# The role of microRNAs in cancer biology and therapy from a systems biology perspective

Xin Lai[1,2,#], Ulf Schmitz[3], Julio Vera[1,2,#]

[1] Laboratory of Systems Tumor Immunology, Department of Dermatology, Friedrich-Alexander-Universität Erlangen-Nürnberg (FAU) and Universitätsklinikum Erlangen, Erlangen, Germany

[2] Deutsches Zentrum Immuntherapie and Comprehensive Cancer Center Erlangen-EMN, Erlangen, Germany

[3] Department of Molecular and Cell Biology, College of Public Health, Medical and Veterinary Sciences, James Cook University, Townsville, Australia

[#]Corresponding Author: xin.lai@uk-erlangen.de; julio.vera-gonzalez@uk-erlangen.de

## Abstract

Since the discovery of microRNA (miRNA) in Caenorhabditis elegans, our understanding of their cellular function has progressed continuously. Today, we have a good understanding of miRNA-mediated gene regulation, miRNA-mediated crosstalk between genes including competing endogenous RNAs, and miRNA-mediated signaling transduction both in normal human physiology and in diseases.

Besides, these non-coding RNAs have shown their value for clinical applications, especially in an oncological context. They can be used as reliable biomarkers for cancer diagnosis and prognosis and attract increasing attention as potential therapeutic targets. Many achievements made in the miRNA field are based on joint efforts from computational and molecular biologists. Systems biology approaches, which integrate computational and experimental methods, have played a fundamental role in uncovering the cellular functions of miRNAs.

In this chapter, we review and discuss the role of miRNAs in oncology from a system biology perspective. We first describe biological facts about miRNA genetics and function. Next, we discuss the role of miRNAs in cancer progression and review the application of

miRNAs in cancer diagnostics and therapy. Finally, we elaborate on the role that miRNAs play in cancer gene regulatory networks. Taken together, we emphasize the importance of systems biology approaches in our continued efforts to study miRNA cancer regulation.

**Keywords:** mRNA destabilization, translation repression, mathematical modelling, network biology, bioinformatics, post-transcriptional gene regulation, oncomir, tumour suppressor miRNA, metastamir, circulating miRNA

## 1 Biological facts about miRNA biogenesis and function

MicroRNAs (miRNAs) are a class of small endogenous non-coding RNAs with a length of around 22 nucleotides (nt). miRNAs are evolutionarily conserved regulatory molecules that, in most cases, modulate the stability and/or translation of target mRNAs through direct binding to the 3' UTR of their target mRNAs (1). miRNAs were first found to be pivotal for Caenorhabditis elegans development (2) and it was soon demonstrated that they play a key role in gene expression regulation in both animals and plants. More recently, there is mounting evidence suggesting that miRNAs and other similar non-coding RNAs are also important in viral and bacterial gene regulation, as well as in the microbe-mediated host gene regulation. Taken together, miRNAs are ubiquitous post-transcriptional regulators of gene expression important in normal cell physiology and function (3).

To date over 2,500 miRNA sequences have been identified in the human genome and registered in the miRBase database (4). These miRNAs are estimated to regulate more than half of all protein-coding genes (5). This indicates their pervasive roles in the regulation of cellular processes, like proliferation, differentiation, and apoptosis. In addition to exerting critical functions during normal development and cellular homeostasis, miRNA dysregulation has been found in many human diseases, like cancer (6). Thus, understanding the function of miRNAs in gene regulation is crucial for unraveling mechanisms underlying human pathogenesis and improving therapeutic approaches in human diseases.

### 1.1 miRNA biogenesis

The miRNA biogenesis pathway is a complex process composed of multiple steps (Figure 1) (7–9). At first, long primary transcripts known as primary miRNAs (pri-miRNAs) are transcribed from miRNA genes by RNA polymerase II (Pol II). pri-miRNA molecules have a 5′-terminal 7-methylguanosine ($m^7G$) cap, which is extended by a hairpin structure with a terminal loop and a ~32 nt long imperfectly base-paired stem and end with a 3' poly(A) tail. Depending on the features of miRNA genes, pri-miRNAs can contain single or multiple miRNA pairs that form hairpin structures. Next, with the help of the complex that includes Drosha and its binding partner DGCR8, pri-miRNAs are processed into

precursor miRNAs (pre-miRNAs), which are ~70 nt long hairpin structures with a characteristic 2 nt 3' overhang. Then, through the recognition of the 2 nt overhangs, exportin 5 in conjunction with the cofactor Ran-GTP exports pre-miRNAs from the nucleus into the cytoplasm. After that, cytoplasmic processing by another complex, which is composed of Dicer, an Argonaute protein (AGO), and a TAR RNA binding protein (TRBP), cleaves the pre-miRNA into a ~22 nt double-stranded miRNA duplex (also known as mature miRNAs). Finally, one strand of the miRNA duplex known as the active strand is loaded into the AGO-containing miRNA-induced silencing complex (miRISC) that will bind to miRNA-specific target mRNAs for subsequent cleavage or translation repression. The complementary strand of the miRNA duplex, known as the passenger strand, will be degraded.

