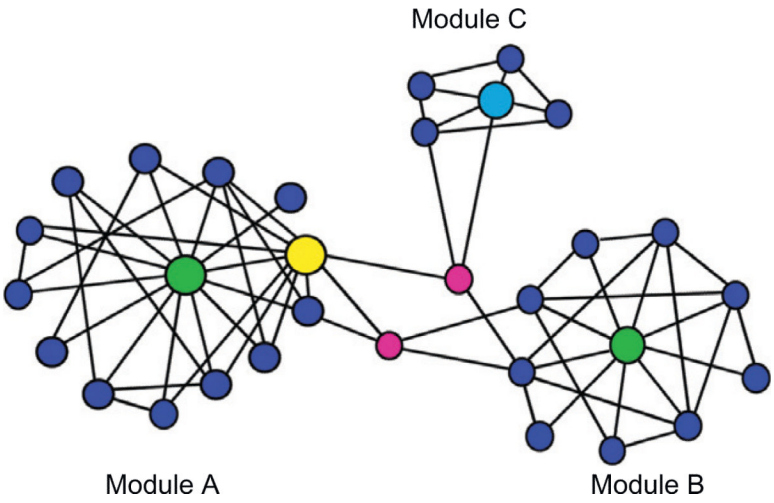

Figure 9.3.
Modular design of biochemical networks. A network with three modules. Modules A and B have hubs (green) and bottlenecks (yellow and red). Module C has a hub–bottleneck (blue).

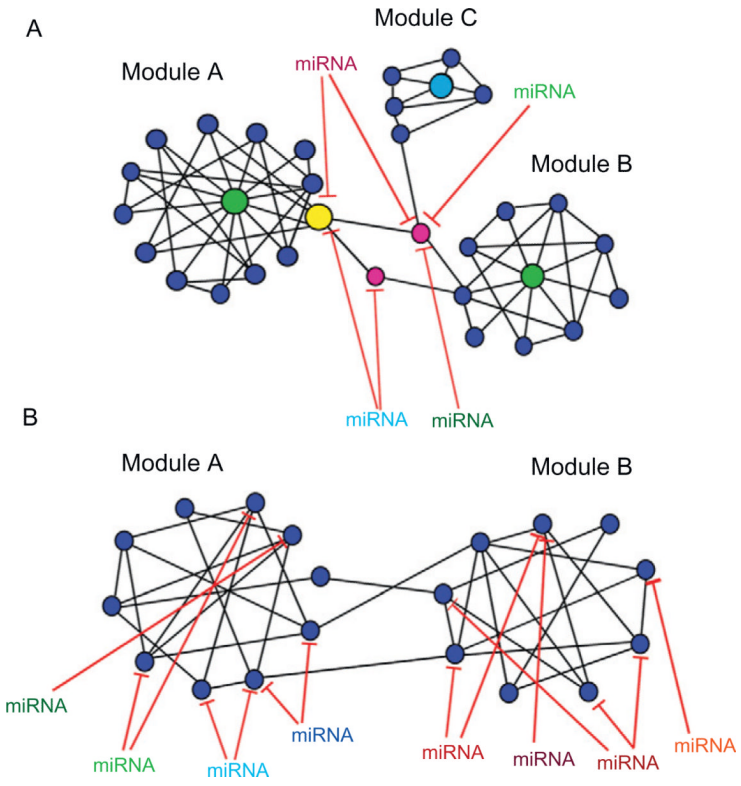

Figure 9.4.
MicroRNAs selectively regulate modules. (A) Various miRNAs preferentially regulate networks through module bottlenecks. (B) Families or classes of miRNAs selectively regulate certain modules in a network. Each miRNA frequently regulates more than one node in a particular module.

