NON-CODING RNA

A network-biology perspective of microRNA function and dysfunction in cancer

Cameron P. Bracken[1,2], Hamish S. Scott[1,2] and Gregory J. Goodall[1,2]

Abstract | MicroRNAs (miRNAs) participate in most aspects of cellular differentiation and homeostasis, and consequently have roles in many pathologies, including cancer. These small non-coding RNAs exert their effects in the context of complex regulatory networks, often made all the more extensive by the inclusion of transcription factors as their direct targets. In recent years, the increased availability of gene expression data and the development of methodologies that profile miRNA targets *en masse* have fuelled our understanding of miRNA functions, and of the sources and consequences of miRNA dysregulation. Advances in experimental and computational approaches are revealing not just cancer pathways controlled by single miRNAs but also intermeshed regulatory networks controlled by multiple miRNAs, which often engage in reciprocal feedback interactions with the targets that they regulate.

MicroRNA
(miRNA). Short (~19–25 nucleotides in length) non-coding RNA that forms the target recognition component of the RNA-induced silencing complex.

RNA-induced silencing complex
(RISC). Ribonucleoprotein complex containing an Argonaute-bound microRNA that enables target recognition and accessory proteins that collectively mediate target destabilization and translational inhibition.

[1]*Centre for Cancer Biology, an alliance of SA Pathology and University of South Australia, Adelaide, South Australia 5000, Australia.*
[2]*Department of Medicine, University of Adelaide, Adelaide, South Australia 5005, Australia.*

Correspondence to G.J.G. and C.P.B.
greg.goodall@sa.gov.au; cameron.bracken@sa.gov.au

doi:10.1038/nrg.2016.134
Published online 31 Oct 2016

The first report of a regulatory microRNA (miRNA) was made in 1993 with the discovery that a *Caenorhabditis elegans* gene, *lin-4*, which controls diverse postembryonic cell lineages, does not encode a protein but rather a small RNA that represses the expression of a target gene (*lin-14*) with which it shares extensive sequence complementarity[1]. Since then, more than 2,500 miRNAs have been annotated in humans (miRBase v. 21, September 2016), and considerable detail is now known about their biogenesis[2], mechanisms of action[3] and functions. miRNAs are short (19–25 nucleotides in length) non-coding RNAs that serve as the target-recognition element of an RNA–protein complex known as the RNA-induced silencing complex (RISC), which contains an Argonaute (AGO) family protein that binds the miRNA, along with a range of accessory components[4]. The 5′ end of the miRNA (the seed region) forms the major target recognition element[5,6], and is of such importance that miRNAs are grouped into families on the basis of shared seed sequences (FIG. 1a). Structural studies show that the 5′ end of the miRNA is stacked in the AGO protein such that the Watson–Crick edges of bases 2–8 are ideally positioned for interaction with target mRNAs[7–9]. When bound to a target mRNA, the RISC complex reduces the rate of translation of the mRNA and accelerates the shortening of the poly(A) tail, resulting in faster mRNA degradation[3].

miRNAs have important roles regulating gene-expression programmes that underlie normal and pathologic cellular processes, including cancer. Some miRNAs act as tumour suppressors, whereas others, when aberrantly overexpressed, can promote tumour initiation, growth and/or progression to metastasis[10,11]. Presumably because of their small size, miRNAs with point mutations are rarely observed; by contrast, however, their dysregulation is common in many cancers. miRNAs can also be globally depleted in tumours relative to normal tissue[12], and cancer growth is accelerated in models in which miRNA biogenesis is disrupted[13]. In such contexts, decreased miRNA levels would cause widespread de-repression of targets and an unbuffering of gene expression, which would be expected to result in increased genetic 'noise' and an associated increase in the clonal heterogeneity of cancer cells[14,15]. This in turn may increase cancer adaptability and survival in a manner analogous to genetic noise increasing the survival of yeast and bacteria in response to changing environmental conditions[16,17]. Multiple reports now also suggest that miRNA expression signatures derived from either tumour tissue or liquid biopsies enable more accurate diagnosis and prognosis to be made in patients with cancer[18–20] and that miRNAs could even represent therapeutic targets in their own right[21].

Our abilities to understand miRNA functions in a network context and to map the consequences that their dysregulation can have in cancer have been greatly expanded by recent advances. These include methods of genome-wide identification of miRNA–target interactions, the application of RNA sequencing to detect consequences of miRNA overexpression or inhibition,

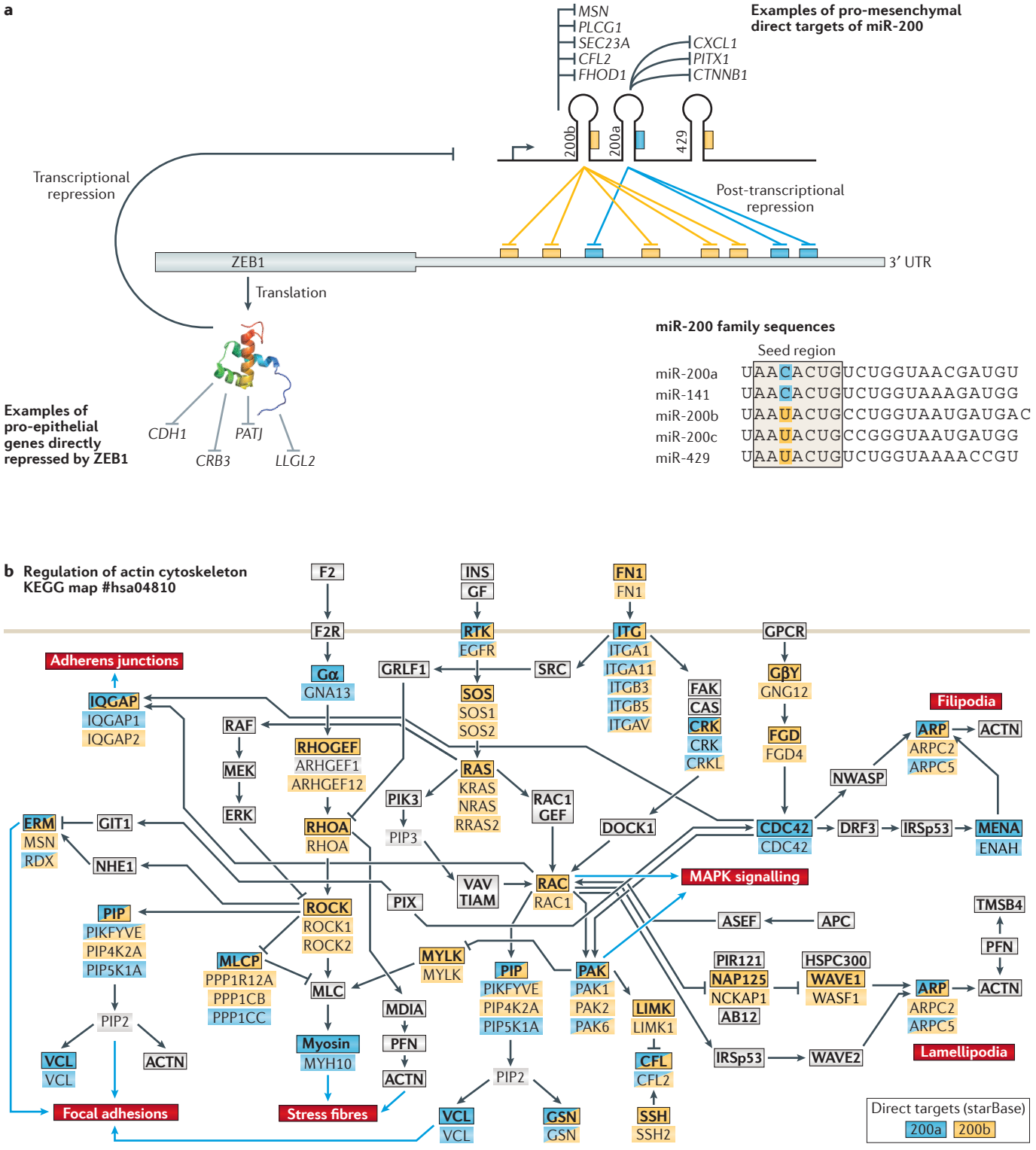


Figure 1 | **An example of microRNAs from a single gene that jointly regulate multiple targets in a network. a** | In epithelial cells, miR-200a and miR-200b strongly repress expression of the transcription factor zinc-finger E-box-binding homeobox 1 (ZEB1) through multiple sites in the ZEB1 3′ untranslated region (3′ UTR). They also repress numerous targets involved in cytoskeletal dynamics, cell migration and stemness. If ZEB1 expression is induced (for example, by transforming growth factor-β), ZEB1 represses the miR-200 gene and numerous other epithelial genes, thereby promoting epithelial–mesenchymal transition. A handful of key post-transcriptional targets are indicated in part **a**, more are indicated in part **b**. **b** | Experimentally verified targets (starBase[217]) of the polycistronically encoded miR-200a and miR-200b are mapped onto the regulation of the actin cytoskeleton pathway, adapted from Kyoto Encyclopedia of Genes and Genomes (KEGG) map #hsa04810. This illustrates the capacity for co-expressed microRNAs to jointly target multiple genes in a pathway. Functionally, the miR-200 family is known to regulate cytoskeletal processes, including adherens junctions, focal adhesion turnover and invadopodia[30]. Blue arrows show functional outcomes. Part **b** is adapted, with permission, from REFS 218, 219, Kanehisa Laboratories.

the availability of gene and miRNA expression data from both cell lines and patients, and the recognition of the role that competing endogenous RNAs may have on the availability of miRNAs to function in a cell.

In this Review, we discuss the operation of individual and co-expressed miRNAs acting at multiple levels in signalling networks, and we highlight the intricate and reciprocal relationship that exists between miRNAs and transcription factors — the largest families of post-transcriptional and transcriptional gene regulators, respectively. We also discuss recent work describing how miRNA dysregulation underlies cancer progression and argue for the importance of using genome-wide experimental and bioinformatic tools to examine miRNA function from a network-biology perspective.

Features of miRNA-regulated networks

Simultaneous regulation of multiple genes. Post-transcriptional gene regulation by miRNAs is mediated through mechanisms of translational inhibition and mRNA destabilization. Conveniently for the study of miRNA function, most targets show evidence of depleted mRNA levels[22,23], and mRNA destabilization is the dominant effect of miRNAs by the time that substantial target repression occurs[24]. However, in the vast majority of cases, the magnitude of miRNA-directed mRNA depletion is mild[22,25–29], which seems surprising given the extensive roles that miRNAs have across different biological processes and pathologies. How is it then possible that such crucial regulators of cellular processes seem to have such modest effects on most of their targets? The answer lies largely with the capacity of individual miRNAs to simultaneously regulate large cohorts of genes, coupled with the effects that arise from the direct suppression of transcriptional regulators (FIG. 1a). If targets are enriched for genes whose products participate in common signalling pathways, then the sum of typically modest interactions — sometimes coupled with a small subset of strongly regulated target genes — can facilitate a stronger response than might be achieved through the direct regulation of any one gene in isolation. One such example is the regulation of cell motility and invasion by miR-200b, which is achieved through the multi-level targeting of cytoskeletal genes that control the formation of such structures as focal adhesions and invadopodia[30] (FIG. 1b). The simultaneous targeting of multiple genes may also facilitate more specific fine-tuning through the regulation of distinct sub-networks.

Polycistrons as cooperative functional units. About two-thirds of miRNAs are encoded in polycistronic clusters; that is, they are co-transcribed with their cluster partners[31]. miRNAs in polycistrons should therefore be regarded as cooperative functional units, and their actions should be considered collectively. Such co-regulated miRNAs expressed from the same cluster have a tendency to target the same gene[32] or target different genes in the same pathways[33]; this reinforces the network-regulating roles of these miRNAs, as exemplified by the co-targeting of the actin cytoskeleton pathway by members of the miR-200 family (FIG. 1). This aspect is considered further below in the section 'Co-regulation of common biological processes by multiple miRNAs'.

Dominant regulatory roles of network hubs. The targeting of many genes by each miRNA, the targeting of individual genes by multiple miRNAs and the downstream effects that result from the miRNA-mediated regulation of transcription factors lead to highly complex networks of miRNAs and their target genes[34] (FIG. 1). The nodes of these networks (which can be individual miRNAs or mRNAs, including transcription factors), are typically connected to many other nodes in the complex regulatory webs. It is useful to identify nodes with atypically high numbers of connections ('hubs') because these represent sites of signalling convergence with potentially large explanatory power for network behaviour or utility for clinical prognosis and therapy[35].

Hub mRNAs tend to possess longer than average 3′ untranslated regions (3′ UTRs) with a higher than average density of target sites, which indicate their evolutionary selection as prominent points of regulation[36]. Conversely, 'housekeeping' and highly expressed tissue-specific genes typically have low densities of target sites for co-expressed miRNAs and are therefore less subject to direct miRNA-mediated suppression[37]. Individual miRNAs that constitute highly connected hubs are predicted to have dominant roles in the gene regulatory web. In a recent study, researchers determined the enrichment of miRNA target sites in groups of genes of similar function and applied network representation to examine connectivity patterns[33]. They found that a relatively small number of miRNA nodes could account for the majority of network connections, an observation that was not explicable simply by differences in the sizes of the predicted target gene sets between miRNAs[33]. Similar findings were gained from the investigation of miRNAs that are thought to be responsible for gene expression changes that occur during cancer progression. For example, in serous ovarian cancer, 89% of genes that identified a mesenchymal cancer subtype associated with poor patient survival were regulated by only eight miRNAs (representing seven distinct seed families)[20]. Similarly, 21 hub miRNAs were predicted to target 70% of the genes that are differentially expressed between grade II and grade III–IV gliomas[38]. Collectively, these studies indicate that (relatively) small numbers of individual miRNAs can play important parts in establishing and maintaining gene expression patterns.

Challenges in understanding miRNA function

Central to understanding miRNA function is the identification of miRNA targets; if the targets of a given miRNA are enriched in a biological process or pathway, then it is reasonable to infer that the regulating miRNA is involved in that process. A number of strategies have been used over the past decade to identify miRNA targets through sequence-based prediction, physical association and/or correlative gene expression (BOX 1). Lists of target genes can then be examined collectively

Argonaute
(AGO). The microRNA-binding protein in RNA-induced silencing complex. Four different AGO proteins, AGO1–AGO4, are present in mammals.

Seed region
The predominant target-recognition region of a microRNA, typically nucleotides 2–8 from the 5′ end. In recognition of the importance of the seed, microRNAs are grouped into families of shared seed sequence.

Liquid biopsies
Analyses of gene expression from circulating tumour cells and cell-free tumour DNA released into the blood or lymphatic system. Used as a means to improve diagnosis and treatment strategy.

Focal adhesion
Dynamic membrane-associated protein complexes through which the internal cell cytoskeleton connects with the surrounding extracellular matrix.

Invadopodia
Actin-rich extensions of the cell membrane that are associated with extracellular matrix degradation in cancer cell invasion.

Polycistronic cluster
Two or more genes or microRNAs that are encoded (and presumably co-expressed) from a single parental transcript.

3′ untranslated regions
(3′ UTRs). The part of the mRNA transcript 3′ to the protein coding region that constitutes the main functional microRNA-targeting region.

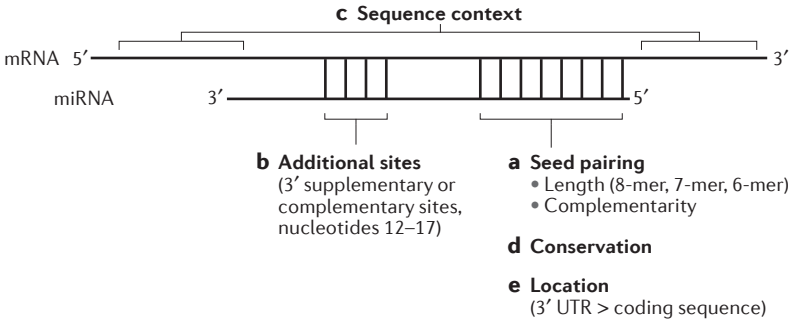
KEGG
(Kyoto Encyclopedia of Genes and Genomes). Database of biological pathways commonly used as a resource for understanding high-level functions of a biological system from gene-level information derived from high-throughput experimental techniques.

in the contexts of KEGG pathways[39–41] (FIG. 1b), protein–protein interaction networks[42–45] and enrichment analysis for common gene ontology terms[46,47]. Collectively, these approaches aim to reveal the biological function of miRNAs, predicated on the assumption that this will be revealed through the high representation of miRNA targets in common pathways, protein complexes or previously annotated roles. Several computational tools for target enrichment analysis facilitate the identification of hierarchical functions of miRNAs in gene regulatory networks (TABLE 1).

The participation of miRNAs in extensively connected genetic networks provides a challenge to understanding their function, because if the level of evidence required to establish miRNA–target relationships is set too low, genuine interactions become obscured by noise.

Box 1 | **Predictive and experimental methods of target identification**

Target-identification strategies are described briefly here and in further detail elsewhere[199–201].



***In silico* target prediction**

In silico prediction programs[39,202–208] differentially weigh various features of microRNA (miRNA)–mRNA target interaction to rank the predicted strength of target suppression. Key determinants include the length and complementarity associated with the primary site of miRNA–target interaction (the seed site; see the figure part **a**), and other factors such as the presence or absence of additional 3′ pairing (see the figure, part **b**); AU richness of the surrounding sequence (see the figure, part **c**); site conservation between species (see the figure, part **d**); and the location of the interaction site (3′ untranslated region (3′ UTR) sites, especially towards the beginning and end of the 3′ UTR, are generally more highly functional than sites in the coding sequence; see the figure, part **e**). Accurate target prediction is important in that it helps the interpretation of genomic data, guides further research and enables the modelling of systems that are difficult to study experimentally. Correctly weighing noise versus sensitivity and the contribution of abundant 'non-canonical', short or otherwise imperfect target sites remains a challenge.

Quantification of gene expression after miRNA manipulation

Reduced levels of target mRNAs, or depletion of target protein levels, are observable after ectopic miRNA expression and can be measured *en masse* using microarrays, RNA sequencing or global proteomic techniques. Techniques are also available to screen candidate 3′ UTR-luciferase reporter genes (and corresponding controls possessing mutated target sites) for targeting by co-transfected miRNAs[209], or to engage the high-throughput screening of miRNAs for the suppression of a directed panel of protein targets[26,210]. Similarly, ribosome profiling[211] or ribosome footprinting[23] can be used to measure the differential translation of genes after miRNA manipulation. These techniques are advantageous in their genome-wide scale and the fact that they attempt to approximate the degree of regulation, although, with the exception of luciferase reporter assays, they identify both direct and indirect targets and fail to precisely identify the target site sequence.

Argonaute and miRNA immunoprecipitation or pulldown methodologies

Several biochemical approaches have been developed to capture and sequence miRNA–target complexes from cells on a global scale. miRNAs, and mRNAs in the process of being regulated, can be co-precipitated with Argonaute (AGO)[211]. The efficiency of this procedure was further enhanced by the use of ultraviolet (UV) light to crosslink RNA–protein complexes and by the incorporation of an RNase step to more precisely identify the miRNA binding site (HITS–CLIP)[212]. Incorporation of photoreactive 4-thiouridine into RNA followed by UVA crosslinking (photoactivatable ribonucleoside-enhanced crosslinked immunoprecipitation (PAR-CLIP)) enhanced the capture of target mRNAs[61]. The further incorporation of a ligase step in the crosslinking, immunoprecipitation and sequencing of hybrids (CLASH) procedure enables the sequencing of miRNA–target mRNA pairs in a single continuous sequencing read, eliminating uncertainty over the identity of the miRNA that is responsible for any given site of AGO binding. The efficiency of this protocol at present, however, is low[60]. Analternative strategy is available whereby miRNA–mRNA complexes are identified through streptavidin pulldown of exogenous biotinylated miRNAs[213]. Collectively, these strategies offer a major advantage in their exclusion of inaccessible sites, and their ability to experimentally identify non-canonical sites, although challenges remain in identifying the interactions of functional significance from among the many interactants detected.

Genetic screening

Target prediction, expression analysis and the identification of physical interactions all serve as indicators of function, yet none directly addresses functional significance. Genetic screening aims to do this, combining the above measures to identify candidate genes of interest, then screening for the suppression of miRNA mutant phenotypes *in vivo* after the individual knockdown of candidate genes[214]. Although the functional significance of such assays is a clear advantage, genetic screening is not discriminatory for direct or indirect targets, is difficult to perform in mammals and is not well attuned to identifying the coordinated activities of multiple genes.

Table 1 | **Web-tools for miRNA target enrichment analysis**

Name	Web site	Refs
miTEA (miRNA Target Enrichment Analysis)	http://cbl-gorilla.cs.technion.ac.il/miTEA	220
DIANA mirPath	http://snf-515788.vm.okeanos.grnet.gr/	221
miRTrail	http://mirtrail.bioinf.uni-sb.de	222
CoMeTa	http://cometa.tigem.it/index.php	105
mirTarVis	http://hcil.snu.ac.kr/~rati/miRTarVis/index	223
miRNet	http://www.mirnet.ca	224
Mirin	http://mirin.ym.edu.tw	225
MAGIA (miRNA and Genes Integrated Analysis)	http://gencomp.bio.unipd.it/magia	226
miEAA (miRNA Enrichment Analysis and Annotation)	http://www.ccb.uni-saarland.de/mieaa_tool	227
TAM (Tool for Annotations of miRNAs)	http://cmbi.bjmu.edu.cn/tam	228
miRSystem	http://mirsystem.cgm.ntu.edu.tw	229
CORNA	http://corna.sf.net	230
MMIA (miRNA and mRNA Integrated Analysis)	http://epigenomics.snu.ac.kr/MMIA/mmia_main.html	231
FAME (Functional Assignment of miRNAs by Enrichment)	http://acgt.cs.tau.ac.il/fame	232
miSEA (miRNA Set Enrichment Analysis)	http://www.baskent.edu.tr/~hogul/misea	233

miRNA, microRNA.

Conversely, establishing overly stringent thresholds may cause the loss of too much information to allow the modelling of sufficiently detailed networks. Several factors add challenges to understanding miRNA function, which we discuss below.

Accuracy of target prediction. Each miRNA has hundreds (or thousands) of potential targets, but the accurate identification of functionally relevant target genes remains challenging and has been beset by high false-positive and false-negative rates[48]. Accurate prediction is hampered by the inability to effectively model secondary structures in RNA that preclude miRNA–target interaction, the influence of RNA binding proteins on target-site accessibility and the effect of competition between potential binding sites[49]. It is made even more difficult by the occasional and unpredictable tolerance of mismatches in the seed region, as well as the potential for non-seed interaction through central and 3′ pairing[50–53] (BOX 2).

However, our ability to predict the contribution of different sequence features to the effectiveness of targeting continues to improve[54] and has been complemented by the advent of experimental transcriptome-wide techniques to identify miRNA–mRNA interactions for specific miRNAs *en masse* through the co-immunoprecipitation of AGO–miRNA–mRNA complexes or the pulldown of biotinylated miRNAs[30,55–58] (BOX 1). Nevertheless, regulatory relationships remain difficult to disentangle owing to the large numbers of binding interactions that can be detected and the failure of many sites of physical interaction to bestow significant target repression at endogenous levels of miRNA expression. This is particularly the case for binding interfaces that are short (~6 nucleotides in length) or contain mismatched nucleotides[30,50,54,59–61].

Indirect effects. miRNAs act on their direct targets at the levels of both transcript stability and translational suppression, but can also initiate indirect effects on gene expression through the downstream activities of miRNA-targeted transcription factors. Such is the importance of miRNA–transcription factor interactions that most changes in mRNA level after miRNA manipulation may actually be due to altered transcription and not the direct transcript-destabilizing effects of the miRNAs themselves[29]. A standout example of this can be seen in the profound effect the miR-200 family has on controlling epithelial–mesenchymal transition (EMT) through its repression of the master EMT regulators ZEB1 (zinc-finger E-box-binding homeobox 1)[62] and SNAI1[63]. The presence of multiple target sites in the ZEB1 3′ UTR bestows unusually strong suppression that is mediated by miR-200, although there are also other, equally prominent examples, such as repression of the *HMGA2* oncogene by the tumour-suppressor miRNA let-7 (REFS 64,65).

Context-dependent effects. The same miRNA can have different, or even opposite, functional outcomes in different contexts. For example, miR-182-5p is reported to behave as an oncogene in breast[56], ovarian[66] and bladder cancer[67], but as a tumour suppressor in lung cancer[68]. Similarly, in hepatocellular and colorectal carcinoma, miR-17-5p is oncogenic[69,70], whereas it behaves as a tumour suppressor in cervical cancer[71]. miR-200 can also either promote[72] or inhibit[73,74] metastasis, depending on the cell model used. These discrepancies are likely to reflect the differential expression of target genes between tissues and serve both as a reminder of the complexities associated with miRNAs and as a cautionary note for the rigid assignment of any given miRNA to one specific role or function.

Epithelial–mesenchymal transition
(EMT). A process regulated by a complex gene-expression programme through which epithelial cells, which normally maintain close contacts with their neighbours through tight junctions, adherens junctions and desmosomes, transition towards a mesenchymal phenotype, whereby cells dissociate from their neighbours and become motile. Carcinomas, the most common form of solid tumours, arise from epithelial cells, with EMT being an important (although controversial) step in the progression to metastasis.

The influence of competitive endogenous RNAs. The effectiveness of a miRNA relies on there being sufficient copies of the miRNA within the cell to bind all its targets. Consequently, miRNA function can potentially be affected by the number and affinity of targets in the transcriptome[75,76] and the 'sponging' activities of specific pseudogenes[77], long non-coding RNAs[78–80], circular RNAs[81,82] and transcripts of protein-coding genes[65] that have high miRNA-binding capacity. However, the extent to which competing endogenous RNAs (ceRNAs) modulate the effects of individual miRNAs is currently controversial (see REFS 83–85 for recent discussions). Susceptibility to competition by ceRNAs may mostly be limited to miRNAs that are at relatively low abundance[86], but might become influential even for abundant miRNAs in some scenarios, such as gene amplification of a target. For example, amplification of the *MYCN* gene in neuroblastomas can attenuate the tumour-suppressor function of the let-7 family of miRNAs[87]. Predicting the effects of the transcriptomic milieu on the activity of individual miRNAs remains a challenge.