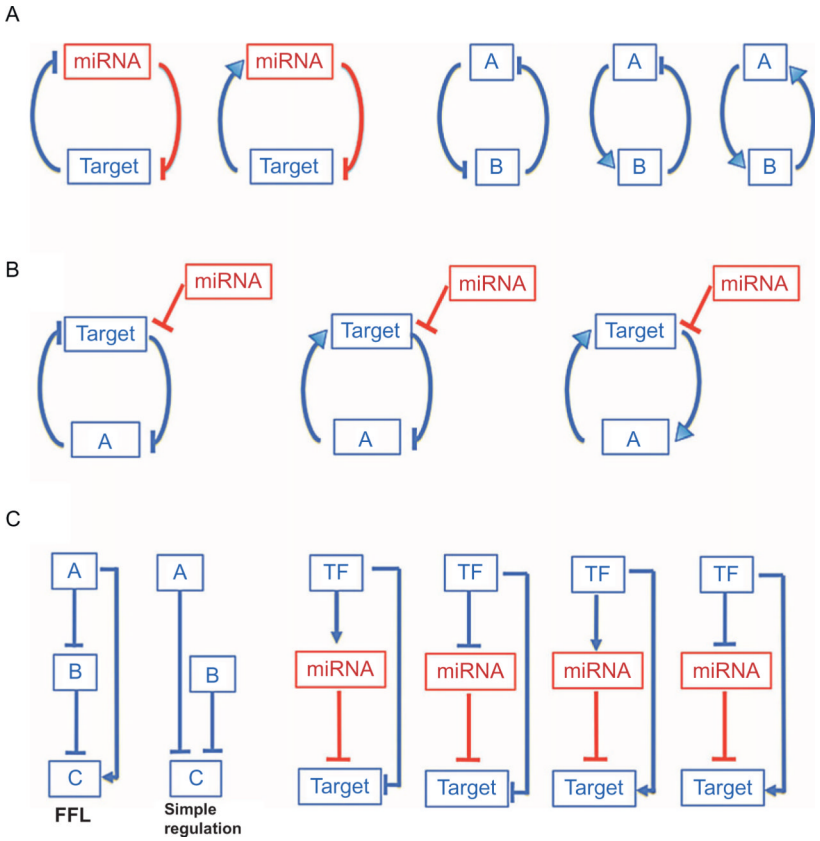

Figure 9.5.

MicroRNAs are components of network circuits. (A) Feedback loops (FBLs) can be double-negative, single-negative, or positive in sign (right). Since miRNAs repress their targets, then miRNAs can form double-negative or single-negative FBLs with targets (left). (B) FBLs of any sign can be regulated by a miRNA that exists outside of the loop. (C) Feedforward loops (FFLs) are distinct from simpler regulatory circuits (left). MicroRNAs can be components of FFLs, as shown on the right, where a miRNA is an intermediate in four different FFLs. Two of the FFLs are coherent, in which each arm of the loop has the same sign. Two of the FFLs are incoherent since each arm of the loop has the opposite sign.

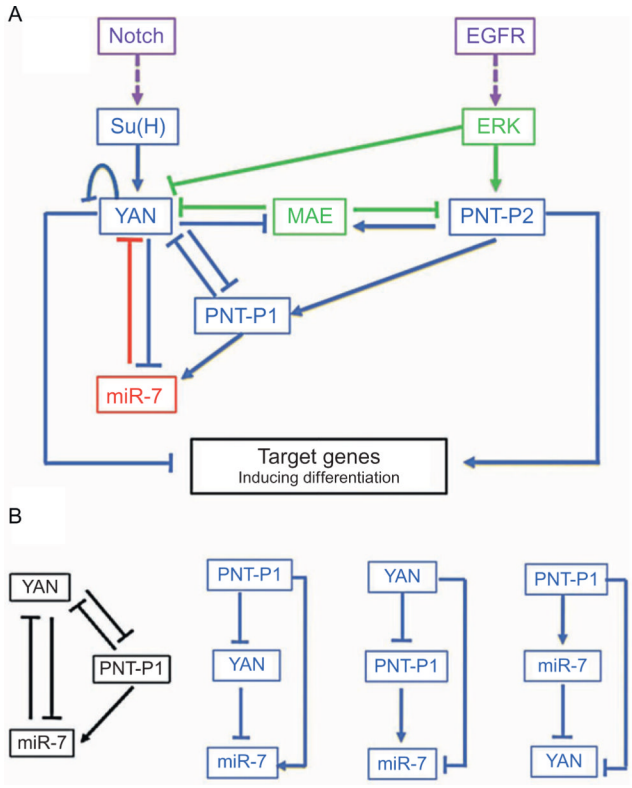

Figure 9.6.

The YAN network. (A) The network is constituted by transcription factors (blue) *YAN*, *Pnt-P1*, *Pnt-2*, the miRNA *miR-7* (red), protein-interactors (green) *MAE* and *ERK*, and inputs from the *Notch* and *EFGR* signaling pathways (purple). The output of the network is the transcriptional regulation of target genes (box) required for differentiation. (B) Coupled FFLs and double-negative FFLs exist in the network. Three three-node FFLs linking *YAN*, *Pnt-P1*, and *miR-7* are interlocked (left, in black). These three FFLs are shown separately (in blue).

Table 9.1

MicroRNAs and network motifs: Experimental evidence

System	Factors	Reference
FBLs		
Human granulocytes	miR-223; NFI-A; CEBP	Fazi *et al.* (2005)
C. elegans ASE neurons	lsy-6; miR-273; die-1; cog-1	Johnston *et al.* (2005)
C. *elegans* VPCs	miR-61; lin-12; vav-1	Yoo and Greenwald (2005)
Drosophila R cells	miR-7; Yan; Pnt-P1	Li and Carthew (2005)
Drosophila SOPs	miR-9a; Sens; Proneural	Li *et al.* (2006)
Cardiomyocytes	miR-1; miR-133l SRF; MEF-2	Chen *et al.* (2006)
FFLs		
Human B cells	miR-17-5p; c-myc; E2F	O'Donnell *et al.* (2005)
Drosophila R cells	miR-7; Yan; Pnt-P1	Li *et al.* (2009)