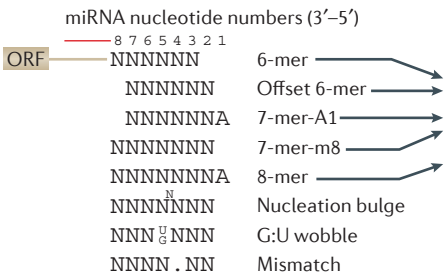
A cautionary note on ontological enrichment. Despite the widespread use and utility of ontological enrichment and pathway mapping as means to identify miRNA function on a global scale, there is a cautionary note with regard to biases that may be introduced in such analyses. These biases include spurious or incomplete assignment of gene function or the over-representation in pathways of better-studied genes[88]. Furthermore, biases in the lists of genes generally predicted to be targeted by miRNAs mean that the assumption of uniform sampling that is required to calculate statistical enrichment may not be reasonable, and it is not helpful to report enriched gene ontology terms for miRNAs of interest if an equally strong enrichment is likely to be obtained for randomly picked miRNAs[89]. To correct for this, an alternative sampling strategy has been proposed that measures enrichment against lists of predicted targets for other miRNAs[89].

Cooperative regulation by miRNAs

Multi-level targeting of cancer pathways by individual miRNAs. There are now many examples of cancer pathways that are regulated at multiple points by miRNAs (FIG. 2). The first experimental demonstration of such targeting involved the promotion of G0/G1 cell cycle arrest by miR-16 (REF. 90). The inhibition of no single gene alone (to a level of inhibition that is equivalent to that mediated by miR-16) fully re-capitulated the cell cycle arrest induced by miR-16 itself. Subsequently, network regulatory functions for other miRNAs have been found; the insulin-like growth factor (IGF), nuclear factor-κB (NF-κB), AKT kinase and receptor tyrosine kinase signalling pathways are regulated at multiple levels by miR-486 (REF. 91), miR-892b[92], miR-542-3p[93] and miR-133a[94], respectively. miR-23b regulates cytoskeletal dynamics[95] and miR-634

Box 2 | Sequence determinants of microRNA target selection

The strength of target repression strongly correlates with the length and degree of complementarity between the microRNA (miRNA) seed region (nucleotides 2–8) and the target[22,30,50,118] (BOX 1). Although the number of potential targets decreases with progressively longer classes of target site, the likelihood that such sites will bestow strong repression of the target increases (see the figure), as shown, for example, by the higher proportion of 8-mer seed match targets that are downregulated by more than twofold at the mRNA level after transient expression of miR-200a. This indicates that not only are longer seed matches more effective but also most targets are not strongly regulated, at least at the mRNA level. Many target-prediction algorithms are largely, or solely, seed-sequence dependent. Despite this, various types of sequence mismatching may be tolerated, including G:U pairing 'wobbles' (REFS 50,208,215,216), single-nucleotide mismatches and the insertion or deletion of single nucleotides in the seed, creating seed 'nucleation bulges' (REF. 53). Furthermore, interaction sites may be seed-independent, as with 'central-pairing' interactions involving nucleotides 4–15 of the miRNA[52], or may utilize additional base pairing in the 3′ regions of the miRNA (especially nucleotides 12–17) (BOX 1). These are termed supplementary or complementary sites depending on whether they function in addition to the seed or serve to strengthen imperfect seed matches[50,51]. Global target identification studies report that these 'non-canonical', imperfectly paired sites are the most abundant, although generally target suppression is weaker than for canonical seed sites, with the continuous pairing of 7–8 nucleotides[22,30,50,118]. This increases the difficulty of interpreting data when considering whether to include such sites in putative miRNA-regulated networks. To further complicate matters, the same targeting 'rules' may not be universally applicable to all miRNAs: for example, physical-interaction data obtained using crosslinking, immunoprecipitation and sequencing of hybrids (CLASH) suggest predominant central-paired and 3′ interaction sites for miR-222 and miR-92a, respectively[60].



	Total number of predicted miR-200a targets	Number of predicted targets downregulated >2× by miR-200a	Percentage of total predicted targets downregulated >2× by miR-200a
6-mer	5,407	23	0.4
7-mer	2,905	29	1.0
8-mer	726	27	3.7

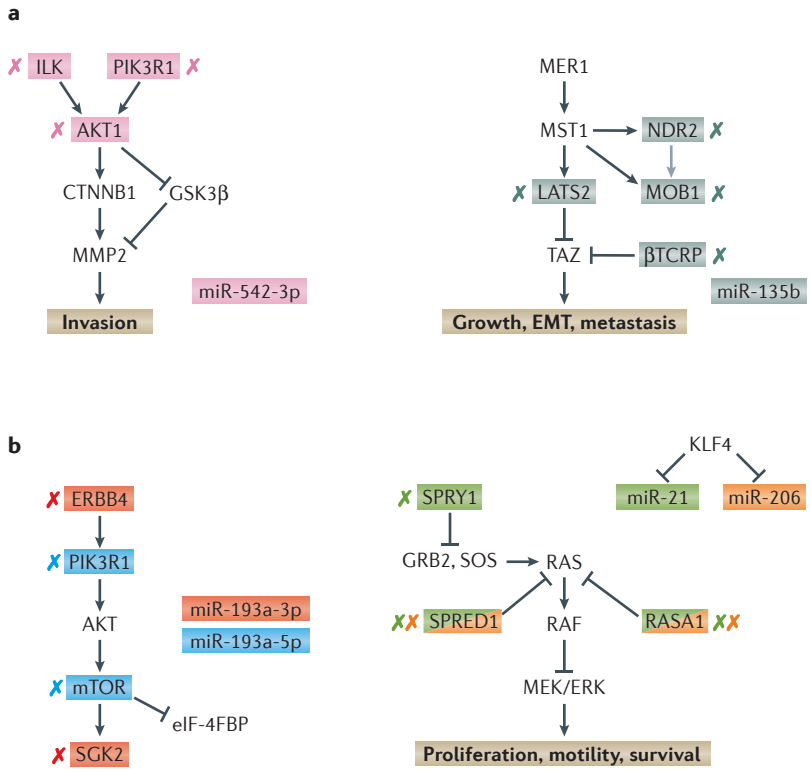


Figure 2 | **Individual and co-expressed microRNAs target multiple genes in common pathways to mediate effects. a, b** | Individual (part **a**) and multiple (part **b**) microRNAs (miRNAs) affect biological processes through multi-level regulation of common pathways. This leads to a stronger degree of regulation than would be possible through the targeting of any single gene or through the actions of any individual miRNA. EMT, epithelial–mesenchymal transition.

enhances chemotherapy-induced cytotoxicity through mitochondrial and apoptotic pathways[96]. In most of the aforementioned examples, the miRNA exerts a tumour-suppressive effect. Conversely, miR-135b was found to promote lung cancer metastasis through multi-level modulation of Hippo signalling[97].

The studies mentioned above all derived miRNA targets from *in silico* prediction, often coupled with expression analysis. As outlined above, target prediction is beset by questions of accuracy, and the incorporation of gene expression data, albeit useful, is complicated by indirect effects and the fact that miRNA targets may not necessarily be strongly regulated at the RNA level. In an effort to overcome such limitations, biochemical techniques have been developed to experimentally identify targets in a comprehensive and unbiased manner through the direct immunoprecipitation of miRNAs or RISC components (BOX 1). One such technique, the pulldown of biotinylated miRNA, was applied to find direct targets of the tumour suppressor miR-34a, revealing an enrichment of factors associated with the cell cycle and growth-factor signalling[55]. The capture of biotinylated miRNAs similarly revealed roles for miR-182-5p in the DNA damage response[56] and for miR-139-5p as a regulator of metastatic signalling[57]. Cancer regulatory roles were also found for miR-522 on the basis of its targets identified through biotinylated-miRNA pulldown[58], which were enriched for genes associated with the cell cycle, proliferation, apoptosis and the EMT-associated processes of morphology, motility and cytoskeletal organization. Importantly, knockdown of individual targets only partially recapitulated aspects of miR-522 expression, strongly suggesting that the phenotypic actions of miR-522 come about as a result of the direct regulation of multiple genes.

Our group drew a similar conclusion when investigating targets of the prominent EMT regulators miR-200a and miR-200b, both of which contribute to coordinated effects on RHO–RHO-associated protein kinase (ROCK) signalling, invadopodia formation and focal adhesions through the regulation of multiple targets[30] (FIG. 1b). Moreover, miR-200c, which has the same seed sequence as miR-200b (FIG. 1a), was found by pulldown of a biotinylated form of the miRNA to interact with an enrichment of components of the transforming growth factor-β (TGFβ) and epidermal growth factor (EGF)–mitogen-activated protein kinase (MAPK) signalling pathways and multiple components of the ZEB1 and SNAI1-containing co-repressor complexes that orchestrate a pro-mesenchymal transcriptional programme[63]. Thus, for the miR-200 family, the influence on cells is particularly multi-level, affecting high level 'master controller' transcription factors[62,98,99] and their repressor-complex partners[63], as well as having extensive effects on the networks that regulate actin cytoskeleton dynamics[30] and growth factor signalling[63].

Multi-level targeting of pathways by naturally occurring miRNA variants. Typically, miRNAs are annotated as a single defined sequence, although many recent RNA sequencing studies show that miRNAs are actually expressed as a range of naturally occurring variants, known as 'isomiRs' (REF. 100). These may arise through variable cleavage activity by Drosha and Dicer in the conventional biogenesis pathway, through the 'nibbling' activities of exonucleases, from the post-transcriptional addition of nucleotides (primarily A and U) to the 3′ termini or from the activity of RNA-editing enzymes (which occurs less frequently). Regardless of the mechanism, sequence variation can lead to differential targeting activities, particularly if this variation is located at the 5′ end of the miRNA, thereby shifting the location and sequence of the seed site.

IsomiRs are generally expressed in similar patterns to their canonical counterparts and drive similar processes[101]. Seed-shifted isomiRs, however, can target different genes, as shown by the miR-142-3p isomiRs, which coordinately regulate the actin cytoskeleton through combinations of shared and different genes in the cytoskeletal network[102]. Intriguingly, different isomiRs can also have opposing roles, as demonstrated by the differential (and probably indirect) effects of seed-shifted miR-183-5p isomiRs on the expression of such genes as *EGFR* and *NRAS*[103]. IsomiRs of miR-183-5p (and miR-375) were also identified as high-ranking hubs in the type 2 diabetes network through the prediction of miRNA targets coupled with the modelling of high-confidence protein–protein interactions[104].

Drosha
The nuclear RNase-type III enzyme in the microprocessor complex (along with DGCR8) that cleaves the precursor microRNA stem–loop from the microRNA primary transcript (pri-miRNA).

Dicer
A second RNase-type III enzyme that operates in the biogenesis pathway downstream of Drosha to cleave precursor microRNAs in the cytoplasm to generate mature microRNAs that are loaded onto Argonaute.

Co-regulation of common biological processes by multiple miRNAs. Different miRNAs can operate in miRNA 'communities', whereby cooperative effects are exerted by the convergent targeting of a common gene or pathway[105]. This is best established for miRNAs co-expressed in polycistrons[31]. These may represent either miRNAs from the same family that target the same or similar subsets of genes owing to their related seed sequences or miRNAs with different targeting specificities that nevertheless target the same pathway or process. Bioinformatic studies suggest that clustered miRNAs co-regulate genes in shared protein–protein interaction networks and that the closer the proximity of proteins in the network, the more likely they are to be targeted by miRNAs from the same cluster[44]. There are a number of experimentally validated examples whereby the coordinate actions of polycistronically encoded miRNAs regulate some aspect of cancer. For example, the miR-192–miR-194–miR-215 clusters (which are spread across two loci) coordinately supress tumour progression in renal cell carcinoma[106,107], and each member of the let7c–miR-99b–miR-125b cluster directly targets interleukin-6 receptor (IL-6R) and other components of the IL-6–signal transducer and activator of transcription 3 (STAT3) signalling pathway to decrease mammosphere growth, invasion and the metastatic spread of tumours in xenograft mouse models[108]. EMT is suppressed by the two polycistronic genes that encode the five members of the miR-200 family[62,98,99,109,110] (FIG. 1a), and by a seven-miRNA cluster that collectively targets various pro-mesenchymal transcription factors, including ZEB1, TWIST and BMI1 (REF. 111). Conversely, EMT and metastasis are promoted by the coordinated actions of miR-96, miR-182 and miR-183 (REF. 112).

A special form of 'polycistronic' production of miRNAs arises when both RNA strands produced by Dicer-mediated cleavage of the pre-miR are incorporated into RISC complexes and hence are functional. For many miRNAs, just one strand is selectively incorporated and the other is degraded, but in some cases either strand can be incorporated, in which case the miRNA name includes '-5p' or '-3p' to designate the strand, based on its location in the pre-miR. These products can have co-regulatory roles, as reported for miR-193a, for which both 5p- and 3p-derived miRNAs downregulate the oestrogen-related receptor-β (ERRB)–AKT pathway to suppress EMT and its accompanying effects on cell invasion and metastasis[113].

Perhaps the best-studied miRNA cluster is the miR-17~92 polycistron, which contains six miRNAs that represent four seed classes. Such is the degree of overexpression of this cluster in haematopoietic malignancies[114,115] and solid tumours[116,117] that it is also known as an oncomiR, specifically 'oncomiR-1'. Several studies demonstrate cooperative targeting. In one study, quantitative mass spectrometry was applied to measure protein response in a tetracycline-inducible model of miR-17~92 expression. Here, with the curious exception of miR-18, genes possessing sites for each of the other miR-17~92 family members were enriched in the pool of downregulated genes. Furthermore, ontology analysis revealed an over-representation of genes associated with TGFβ, RAS and oestrogen signalling pathways among the targets[118]. In another study, targeted deletions of single or multiple miRNAs from this cluster in mouse models indicated functionally cooperative roles[28]. For example, axial patterning was predominantly disrupted by the loss of miR-17, but further exacerbated by the additional loss of miR-18, whereas the loss of miR-19a and miR-19b impaired MYC-driven tumorigenesis. Cooperativity was further seen at the gene-expression level: the total number of genes disrupted by deleting the entire miR-17~92 cluster was far higher than the total number of genes disrupted by individual deletion of the corresponding individual miRNAs[28].

Similar cooperativity is also reported for non-polycistronic miRNAs, including a small subset of miRNAs that mediate the cell cycle re-entry of terminally differentiated myotubes in response to the *E1A* oncogene[119]. Here, cooperativity was shown by the absence of a phenotypic impact when low levels of individual miRNAs were transfected; additionally, co-transfection of multiple miRNAs (at equally low levels) facilitated a decrease in proliferation and increased differentiation. In other studies, the use of antisense inhibitors to miR-21, miR-23a and miR-27a together showed synergistic effects on reducing the proliferation of cells in culture and the growth of xenograft tumours in mice to a greater extent than the inhibition of single miRNAs alone[120]. Similarly, the transcription factor Krüppel-like factor 4 (KLF4) activates the expression of miR-21 and miR-206, both of which target RAS GTPase-activating protein 1 (RASA1) and sprouty-related EVH1 domain-containing protein 1 (SPRED1) to de-repress RAS–extracellular signal-related kinase (ERK) signalling[121] (FIG. 2). Cooperative effects of at least pairs of miRNAs on their mutual targets may represent a frequent mechanism for fine-tuning target-gene expression: a computational modelling approach has identified thousands of putative targets of such cooperative gene regulation[122].

miRNA–transcription factor co-regulation

Increasing evidence suggests that a complex interplay exists between the two largest classes of transcriptional and post-transcriptional regulators — transcription factors and miRNAs — to buffer gene expression and/or potentiate signalling. From the early days of miRNA-network biology, it was observed that predicted miRNA targets were enriched for transcriptional regulators[123,124] and that many miRNA target hubs in genetic networks are themselves transcription factors[36]. Furthermore, reciprocal feedback loops in which a miRNA and transcription factor co-regulate the expression of one another constitute a recurring network motif that occurs more often than predicted by chance[125]. Other network motifs between miRNAs and transcription factors are also common, including coherent and incoherent feedforward loops, whereby miRNAs and transcription factors regulate common targets[126,127] (FIG. 3). In coherent feedforward loops, targets are regulated in the same direction (coordinated repression) such that the activities of both the miRNA and transcription factor reinforce each

Mammosphere
Spherical structures that are formed from the clonal growth of mammary-derived cells that have stem cell-like properties.

Xenograft
Cell, tissue or organ transplant from the donor of one species into a recipient of another species.

OncomiR
A microRNA that has been functionally associated with the promotion of cancer.

Axial patterning
Control of body morphology through the actions of homeotic genes.

Network motif
Recurrent and statistically significant patterns of genetic interconnections in complex biological networks.

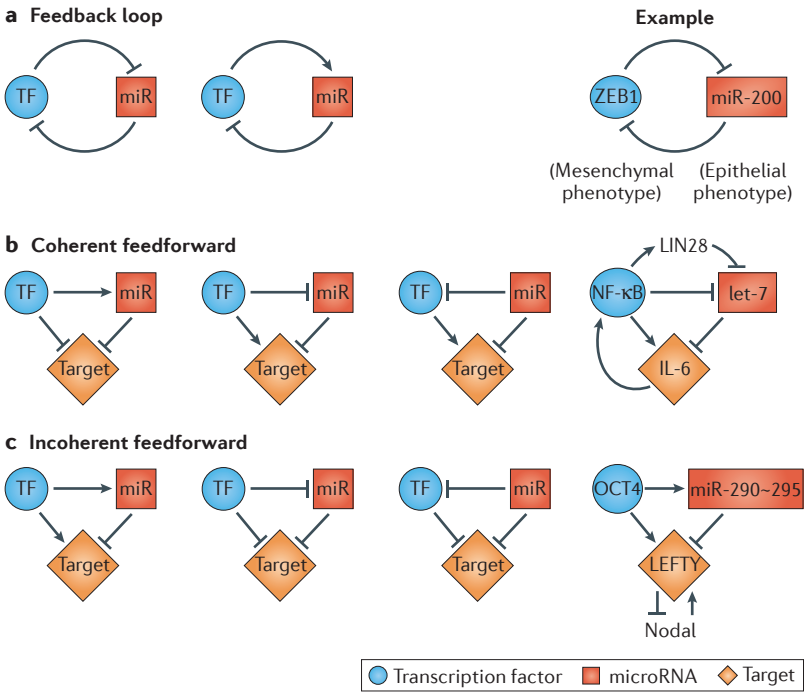


Figure 3 | **Schematic representation of common microRNA–transcription factor auto-regulatory network motifs.** Three major classes of microRNA (miRNA)-associated signalling feedback loops are represented. **a** | Direct reciprocal feedback between miRNAs and transcription factors (TFs). **b** | Coherent feedforward in which a transcription factor and miRNA regulate a target in a complementary direction (either activating or repressing). **c** | Incoherent feedforward, whereby the transcription factors and miRNAs have opposing (buffering) effects. Prominent known examples of each class are shown.

other. In incoherent feedforward loops, the miRNA and transcription factor carry out opposing functions, which enables precise modulation of gene expression to reduce noise and confer stability[15,128,129] (FIG. 3). Genes encoding transcription factors that are highly connected in the overall gene network tend to regulate miRNAs more extensively than they regulate other genes, and are themselves more likely than other genes to be regulated by these same miRNAs[130,131]. miRNAs are also more likely to regulate a pair of transcription factors if the transcription factors physically interact, further underscoring the function of miRNAs in downmodulating entire functional units[131]. Accordingly, numerous reports identify linked miRNA–transcription factor pairs in establishing and maintaining cell phenotype[109,132–139].

To elucidate the mechanisms that control cell responses, high-throughput transcriptomic data can be combined with known or predicted interactions between miRNAs, transcription factors and target genes, thereby uncovering regulatory networks in a way that would not be possible through the consideration of individual targets in isolation. In one example of this approach, chromatin immunoprecipitation followed by sequencing (ChIP–seq) experiments examining the binding sites of 119 different transcription factors were coupled with gene-expression and miRNA-target-site prediction to reveal thousands of putative miRNA–transcription factor–target interactions[131]. In a separate approach, experimental and predicted regulatory relationships drawing on 25 separate databases were combined to identify network motifs between miRNAs, transcription factors and target genes in both human and mouse[140]. In both studies, specific subtypes of feedforward motifs were enriched, which may prove useful in future for identifying the types of regulatory relationships of highest importance in biological systems.

A striking recent report describes the extent to which the influence of miRNAs is mediated not just directly through their primary targets but also indirectly through the action of the transcription factors they regulate[29]. When comparing the profiles of wild-type and Dicer-knockout fibroblasts (which are consequently depleted of most miRNAs), it was found that, as expected, the direct targets that were identified by HITS–CLIP were regulated post-transcriptionally. However, most of the overall gene expression changes after miRNA perturbation occurred at the level of transcription, with these changes being greater in both number and magnitude than post-transcriptional changes[29]. Solely characterizing direct targets therefore fails to capture the impact that miRNAs have on regulatory networks. Only effects mediated by miRNA-regulated transcription factors were considered in this study, though there remains an intriguing possibility that miRNAs themselves may also directly regulate some of these transcriptional effects in the nucleus, modulating transcription through direct binding to promoters[141,142]. Regardless of the mechanism (or mechanisms), it is clear that one must consider the transcriptional outcomes of miRNA manipulation when considering miRNA function. The propagation of signal through transcription-factor interactions also provides further explanation as to how miRNAs can have a major impact on cell behaviour, yet only modestly regulate most of their direct targets.

Mechanisms of miRNA dysregulation in cancer

The mechanisms responsible for the dysregulation of miRNAs in cancer are numerous and varied, as cancer cells seem capable of commandeering almost every step of the miRNA biogenesis pathway to promote dysregulated expression (for detailed reviews, see REFS 2,143). Many human miRNA genes are located at fragile sites or in other genomic regions that are subject to mutation, deletion, amplification or translocation in cancer[144,145]. Increased transcription of oncomeric miRNAs, such as activation of the miR-17~92 cluster by MYC[146,147], can promote cancer, as can hypermethylation of the promoters of tumour-suppressive miRNAs, which results in their aberrant epigenetic silencing[148]. Transcriptional regulators can also have surprising post-transcriptional roles. For example, receptor-regulated SMADs (R-SMADs), which are signal transducers of TGFβ and BMP signalling, associate with a sequence element in the stem loop of the miR-21 oncomiR to increase its processing by Drosha[149]. Similarly, the RNA-binding protein KH-type splicing regulatory protein (KSRP; also known as FUSE-binding protein 2) binds the terminal loop of a subset of primary miRNAs (pri-miRNAs) to promote their processing by Drosha[150]. By contrast, the Drosha processing step is inhibited by Yes-associated protein 1

HITS–CLIP
(High-throughput sequencing of RNAs isolated by crosslinking immunoprecipitation). Methodology by which Argonaute–microRNA–mRNA targets are crosslinked (by ultraviolet light) and purified (by Argonaute immunoprecipitation) to reveal microRNA targets on a global scale using high-throughput sequencing.

Fragile sites
Specific and heritable chromosomal locations that are prone to breakage on replication stress, especially in cancer.

(YAP1), a downstream product of the Hippo signalling pathway that binds and sequesters the Drosha-associated RNA helicase p72 (REF. 151).

Most typically, it is the loss of Drosha or Dicer that is implicated in a diverse range of cancers[152–159], although Drosha overexpression is associated with advanced oesophageal and cervical cancers, and Dicer is overexpressed in prostate-cancer metastasis[152]. Perhaps the most direct and convincing evidence that miRNAs have a marked functional role in cancer is the finding that germline heterozygous loss-of-function mutations in Dicer cause an autosomal-dominant familial tumour predisposition syndrome, often referred to as Dicer syndrome[152,156,160]. Given that disruption of Dicer or Drosha will affect the production of all canonically produced miRNAs, the specific miRNAs responsible for cancer progression in the context of Drosha or Dicer mutations are likely to be numerous and varied. Nonetheless, one study demonstrates a crucial role for a fibroblast growth factor 9 (FGF9)–miR-140 signalling axis in pleuropulmonary blastoma, a rare cancer that originates in the lung or plural cavity and that features cysts lined with both benign-appearing epithelium and mesenchymal cells that are susceptible to malignant transformation[156]. Yin *et al.*[161] demonstrated that miR-140, which is expressed in the lung epithelium, directly regulates the expression of FGF9, a growth factor that becomes overexpressed in the lung epithelium of mice lacking epithelial Dicer (and hence lacking miR-140) in the initial stage of pleuropulmonary blastoma. Increased levels of secreted FGF9 then promote mesenchymal-cell transformation in a cell-autonomous manner to further drive cancer progression.

It should, however, be noted that miRNA-independent roles have been described for Drosha, Dicer and the AGO proteins in the direct miRNA-independent cleavage of mRNA targets[162–165], ribosomal RNA processing[166,167] and the production of various small RNAs[168,169]. It is therefore possible that some aspects of the knockout and mutation phenotypes of these genes may also reflect such miRNA-independent functions.

Circumventing miRNA regulation in cancer

Given that miRNAs have widespread homeostatic roles, it is not surprising that cancer cells can acquire mechanisms that result in not only the dysregulation of miRNA expression but also the capacity to circumvent miRNA-mediated regulation. Proliferating cancer cells express substantial amounts of mRNA isoforms with shortened 3′ UTRs that result from alternative cleavage and polyadenylation[170,171]. The shortening of the 3′ UTR can lead to a loss of miRNA-mediated repression, increased protein production and the increased efficiency of oncogenic transformation, as proto-oncogenes with shortened 3′ UTRs are expressed at higher levels relative to their longer 3′ UTR counterparts[170]. This is likely to result in far stronger effects than individual target-site mutations, as 3′ shortening simultaneously removes multiple miRNA binding sites, which have a combinatorial effect on protein output[22]. 3′ UTR shortening of the programmed cell death 1 ligand 1 (PDL1) transcript has recently been reported to increase the survival of a diverse range of cancers through the upregulation of this immunity-suppressing protein[172]. Although avoidance of miRNA-mediated regulation was not demonstrated, it is noteworthy that the shortened 3′ UTR loses target sites for miRNAs such as miR-34 and miR-200. 3′ UTR shortening as a generalized phenomenon also occurs during T cell activation[173], neuronal activation[174] and early in embryonic development[175].

Single-nucleotide polymorphisms (SNPs) have also been extensively characterized in the genes required for miRNA biogenesis and in miRNA genes themselves, and they may affect the transcription, processing or target specificity of miRNAs[176–180]. Furthermore, correlative and causative SNPs have been identified in the 3′ UTRs of cancer-associated genes that create or disrupt miRNA binding sites to promote carcinogenesis. Mutation of the let-7 regulatory site of KRAS in non-small cell lung carcinoma[181,182], the miR-199a site of hypoxia-inducible factor 1α (HIF1A) in pancreatic ductal carcinoma[183] and the miR-367 regulatory site of ryanodine receptor 3 (RYR3)[184] in breast cancer are prominent examples of how cancers escape miRNA-mediated regulation. Evolutionarily, there is negative selection against 3′ UTR SNPs in predicted miRNA binding sites compared with the background frequency of SNPs in 3′ UTRs[185].

Concluding remarks

Although miRNAs typically only have a mild effect on individual targets, combinatorial miRNA–target networks have been shaped by evolution to produce profound effects of miRNAs on cellular properties, including their regulation of many processes whose dysregulation leads to cancer. The widespread occurrence of aberrations of miRNA expression or action in cancer can result from genetic or epigenetic changes in the miRNA genes, aberrations in their regulators or changes in their targets. Our further understanding of miRNA function rests on the successful identification of their many target genes, which has been facilitated by the development of methodologies to profile targets *en masse*. However, as the technologies to identify these targets have improved, the task of accurately modelling the interactions that exist between these genes has become ever more complex. This is especially true when one considers the close relationships between miRNAs and transcription factors: indeed, the number of gene-expression changes caused by miRNA-mediated effects on transcription factors rivals the number of changes that result from direct miRNA interaction[29]. Furthermore, many miRNAs are subject to epigenetic regulation and/or act on epigenetic regulators[186–188], with important consequences for development, cancer and other diseases. Comprehending this complexity will require transcriptome-wide and proteome-wide analyses along with network-biology and mathematical-modelling[189] approaches to the integration of data.

A move towards clinically relevant discoveries will be further aided through the integration of data from such resources as The Cancer Genome Atlas and the International Cancer Genome Consortium, which have or are currently generating staggering amounts of

mRNA, miRNA and epigenetic sequencing data derived from hundreds of cancer types and thousands of samples[190]. These data are publically available in different formats and can be analysed and accessed through numerous public portals[191,192]. They have already been used for differential expression studies, to find diagnostic and prognostic miRNA signatures, to identify targets through correlative expression with mRNAs and to identify miRNA mutations in cancer[193]. System approaches will be required to harness this wealth of data, which should lead to testable predictions of the structures of regulatory networks that miRNAs participate in, and to the identification of both miRNA and mRNA signalling hubs that may be amenable to targeting to inhibit cancer growth or progression. The use of miRNA mimics and miRNA inhibitors as therapeutics has promise but, aside from a few exceptions[194–198], still awaits the development of efficient delivery systems to become a clinical reality. However, we envision that network-based approaches will be used to guide the identification of combinations of genes for therapeutic targeting, using combinations of drugs at low doses that act with synergistic effect.

1. Lee, R. C., Feinbaum, R. L. & Ambros, V. The *C. elegans* heterochronic gene *lin-4* encodes small RNAs with antisense complementarity to *lin-14*. *Cell* **75**, 843–854 (1993).
2. Lin, S. & Gregory, R. I. MicroRNA biogenesis pathways in cancer. *Nat. Rev. Cancer* **15**, 321–333 (2015).
3. Jonas, S. & Izaurralde, E. Towards a molecular understanding of microRNA-mediated gene silencing. *Nat. Rev. Genet.* **16**, 421–433 (2015).
4. Kawamata, T. & Tomari, Y. Making, RISC. *Trends Biochem. Sci.* **35**, 368–376 (2010).
5. Lai, E. C. Micro RNAs are complementary to 3′ UTR sequence motifs that mediate negative post-transcriptional regulation. *Nat. Genet.* **30**, 363–364 (2002).
6. Lewis, B. P., Burge, C. B. & Bartel, D. P. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell* **120**, 15–20 (2005).
7. Wang, Y. *et al.* Structure of an argonaute silencing complex with a seed-containing guide DNA and target RNA duplex. *Nature* **456**, 921–926 (2008).
8. Elkayam, E. *et al.* The structure of human argonaute-2 in complex with miR-20a. *Cell* **150**, 100–110 (2012).
9. Schirle, N. T., Sheu-Gruttadauria, J. & MacRae, I. J. Structural basis for microRNA targeting. *Science.* **346**, 608–613 (2014).
10. Croce, C. M. Causes and consequences of microRNA dysregulation in cancer. *Nat. Rev. Genet.* **10**, 704–714 (2009).
11. Adams, B. D., Kasinski, A. L. & J., S. F. Aberrant regulation and function of microRNAs in cancer. *Curr. Biol.* **24**, R762–R776 (2014).
12. Lu, J. *et al.* MicroRNA expression profiles classify human cancers. *Nature* **435**, 834–838 (2005).
13. Kumar, M. S., Lu, J., Mercer, K. L., Golub, T. R. & Jacks, T. Impaired microRNA processing enhances cellular transformation and tumorigenesis. *Nat. Genet.* **39**, 673–677 (2007).
14. Brock, A., Krause, S. & Ingber, D. E. Control of cancer formation by intrinsic genetic noise and microenvironmental cues. *Nat. Rev. Cancer* **15**, 499–509 (2015).
15. Ebert, M. S. & Sharp, P. A. Roles for microRNAs in conferring robustness to biological processes. *Cell* **149**, 515–524 (2012).
16. Acar, M., Mettetal, J. T. & van Oudenaarden, A. Stochastic switching as a survival strategy in fluctuating environments. *Nat. Genet.* **40**, 471–475 (2008).
17. Çağatay, T., Turcotte, M., Elowitz, M. B., Garcia-Ojalvo, J. & Süel, G. M. Architecture-dependent noise discriminates functionally analogous differentiation circuits. *Cell* **139**, 512–522 (2009).
18. Schwarzenbach, H. Circulating nucleic acids as biomarkers in breast cancer. *Breast Cancer Res.* **15**, 211 (2013).
19. Hayes, J., Peruzzi, P. P. & Lawler, S. MicroRNAs in cancer: biomarkers, functions and therapy. *Trends Mol. Med.* **20**, 460–469 (2014).
20. Yang, D. *et al.* Integrated analyses identify a master microRNA regulatory network for the mesenchymal subtype in serous ovarian cancer. *Cancer Cell* **23**, 186–199 (2013).
This study identifies a small subset of crucial hub miRNAs that regulate a gene-expression network that defines a mesenchymal subtype associated with poor survival.
21. Shah, M. Y. & Calin, G. A. MicroRNAs as therapeutic targets in human cancers. *Wiley Interdiscip. Rev. RNA* **5**, 537–548 (2014).
22. Baek, D. *et al.* The impact of microRNAs on protein output. *Nature* **455**, 64–71 (2009).
23. Guo, H., Ingolia, N. T., Weissman, J. S. & Bartel, D. P. Mammalian microRNAs predominantly act to decrease target mRNA levels. *Nature* **466**, 835–840 (2010).
24. Eichhorn, S. W. *et al.* mRNA destabilization is the dominant effect of mammalian microRNAs by the time substantial repression ensues. *Mol. Cell* **56**, 104–115 (2014).
25. Selbach, M. *et al.* Widespread changes in protein synthesis induced by microRNAs. *Nature* **455**, 58–63 (2008).
These authors show that individual miRNAs repress hundreds of proteins, but do so at typically modest levels.
26. Uhlmann, S. *et al.* Global microRNA level regulation of EGFR-driven cell-cycle protein network in breast cancer. *Mol. Syst. Biol.* **8**, 570 (2012).
This study demonstrates that multiple miRNAs simultaneously regulate an EGF-driven network, including miRNAs that have opposing effects to balance and fine-tune responses.
27. Du, N. H., Arpat, A. B., De Matos, M. & Gatfield, D. MicroRNAs shape circadian hepatic gene expression on a transcriptome-wide scale. *eLife* **2014**, 1–29 (2014).
28. Han, Y.-C. *et al.* An allelic series of miR-17 ~ 92–mutant mice uncovers functional specialization and cooperation among members of a microRNA polycistron. *Nat. Genet.* **47**, 766–775 (2015).
An elegant demonstration of unique and cooperative roles of polycistronically encoded miRNAs, as assessed by both mouse knockout phenotype and gene expression.
29. Gosline, S. J. C. *et al.* Elucidating microRNA regulatory networks using transcriptional, post-transcriptional, and histone modification measurements. *Cell Rep.* **14**, 310–319 (2016).
This paper shows that gene-expression changes mediated by miRNA-regulated transcription factors are major contributors to the cell response to miRNA manipulation.
30. Bracken, C. P. *et al.* Genome-wide identification of miR-200 targets reveals a regulatory network controlling cell invasion. *EMBO J.* **33**, 2040–2056 (2014).
31. Chiang, H. *et al.* Mammalian microRNAs: experimental evaluation of novel and previously annotated genes. *Genes Dev.* **24**, 992–1009 (2010).
32. Grün, D., Wang, Y.-L., Langenberger, D., Gunsalus, K. C. & Rajewsky, N. microRNA target predictions across seven *Drosophila* species and comparison to mammalian targets. *PLoS Comput. Biol.* **1**, e13 (2005).
33. Tsang, J. Ebert, M. & van Oudenaarden, A. Genome-wide dissection of microRNA functions and cotargeting networks using gene set signatures. *Mol. Cell* **38**, 140–153 (2010).
These authors introduce 'miR-bridge', a computational method to identify miRNA target sites enriched for groups of genes of known function. They find that small numbers of hub miRNAs are disproportionately over-represented in co-targeting relationships.
34. Ooi, C. H. *et al.* A densely interconnected genome-wide network of microRNAs and oncogenic pathways revealed using gene expression signatures. *PLoS Genet.* **7**, e1002415 (2011).
A study showing that co-expressed miRNAs are likely to exhibit functional redundancy in targeting similar sets of downstream genes.
35. Barabási, A.-L., Gulbahce, N. & Loscalzo, J. Network medicine: a network-based approach to human disease. *Nat. Rev. Genet.* **12**, 56–68 (2011).
36. Shalgi, R., Lieber, D., Oren, M. & Pilpel, Y. Global and local architecture of the mammalian microRNA-transcription factor regulatory network. *PLoS Comput. Biol.* **3**, 1291–1304 (2007).
These authors demonstrate that miRNAs coordinately regulate key hub genes, which are often transcriptional regulators.
37. Cheng, C., Bhardwaj, N. & Gerstein, M. The relationship between the evolution of microRNA targets and the length of their UTRs. *BMC Genomics* http://dx.doi.org/10.1186/1471-2164-10-431 (2009).
38. Li, Y. *et al.* Comprehensive analysis of the functional microRNA–mRNA regulatory network identifies miRNA signatures associated with glioma malignant progression. *Nucleic Acids Res.* **41**, e203 (2013).
39. Gaidatzis, D., van Nimwegen, E., Hausser, J. & Zavolan, M. Inference of miRNA targets using evolutionary conservation and pathway analysis. *BMC Bioinformatics* **8**, 69 (2007).
40. Maragkakis, M. *et al.* DIANA-microT web server: elucidating microRNA functions through target prediction. *Nucleic Acids Res.* **37**, 273–276 (2009).
41. Shirdel, E. A., Xie, W., Mak, T. W. & Jurisica, I. NAViGaTing the micronome — using multiple microRNA prediction databases to identify signalling pathway-associated microRNAs. *PLoS ONE* **6**, e17429 (2011).
42. Liang, H. & Li, W. MicroRNA regulation of human protein — protein interaction network. *RNA* **13**, 1402–1408 (2007).
43. Hsu, C. W., Juan, H. F. & Huang, H. C. Characterization of microRNA-regulated protein-protein interaction network. *Proteomics* **8**, 1975–1979 (2008).
44. Yuan, X. *et al.* Clustered microRNAs' coordination in regulating protein-protein interaction network. *BMC Syst. Biol.* **3**, 65 (2009).
45. Sass, S. *et al.* MicroRNAs coordinately regulate protein complexes. *BMC Syst. Biol.* **5**, 136 (2011).
46. Ashburner, M. *et al.* Gene ontology: tool for the unification of biology. *Nat. Genet.* **25**, 25–29 (2000).
47. Hung, J. H., Yang, T. H., Hu, Z., Weng, Z. & DeLisi, C. Gene set enrichment analysis: performance evaluation and usage guidelines. *Brief. Bioinform.* **13**, 281–291 (2011).
48. Ritchie, W., Flamant, S. & Rasko, J. E. J. Predicting microRNA targets and functions: traps for the unwary. *Nat. Methods* **6**, 397–398 (2009).
49. Bartel, D. P. MicroRNAs: target recognition and regulatory functions. *Cell* **136**, 215–233 (2009).
50. Grimson, A. *et al.* MicroRNA targeting specificity in mammals: determinants beyond seed pairing. *Mol. Cell* **27**, 91–105 (2007).
A seminal investigation of seed and non-seed determinants of miRNA targeting efficiency.
51. Brennecke, J., Stark, A., Russell, R. B. & Cohen, S. M. Principles of microRNA-target recognition. *PLoS Biol.* **3**, 0404–0418 (2005).
52. Shin, C. *et al.* Expanding the microRNA targeting code: functional sites with centred pairing. *Mol. Cell* **38**, 789–802 (2010).
53. Chi, S. W., Hannon, G. J. & Darnell, R. B. An alternative mode of microRNA target recognition. *Nat. Struct. Mol. Biol.* **19**, 321–327 (2012).
54. Agarwal, V., Bell, G. W., Nam, J. W. & Bartel, D. P. Predicting effective microRNA target sites in mammalian mRNAs. *eLife* **4**, e05005 (2015).
This study shows that non-canonical targets, identified extensively in global miRNA–target pulldown strategies, have little functional impact.

55. Lal, A. *et al.* Capture of microRNA-bound mRNAs identifies the tumor suppressor miR-34a as a regulator of growth factor signaling. *PLoS Genet.* **7**, 19–21 (2011).
A study demonstrating that miR-34a targets an extensive network of genes associated with cell cycle progression and growth factor signalling both directly and indirectly.
56. Krishnan, K. *et al.* MicroRNA-182-5p targets a network of genes involved in DNA repair. *RNA* **19**, 230–242 (2013).
57. Krishnan, K. *et al.* miR-139-5p is a regulator of metastatic pathways in breast cancer. *RNA* **19**, 1767–1780 (2013).
58. Tan, S. M. *et al.* Sequencing of captive target transcripts identifies the network of regulated genes and functions of primate-specific miR-522. *Cell Rep.* **8**, 1225–1239 (2014).
These authors show that pulldown of miR-522 targets reveals networks of genes underlying epithelial–mesenchymal transition. Knockdown of individual genes only partially replicated the effect of miR-522, indicating the importance of network targeting by the miRNA.
59. Wang, X. Composition of seed sequence is a major determinant of microRNA targeting patterns. *Bioinformatics* **30**, 1377–1383 (2014).
60. Helwak, A., Kudla, G., Dudnakova, T. & Tollervey, D. Mapping the human miRNA interactome by CLASH reveals frequent noncanonical binding. *Cell* **153**, 654–665 (2013).
61. Hafner, M. *et al.* Transcriptome wide identification of RNA binding protein and microRNA target sites by PAR-CLIP. *Cell* **141**, 129–141 (2010).
62. Gregory, P. A. *et al.* The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1. *Nat. Cell Biol.* **10**, 593–601 (2008).
63. Perdigão-Henriques, R. *et al.* miR-200 promotes the mesenchymal to epithelial transition by suppressing multiple members of the Zeb2 and Snail1 transcriptional repressor complexes. *Oncogene* **35**, 158–172 (2016).
64. Lee, Y. S. & Dutta, A. The tumor suppressor microRNA *let-7* represses the HMGA2 oncogene. *Genes Dev.* **21**, 1025–1030 (2007).
65. Mayr, C., Hemann, M. T. & Bartel, D. P. Disrupting the pairing between *let-7* and *Hmga2* enhances oncogenic transformation. *Science.* **315**, 1576–1579 (2007).
66. Liu, Z. *et al. MiR-182* overexpression in tumourigenesis of high-grade serous ovarian carcinoma. *J. Pathol.* **228**, 204–215 (2012).
67. Hirata, H. *et al.* Oncogenic miRNA-182-5p targets Smad4 and RECK in human bladder cancer. *PLoS ONE* **7**, 1–8 (2012).
68. Sun, Y. *et al.* Hsa-mir-182 suppresses lung tumorigenesis through down regulation of RGS17 expression *in vitro. Biochem. Biophys. Res. Commun.* **396**, 501–507 (2010).
69. Ma, Y. *et al.* Elevated oncofoetal miR-17-5p expression regulates colorectal cancer progression by repressing its target gene *P130*. *Nat. Commun.* **3**, 1291 (2012).
70. Shan, S. W. *et al.* Mature miR-17-5p and passenger miR-17-3p induce hepatocellular carcinoma by targeting PTEN, GalNT7 and vimentin in different signal pathways. *J. Cell Sci.* **126**, 1517–1530 (2013).
71. Wei, Q., Li, Y.-X., Liu, M., Li, X. & Tang, H. MiR-17-5p targets TP53INP1 and regulates cell proliferation and apoptosis of cervical cancer cells. *IUBMB Life* **64**, 697–704 (2012).
72. Korpal, M. *et al.* Direct targeting of Sec23a by miR-200s influences cancer cell secretome and promotes metastatic colonization. *Nat. Med.* **17**, 1101–1108 (2011).
73. Gibbons, D. L. *et al.* Contextual extracellular cues promote tumor cell EMT and metastasis by regulating miR-200 family expression. *Genes Dev.* **23**, 2140–2151 (2009).
74. Li, X. *et al.* MiR-200 can repress breast cancer metastasis through ZEB1-independent but moesin-dependent pathways. *Oncogene* **33**, 4077–4088 (2014).
75. Arvey, A., Larsson, E., Sander, C., Leslie, C. S. & Marks, D. S. Target mRNA abundance dilutes microRNA and siRNA activity. *Mol. Syst. Biol.* **6**, 363 (2010).
76. Garcia, D. M. *et al.* Weak seed-pairing stability and high target-site abundance decreases the proficiency of *isy-6* and other miRNA's. *Nat. Struct. Mol. Biol.* **18**, 1139–1146 (2011).
77. Poliseno, L., Salmeda, J., Zhang, L., Haveman, W. & Pandolfi, P. A coding-independent function of gene and pseudogene mRNAs regulates tumour biology. *Nature* **465**, 1033–1038 (2010).
78. Wang, Y. *et al.* Endogenous miRNA sponge lincRNA-RoR regulates Oct4, Nanog, and Sox2 in human embryonic stem cell self-renewal. *Dev. Cell* **25**, 69–80 (2013).
79. Du, Z. *et al.* Integrative analyses reveal a long noncoding RNA-mediated sponge regulatory network in prostate cancer. *Nat. Commun.* **7**, 10982 (2016).
80. Paci, P., Colombo, T. & Farina, L. Computational analysis identifies a sponge interaction network between long non-coding RNAs and messenger RNAs in human breast cancer. *BMC Syst. Biol.* **8**, 83 (2014).
81. Hansen, T. B. *et al.* Natural RNA circles function as efficient microRNA sponges. *Nature* **495**, 384–388 (2013).
82. Memczak, S. *et al.* Circular RNAs are a large class of animal RNAs with regulatory potency. *Nature* **495**, 333–338 (2013).
83. Thomson, D. W. & Dinger, M. E. Endogenous microRNA sponges: evidence and controversy. *Nat. Rev. Genet.* **17**, 272–283 (2016).
84. Ebert, M. S. & Sharp, P. A. Emerging roles for natural microRNA sponges. *Curr. Biol.* **20**, R858–R861 (2014).
85. Denzler, R., Agarwal, V., Stefano, J., Bartel, D. & Stoffel, M. Assessing the ceRNA hypothesis with quantitative measurements of miRNA and target abundance. *Mol. Cell* **54**, 766–776 (2015).
86. Bosson, A. D., Zamudio, J. R. & Sharp, P. A. Endogenous miRNA and target concentrations determine susceptibility to potential ceRNA competition. *Mol. Cell* **56**, 347–359 (2014).
87. Powers, J. T. *et al.* Multiple mechanisms disrupt the *let-7* microRNA family in neuroblastoma. *Nature* **535**, 246–251 (2016).
88. Godard, P. & van Eyll, J. Pathway analysis from lists of microRNAs: common pitfalls and alternative strategy. *Nucleic Acids Res.* **43**, 3490–3497 (2015).
89. Bleazard, T., Lamb, J. A. & Griffiths-Jones, S. Bias in microRNA functional enrichment analysis. *Bioinformatics* **31**, 1592–1598 (2014).
90. Linsley, P. S. *et al.* Transcripts targeted by the microRNA-16 family cooperatively regulate cell cycle progression. *Mol. Cell. Biol.* **27**, 2240–2252 (2007).
91. Peng, Y. *et al.* Insulin growth factor signaling is regulated by microRNA-486, an underexpressed microRNA in lung cancer. *Proc. Natl Acad. Sci. USA* **110**, 15043–15048 (2013).
92. Jiang, L. *et al.* miR-892b silencing activates NF-κB and promotes aggressiveness in breast cancer. *Cancer Res.* **76**, 1101–1112 (2016).
93. Cai, J. *et al.* MicroRNA-542-3p suppresses tumor cell invasion via targeting AKT pathway in human astrocytoma. *J. Biol. Chem.* **290**, 24678–24688 (2015).
94. Wang, L. K. *et al.* MicroRNA-133a suppresses multiple oncogenic membrane receptors and cell invasion in non-small cell lung carcinoma. *PLoS ONE* **9**, e96765 (2014).
95. Pellegrino, L. *et al.* miR-23b regulates cytoskeletal remodeling, motility and metastasis by directly targeting multiple transcripts. *Nucleic Acids Res.* **41**, 5400–5412 (2013).
96. Fujiwara, N. *et al. miR-634* activates the mitochondrial apoptosis pathway and enhances chemotherapy-induced cytotoxicity. *Cancer Res.* **75**, 3890–3901 (2015).
97. Lin, C. W. *et al.* MicroRNA-135b promotes lung cancer metastasis by regulating multiple targets in the Hippo pathway and LZTS1. *Nat. Commun.* **4**, 1877 (2013).
98. Park, S. M., Gaur, A. B., Lengyel, E. & Peter, M. E. The miR-200 family determines the epithelial phenotype of cancer cells by targeting the E-cadherin repressors ZEB1 and ZEB2. *Genes Dev.* **22**, 894–907 (2008).
99. Burk, U. *et al.* A reciprocal repression between ZEB1 and members of the miR-200 family promotes EMT and invasion in cancer cells. *EMBO Rep.* **9**, 582–589 (2008).
100. Neilsen, C. T., Goodall, G. J. & Bracken, C. P. IsomiRs — the overlooked repertoire in the dynamic microRNAome. *Trends Genet.* **28**, 544–549 (2012).
101. Cloonan, N. *et al.* MicroRNAs and their isomiRs function cooperatively to target common biological pathways. *Genome Biol.* **12**, R126 (2011).
102. Manzano, M., Forte, E., Raja, A. N., Schipma, M. J. & Gottwein, E. Divergent target recognition by coexpressed 5′-isomiRs of miR-142-3p and selective viral mimicry. *RNA* **21**, 1606–1620 (2015).
103. Telonis, A. G., Loher, P., Jing, Y., Londin, E. & Rigoutsos, I. Beyond the one-locus-one-miRNA paradigm: microRNA isoforms enable deeper insights into breast cancer heterogeneity. *Nucleic Acids Res.* **43**, 9158–9175 (2015).
104. Baran-Gale, J., Fannin, E. E., Kurtz, C. L. & Sethupathy, P. Beta cell 5′-shifted isomiRs are candidate regulatory hubs in type 2 diabetes. *PLoS ONE* **8**, e73240 (2013).
105. Gennarino, V. A. *et al.* Identification of microRNA-regulated gene networks by expression analysis of target genes. *Genome Res.* **22**, 1163–1172 (2012).
106. Khella, H. W. Z. *et al.* mir-192, mir-194 and mir-215: a convergent microRNA network suppressing tumor progression in renal cell carcinoma. *Carcinogenesis* **34**, 2231–2239 (2013).
107. Senanayake, U. *et al.* miR-192, miR-194, miR-215, miR-200c and miR-141 are downregulated and their common target ACVR2B is strongly expressed in renal childhood neoplasms. *Carcinogenesis* **33**, 1014–1021 (2012).
108. Lin, K. *et al.* Genome-wide screen identified let-7c/miR-99a/miR-125b regulating tumor progression and stem-like properties in cholangiocarcinoma. *Oncogene* **35**, 3376–3386 (2016).
109. Bracken, C. P. *et al.* A double-negative feedback loop between ZEB1-SIP1 and the microRNA-200 family regulates epithelial-mesenchymal transition. *Cancer Res.* **68**, 7846–7854 (2008).
110. Korpal, M., Lee, E. S., Hu, G. & Kang, Y. The miR-200 family inhibits transition and cancer cell migration by direct targeting of E-cadherin transcriptional repressors *ZEB1* and *ZEB2*. *J. Biol. Chem.* **283**, 14910–14914 (2008).
111. Haga, C. L. & Phinney, D. G. MicroRNAs in the imprinted *DLK1-DIO3* region repress the epithelial-to-mesenchymal transition by targeting the TWIST1 protein signaling network. *J. Biol. Chem.* **287**, 42695–42707 (2012).
112. Zhang, W. *et al.* Autocrine/paracrine human growth hormone-stimulated microRNA 96-182-183 cluster promotes epithelial-mesenchymal transition and invasion in breast cancer. *J. Biol. Chem.* **290**, 13812–13829 (2015).
113. Yu, T. *et al. MicroRNA-193a-3p* and *-5p* suppress the metastasis of human non-small-cell lung cancer by downregulating the ERBB4/PIK3R3/mTOR/S6K2 signaling pathway. *Oncogene* **34**, 413–423 (2014).
114. Ota, A. Identification and characterization of a novel gene, *C13orf25*, as a target for 13q31-q32 amplification in malignant lymphoma. *Cancer Res.* **64**, 3087–3095 (2004).
115. He, L. *et al.* A microRNA polycistron as a potential human oncogene. *Nature* **435**, 828–833 (2005).
116. Hayashita, Y. *et al.* A polycistronic microRNA cluster, *miR-17-92*, is overexpressed in human lung cancers and enhances cell proliferation. *Cancer Res.* **65**, 9628–9632 (2005).
117. Lanza, G. *et al.* mRNA/microRNA gene expression profile in microsatellite unstable colorectal cancer. *Mol. Cancer* **6**, 54 (2007).
118. Mestdagh, P. *et al.* The miR-17-92 microRNA cluster regulates multiple components of the TGF-β pathway in neuroblastoma. *Mol. Cell* **40**, 762–773 (2010).
119. Marzi, M. J. *et al.* Differentiation-associated microRNAs antagonize the Rb-E2F pathway to restrict proliferation. *J. Cell Biol.* **199**, 77–95 (2012).
120. Frampton, A. E. *et al.* MicroRNAs cooperatively inhibit a network of tumor suppressor genes to promote pancreatic tumor growth and progression. *Gastroenterology* **146**, 268–277 (2014).
This study demonstrates the synergistic activity of multiple miRNAs in cancer progression through the use of antisense miRNA inhibitors.
121. Sharma, S. B. *et al.* MicroRNAs 206 and 21 cooperate to promote RAS-extracellular signal-regulated kinase signaling by suppressing the translation of *RASA1* and *SPRED1*. *Mol. Cell. Biol.* **34**, 4143–4164 (2014).
122. Schmitz, U. *et al.* Cooperative gene regulation by microRNA pairs and their identification using a computational workflow. *Nucleic Acids Res.* **42**, 7539–7552 (2014).
123. Lewis, B. P., Shih, I.-H., Jones-Rhoades, M. W. & Bartel, D. P. Prediction of mammalian microRNA targets. *Cell* **115**, 787–798 (2003).
124. Cui, Q., Yu, Z., Purisima, E. O. & Wang, E. Principles of microRNA regulation of a human cellular signaling network. *Mol. Syst. Biol.* **2**, 46 (2006).

125. Martinez, N. *et al.* A *C. elegans* genome-scale microRNA network contains composite feedback motifs with high flux-capacity. *Genes Dev.* **22**, 2535–2549 (2008).
126. Re, A., Cora, D., Taverna, D. & Caselle, M. Genome-wide survey of microRNA-transcription factor feed-forward regulatory circuits in human. *Mol. Biosyst.* **5**, 854–867 (2009).
127. Friard, O., Re, A., Taverna, D., De Bortoli, M. & Corá, D. CircuitsDB: a database of mixed microRNA/transcription factor feed-forward regulatory circuits in human and mouse. *BMC Bioinformatics* **11**, 435 (2010).
128. Tsang, J., Zhu, J. & van Oudenaarden, A. MicroRNA-mediated feedback and feedforward loops are recurrent network motifs in mammals. *Mol. Cell* **26**, 753–767 (2007).
129. Gurtan, A. M. & Sharp, P. A. The role of miRNAs in regulating gene expression networks. *J. Mol. Biol.* **425**, 3582–3600 (2013).
130. Su, W. L., Kleinhanz, R. R. & Schadt, E. E. Characterizing the role of miRNAs within gene regulatory networks using integrative genomics techniques. *Mol. Syst. Biol.* **7**, 490 (2011).
131. Gerstein, M. *et al.* Architecture of the human regulatory network derived from ENCODE data. *Nature* **489**, 91–100 (2012).
This assessment of relationships between transcription factors and miRNAs from large-scale genomic data reveals extensive co-regulation and structures of regulatory network motifs.
132. Ben-Ami, O., Pencovich, N., Lotem, J., Levanon, D. & Groner, Y. A regulatory interplay between miR-27a and Runx1 during megakaryopoiesis. *Proc. Natl Acad. Sci. USA* **106**, 238–243 (2009).
133. Lu, L. *et al.* A novel YY1-miR-1 regulatory circuit in skeletal myogenesis revealed by genome-wide prediction of YY1-miRNA network. *PLoS ONE* **7**, e27596 (2012).
134. Li, Q.-Q. *et al.* Involvement of NF-κB/miR-448 regulatory feedback loop in chemotherapy-induced epithelial-mesenchymal transition of breast cancer cells. *Cell Death Differ.* **18**, 16–25 (2011).
135. Weng, W. *et al.* YY1-C/EBPα-miR34a regulatory circuitry is involved in renal cell carcinoma progression. *Oncol. Rep.* **31**, 1921–1927 (2014).
136. Liu, J. J. *et al.* A novel AP-1/miR-101 regulatory feedback loop and its implication in the migration and invasion of hepatoma cells. *Nucleic Acids Res.* **42**, 12041–12051 (2014).
137. Kim, N. H. *et al.* A p53/miRNA-34 axis regulates Snail1-dependent cancer cell epithelial-mesenchymal transition. *J. Cell Biol.* **195**, 417–433 (2011).
138. Siemens, H. *et al.* miR-34 and SNAIL form a double-negative feedback loop to regulate epithelial-mesenchymal transitions. *Cell Cycle* **10**, 4256–4271 (2011).
139. Aguda, B. D., Kim, Y., Piper-Hunter, M. G., Friedman, A. & Marsh, C. B. MicroRNA regulation of a cancer network: consequences of the feedback loops involving miR-17-92, E2F, and Myc. *Proc. Natl Acad. Sci. USA* **105**, 19678–19683 (2008).
140. Liu, Z., Wu, C., Miao, H. & Wu, H. RegNetwork: an integrated database of transcriptional and post-transcriptional regulatory networks in human and mouse. *Database* http://dx.doi.org/10.1093/database/bav095 (2015).
141. Salmanidis, M., Pillman, K., Goodall, G. & Bracken, C. Direct transcriptional regulation by nuclear microRNAs. *Int. J. Biochem. Cell Biol.* **54**, 304–311 (2014).
142. Kalantari, R., Chiang, C. & Corey, D. R. Regulation of mammalian transcription and splicing by nuclear RNAi. *Nucleic Acids Res.* **44**, 524–537 (2016).
143. Hata, A. & Lieberman, J. Dysregulation of microRNA biogenesis and gene silencing in cancer. *Sci. Signal.* **8**, re3 (2015).
144. Calin, G. A. *et al.* Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers. *Proc. Natl Acad. Sci. USA* **101**, 2999–3004 (2004).
145. Zhang, L. *et al.* microRNAs exhibit high frequency genomic alterations in human cancer. *Proc. Natl Acad. Sci. USA* **103**, 9136–9141 (2006).
146. O'Donnell, K. A., Wentzel, E. A., Zeller, K. I., Dang, C. V. & Mendell, J. T. c-Myc-regulated microRNAs modulate E2F1 expression. *Nature* **435**, 839–843 (2005).
147. Dews, M. *et al.* Augmentation of tumor angiogenesis by a Myc-activated microRNA cluster. *Nat. Genet.* **38**, 1060–1065 (2006).
148. Lujambio, A. *et al.* A microRNA DNA methylation signature for human cancer metastasis. *Proc. Natl Acad. Sci. USA* **105**, 13556–13561 (2008).
149. Davis, B. N., Hilyard, A. C., Nguyen, P. H., Lagna, G. & Hata, A. Smad proteins bind a conserved RNA sequence to promote microRNA maturation by Drosha. *Mol. Cell* **39**, 373–384 (2010).
150. Trabucchi, M. *et al.* The RNA-binding protein KSRP promotes the biogenesis of a subset of miRNAs. *Nat. Genet.* **459**, 1010–1014 (2009).
151. Mori, M. *et al.* Hippo signaling regulates microprocessor and links cell density-dependent miRNA biogenesis to cancer. *Cell* **156**, 893–906 (2015).
152. Foulkes, W. D., Priest, J. R. & Duchaine, T. F. DICER1: mutations, microRNAs and mechanisms. *Nat. Rev. Cancer* **14**, 662–672 (2014).
153. Doros, L. *et al. DICER1* mutations in embryonal rhabdomyosarcomas from children with and without familial PPB-tumor predisposition syndrome. *Pediatr. Blood Cancer* **59**, 558–560 (2012).
154. Tomiak, E., de Kock, L., Grynspan, D., Ramphal, R. & Foulkes, W. D. *DICER1* mutations in an adolescent with cervical embryonal rhabdomyosarcoma (cERMS). *Pediatr. Blood Cancer* **61**, 568–569 (2014).
155. Rakheja, D. *et al.* Somatic mutations in *DROSHA* and *DICER1* impair microRNA biogenesis through distinct mechanisms in Wilms tumors. *Nat. Commun.* **2**, 4802 (2015).
156. Hill, D. A. *et al. DICER1* mutations in familial pleuropulmonary blastoma. *Science.* **325**, 965 (2009).
157. Torrezan, G. T. *et al.* Recurrent somatic mutation in *DROSHA* induces microRNA profile changes in Wilms tumour. *Nat. Commun.* **5**, 4039 (2014).
158. Karube, Y. *et al.* Reduced expression of *Dicer* associated with poor prognosis in lung cancer patients. *Cancer Sci.* **96**, 111–115 (2005).
159. Merritt, W. M. *et al.* Dicer, Drosha, and outcomes in patients with ovarian cancer. *N. Engl. J. Med.* **359**, 2641–2650 (2008).
160. Dehner, L. P. *et al.* Pleuropulmonary blastoma: evolution of an entity as an entry into a familial tumor predisposition syndrome. *Pediatr. Dev. Pathol.* **18**, 504–511 (2015).
161. Yin, Y. *et al.* Fibroblast growth factor 9 regulation by microRNAs controls lung development and links *DICER1* loss to the pathogenesis of pleuropulmonary blastoma. *PLoS Genet.* **11**, e1005242 (2015).
162. Han, J. *et al.* Posttranscriptional crossregulation between Drosha and DGCR8. *Cell* **136**, 75–84 (2009).
163. Pinder, B. D. & Smibert, C. A. MicroRNA-independent recruitment of Argonaute 1 to *nanos* mRNA through the Smaug RNA-binding protein. *EMBO Rep.* **14**, 80–86 (2012).
164. Knuckles, P. *et al.* Drosha regulates neurogenesis by controlling Neurogenin 2 expression independent of microRNAs. *Nat. Neurosci.* **15**, 962–969 (2012).
165. Kaneko, H. *et al.* DICER1 deficit induces *Alu* RNA toxicity in age-related macular degeneration. *Nature* **471**, 325–332 (2011).
166. Wu, H., Xu, H., Miraglia, L. J. & Crooke, S. T. Human RNase III is a 160-kDa protein involved in preribosomal RNA processing. *J. Biol. Chem.* **275**, 36957–36965 (2000).
167. Oskowitz, A. Z., Penfornis, P., Tucker, A., Prockop, D. J. & Pochampally, R. Drosha regulates hMSCs cell cycle progression through a miRNA independent mechanism. *Int. J. Biochem. Cell Biol.* **43**, 1563–1572 (2011).
168. Johanson, T. M., Lew, A. M. & Chong, M. M. W. MicroRNA-independent roles of the RNase III enzymes Drosha and Dicer. *Open Biol.* **3**, 130144 (2013).
169. Chong, M. M. W. *et al.* Canonical and alternate functions of the microRNA biogenesis machinery. *Genes Dev.* **24**, 1951–1960 (2010).
170. Mayr, C. & Bartel, D. P. Widespread shortening of 3′UTRs by alternative cleavage and polyadenylation activates oncogenes in cancer cells. *Cell* **138**, 673–684 (2010).
This study shows that mRNAs escape miRNA regulation through progressive 3′ UTR shortening during cancer progression.
171. Lai, D.-P. *et al.* Genome-wide profiling of polyadenylation sites reveals a link between selective polyadenylation and cancer metastasis. *Hum. Mol. Genet.* **24**, 3410–3417 (2015).
172. Kataoka, K. *et al.* Aberrant *PD-L1* expression through 3′-UTR disruption in multiple cancers. *Nature* **534**, 402–406 (2016).
173. Sandberg, R., Neilson, J. R., Sarma, A., Sharp, P. A. & Burge, C. B. Proliferating cells express mRNAs with shortened 3′UTRs and fewer microRNA target sites. *Science.* **320**, 1643–1647 (2008).
174. Flavell, S. W. *et al.* Genome-wide analysis of MEF2 transcriptional program reveals synaptic target genes and neuronal activity-dependent polyadenylation site selection. *Neuron* **60**, 1022–1038 (2008).
175. Ji, Z., Lee, J. Y., Pan, Z., Jiang, B. & Tian, B. Progressive lengthening of 3′ untranslated regions of mRNAs by alternative polyadenylation during mouse embryonic development. *Proc. Natl Acad. Sci. USA* **106**, 7028–7033 (2009).
176. Iawi, N. & Naraba, H. Polymorphisms in human pre-miRNAs. *Biochem. Biophys. Res. Commun.* **331**, 1439–1444 (2005).
177. Saunders, M. a, Liang, H. & Li, W.-H. Human polymorphism at microRNAs and microRNA target sites. *Proc. Natl Acad. Sci. USA* **104**, 3300–3305 (2007).
178. Duan, R., Pak, C. H. & Jin, P. Single nucleotide polymorphism associated with mature miR-125a alters the processing of pri-miRNA. *Hum. Mol. Genet.* **16**, 1124–1131 (2007).
179. Dzikiewicz-Krawczyk, A. MicroRNA polymorphisms as markers of risk, prognosis and treatment response in hematological malignancies. *Crit. Rev. Oncol. Hematol.* **93**, 1–17 (2015).
180. Wojcicka, A., de la Chapelle, A. & Jazdzewski, K. MicroRNA-related sequence variations in human cancers. *Hum. Genet.* **133**, 463–469 (2014).
181. Chin, L. J. *et al.* A SNP in a *let-7* microRNA complementary site in the *KRAS* 3′ untranslated region increases non-small cell lung cancer risk. *Cancer Res.* **68**, 8535–8540 (2008).
182. Kundu, S. T. *et al.* KRAS alleles: the LCS6 3′UTR variant and KRAS coding sequence mutations in the NCI-60 panel. *Cell Cycle* **11**, 361–366 (2012).
183. Wang, X. *et al.* Single nucleotide polymorphism in the microRNA-199a binding site of HIF1A gene is associated with pancreatic ductal adenocarcinoma risk and worse clinical outcomes. *Oncotarget* **7**, 13717–13729 (2016).
184. Zhang, L. *et al.* Functional SNP in the microRNA-367 binding site in the 3′UTR of the calcium channel ryanodine receptor gene 3 (*RYR3*) affects breast cancer risk and calcification. *Proc. Natl Acad. Sci. USA* **108**, 13653–13658 (2011).
185. Yu, Z. *et al.* Aberrant allele frequencies of the SNPs located in microRNA target sites are potentially associated with human cancers. *Nucleic Acids Res.* **35**, 4535–4541 (2007).
186. Sato, F., Tsuchiya, S., Meltzer, S. J. & Shimizu, K. MicroRNAs and epigenetics. *FEBS Lett.* **278**, 1598–1609 (2011).
187. Gruber, A. J. & Zavolan, M. Modulation of epigenetic regulators and cell fate decisions by miRNAs. *Epigenomics* **5**, 671–683 (2013).
188. Piletič, K. & Kunej, T. MicroRNA epigenetic signatures in human disease. *Arch. Toxicol.* http://dx.doi.org/10.1007/s00204-016-1815-7 (2016).
189. Lai, X., Wolkenhauer, O. & Vera, J. Understanding microRNA-mediated gene regulatory networks through mathematical modelling. *Nucleic Acids Res.* **44**, 6019–6035 (2016).
190. Chu, A. *et al.* Large-scale profiling of microRNAs for The Cancer Genome Atlas. *Nucleic Acids Res.* **44**, e3 (2016).
191. Klonowska, K., Czubak, K. & Wojciechowska, M. Oncogenomic portals for the visualization and analysis of genome-wide cancer data. *Oncotarget* **7**, 176–192 (2016).
192. Plass, C., Pfister, S. M., Lindroth, A. M. & Bogatyrova, O. Mutations in regulators of the epigenome and their connections to global chromatin patterns in cancer. *Nat. Rev. Genet.* **14**, 765–780 (2013).
193. Hezaveh, K. *et al.* Alterations of miRNAs and miRNA-regulated mRNA expression in GC B cell lymphomas determined by integrative sequencing analysis. *Haematologica* http://dx.doi.org/10.3324/HAEMATOL.2016.143891 (2016).
194. Kasinski, A. L. & Slack, F. J. MicroRNAs en route to the clinic: progress in validating and targeting microRNAs for cancer therapy Andrea. *Nat. Rev. Cancer* **11**, 849–864 (2011).
195. Kasinski, A. L. *et al.* A combinatorial microRNA therapeutics approach to suppressing non-small cell lung cancer. *Oncogene* **34**, 3547–3555 (2015).
196. Janssen, H. L. *et al.* Treatment of HCV infection by targeting microRNA. *N. Engl. J. Med.* **368**, 1685–1694 (2013).

197. Lanford, R. E. *et al.* Therapeutic silencing of microRNA-122 in primates with chronic hepatitis C virus infection. *Science.* **327**, 198–201 (2012).
198. Monroig-Bosque, P. D. C., Rivera, C. A. & Calin, G. A. MicroRNAs in cancer therapeutics: "from the bench to the bedside". *Expert Opin. Biol. Ther.* **15**, 1381–1385 (2015).
199. Hausser, J. & Zavolan, M. Identification and consequences of miRNA-target interactions — beyond repression of gene expression. *Nat. Rev. Genet.* **15**, 599–612 (2014).
200. Thomson, D. W., Bracken, C. P. & Goodall, G. J. Experimental strategies for microRNA target identification. *Nucleic Acids Res.* **39**, 6845–6853 (2011).
201. Darnell, R. B. HITS-CLIP: panoramic views of protein-RNA regulation in living cells. *Wiley Interdiscip. Rev. RNA* **1**, 266–286 (2010).
202. Friedman, R. C., Farh, K. K. H., Burge, C. B. & Bartel, D. P. Most mammalian mRNAs are conserved targets of microRNAs. *Genome Res.* **19**, 92–105 (2009).
203. Paraskevopoulou, M. D. *et al.* DIANA-LncBase: experimentally verified and computationally predicted microRNA targets on long non-coding RNAs. *Nucleic Acids Res.* **41**, 239–245 (2013).
204. Betel, D., Koppal, A., Agius, P., Sander, C. & Leslie, C. Comprehensive modeling of microRNA targets predicts functional non-conserved and non-canonical sites. *Genome Biol.* **11**, R90 (2010).
205. Wong, N. & Wang, X. miRDB: an online resource for microRNA target prediction and functional annotations. *Nucleic Acids Res.* **43**, D146–D152 (2015).
206. Bandyopadhyay, S. & Mitra, R. TargetMiner: microRNA target prediction with systematic identification of tissue-specific negative examples. *Bioinformatics* **25**, 2625–2631 (2009).
207. Krek, A. *et al.* Combinatorial microRNA target predictions. *Nat. Genet.* **37**, 495–500 (2005).
208. Miranda, K. C. *et al.* A pattern-based method for the identification of microRNA binding sites and their corresponding heteroduplexes. *Cell* **126**, 1203–1217 (2006).
209. Boutz, D. R. *et al.* Two-tiered approach identifies a network of cancer and liver disease-related genes regulated by miR-122. *J. Biol. Chem.* **286**, 18066–18078 (2011).
210. Leivonen, S.-K. *et al.* Protein lysate microarray analysis to identify microRNAs regulating estrogen receptor signaling in breast cancer cell lines. *Oncogene* **28**, 3926–3936 (2009).
211. Hendrickson, D. G. *et al.* Concordant regulation of translation and mRNA abundance for hundreds of targets of a human microRNA. *PLoS Biol.* **7**, 25–29 (2009).
212. Chi, S. W., Zang, J. B., Mele, A. & Darnell, R. B. Argonaute HITS–CLIP decodes microRNA–mRNA interaction maps. *Nature* **460**, 479–486 (2009).
213. Orom, U. & Lund, A. Isolation of microRNA targets using biotinylated synthetic microRNAs. *Methods* **43**, 162–165 (2007).
214. Hunter, S. E. *et al.* Functional genomic analysis of the *let-7* regulatory network in *Caenorhabditis elegans*. *PLoS Genet.* **9**, e1003353 (2013).
215. Didiano, D. & Hobert, O. Perfect seed pairing is not a generally reliable predictor for miRNA-target interactions. *Nat. Struct. Mol. Biol.* **13**, 849–851 (2006).
216. Doench, J. G. & Sharp, P. A. Specificity of microRNA target selection in translational repression. *Genes (Basel)* **504**, 504–511 (2004).
217. Yang, J. H. *et al.* StarBase: a database for exploring microRNA–mRNA interaction maps from Argonaute CLIP-seq and Degradome-seq data. *Nucleic Acids Res.* **39**, 202–209 (2011).
218. Kanehisa, M., Sato, Y., Kanwashima, M., Furumichi, M. & Tanabe, M., KEGG as a reference resource for gene and protein annotation. *Nucleic Acids Res.* **44**, D457–D462 (2016).
219. Kanehisa, M. & Goto, S. KEGG: Kyoto Encyclopedia of Genes and Genomes. *Nucleic Acids Res.* **28**, 27–30 (2000).
220. Steinfeld, I., Navon, R., Ach, R. & Yakhini, Z. miRNA target enrichment analysis reveals directly active miRNAs in health and disease. *Nucleic Acids Res.* **41**, e45 (2013).
221. Vlachos, I. S. *et al.* DIANA miRPath v.2.0: investigating the combinatorial effect of microRNAs in pathways. *Nucleic Acids Res.* **40**, 498–504 (2012).
222. Laczny, C. *et al.* miRTrail — a comprehensive webserver for analyzing gene and miRNA patterns to enhance the understanding of regulatory mechanisms in diseases. *BMC Bioinformatics* **13**, 36 (2012).
223. Jung, D. *et al.* miRTarVis: an interactive visual analysis tool for microRNA–mRNA expression profile data. *BMC Proc.* **9**, S2 (2015).
224. Fan, Y. *et al.* miRNet — dissecting miRNA-target interactions and functional associations through network-based visual analysis. *Nucleic Acids Res.* **1**, W135–W141 (2016).
225. Yang, K., Hsu, C., Lin, C., Juan, H. & Huang, H. Mirin: identifying microRNA regulatory modules in protein–protein interaction networks. *Bioinformatics* **30**, 2527–2528 (2014).
226. Sales, G. *et al.* MAGIA, a web-based tool for miRNA and genes integrated analysis. *Nucleic Acids Res.* **38**, 352–359 (2010).
227. Backes, C., Khaleeq, Q. T., Meese, E. & Keller, A. miEAA: microRNA enrichment analysis and annotation. *Nucleic Acids Res.* **44**, 110–116 (2016).
228. Lu, M., Shi, B., Wang, J., Cao, Q. & Cui, Q. TAM: a method for enrichment and depletion analysis of a microRNA category in a list of microRNAs. *Bioinformatics* **11**, 419 (2010).
229. Lu, T. *et al.* miRSystem: an integrated system for characterizing enriched functions and pathways of microRNA targets. *PLoS ONE* **7**, e42390 (2012).
230. Wu, X. & Watson, M. CORNA: testing gene lists for regulation by microRNAs. *Bioinformatics* **25**, 832–833 (2009).
231. Nam, S. *et al.* MicroRNA and mRNA Integrated Analysis (MMIA): a web tool for examining biological functions of microRNA expression. *Nucleic Acids Res.* **37**, 356–362 (2009).
232. Ulitsky, I., Laurent, L. C. & Shamir, R. Towards computational prediction of microRNA function and activity. *Nucleic Acids Res.* **38**, e160 (2010).
233. Corapcioglu, M., E. & Hasan, O. BioSystems miSEA: microRNA set enrichment analysis. *Biosystems* **134**, 37–42 (2015).

Acknowledgements

C.P.B. is supported by a Florey Fellowship from the Royal Adelaide Hospital Research Foundation, and H.S.S. and G.J.G. are supported by fellowships from the Australian National Health and Medical Research Council (GNT1023059 and GNT1026191). C.P.B., H.S.S. and G.J.G. acknowledge grant funding from the Australian National Health and Medical Research Council (GNT1034633 and GNT1069128 to G.J.G. and C.P.B., and GNT1068773 to G.J.G.) and the Australian National Breast Cancer Foundation.

Competing interests statement

The authors declare no competing interests.

DATABASES

miRBase: http://www.mirbase.org

ALL LINKS ARE ACTIVE IN THE ONLINE PDF