In addition to the canonical miRNA biogenesis pathway described above, mature and functional miRNAs can also be produced via alternative pathways. These pathways can be classified into Drosha- and Dicer-independent pathways (Figure 1)(10). In the Drosha-independent pathway a class of miRNA genes, which originates from pre-miRNA-sized short introns (termed as mirtrons), can be directly processed into pre-miRNA hairpins without the participation of Drosha. These pre-miRNAs are further cleaved by Dicer in the cytoplasm to produce mature miRNAs (11). In the Dicer-independent pathway, following normal nuclear processing, the pre-miRNA is not cleaved into a miRNA duplex by Dicer but instead by the AGO catalytic center. For example, miR-451 is produced through an AGO-dependent maturation pathway (10).

### 1.2 miRNA function

After the maturation of miRNAs, in most cases the active strands act as guides and direct miRISCs to bind to the 3' UTR of target mRNAs, resulting in the repression of target genes at the post-transcriptional level (Figure 2). Some miRNAs can exert a repressive function on target genes even when their binding sites are located in the 5' UTR or the coding regions of target mRNAs (12). In addition, a few miRNAs can bind to the 5' UTR of their target mRNA and enhance its translation (13). The mechanism by which target mRNAs are regulated is determined by the degree of complementarity between miRNAs and their target mRNAs. When a miRNA perfectly or near-perfectly pairs with its target mRNA,

mostly occurring in plants, target mRNA cleavage is triggered. Imperfect base pairing between a miRNA and its target, predominating in animals, leads to translation repression or destabilization of the target mRNA (1). Based on experimental evidence and bioinformatics analyses in animals, several miRNA seed binding motifs have been identified including 8-mer, 7-mer and 6-mer seed binding (14). These miRNA binding motifs are defined by the number of continuous base pairings in the seed region of miRNAs; for example, 7-mer means that in the seed region of a miRNA there are 7 continuous base pairings between the miRNA and its target mRNA (14). The repression efficiency exerted via these binding motifs can be further enhanced by additional base pairing between the 3' complementarity region of the miRNA and its target (Figure 2)(8). The following subsequences can influence target regulation efficiency: (i) The seed region (miRNA nucleotides 2-8). A continuous seed region base pairing (miRNA nucleotides 2-8) is crucial for assuring effective target repression. If there are G-U pairs (guanine-uracil) or mismatches in this region, the target repression will be greatly affected. However, the appearance of an A (adenine) at position 1 of the miRNA and an A or U appearing at position 9 can improve the repressive efficiency, although they are not required to base pair with the target mRNA. (ii) The central region (miRNA nucleotides 10-12). In this region, bulges or mismatches must be present. (iii) The complementary region (miRNA nucleotides 13 to last). The base pairing between the miRNA and its target mRNA is typically quite loose in this region. Thus, good complementarity, particularly for miRNA nucleotides 13-16, becomes important when mismatches or bulges appear in the seed region.

In addition to the binding motifs, other factors can also affect miRNA repression efficiency. For example, multiple miRNA binding sites in close proximity in the 3' UTR of a single mRNA can enhance the repression of the target (15, 16). RNA-binding proteins (RBPs), which can interact with miRISCs on the 3' UTR of target mRNAs, can either facilitate or counteract miRNA-mediated repression (9).

In mammalian cells, most miRNA-target interactions are based on imperfect base pairing, which can result in two main mechanisms by which miRNAs reduce protein production. The two mechanisms are translation repression and destabilization of the target mRNAs. More particularly, miRNAs can inhibit the translation of target mRNAs by affecting the

initiation or post-initiation stage of mRNA translation (17). At the initiation stage, the miRISC can inhibit translation by interfering with eIF4E-cap recognition and recruitment of 40S small ribosomal subunit or by antagonizing 60S subunit joining and preventing the formation of 80S ribosomal complex (Figure 3A). At the post-initiation stage, the miRISC can inhibit translation by blocking ribosome elongation, inducing ribosome drop-off, or facilitating proteolysis of the nascent polypeptides (Figure 3B). For miRNA-mediated mRNA degradation, with the participation of GW182 and PABP the miRISC induces deadenylation of the poly (A) tail by interacting with the CCR4-NOT deadenylase complex. Then, the 5' terminal $m^7G$ cap is removed by the DCP1-DCP2 decapping complex, resulting in the degradation of the target mRNA (Figure 3C).

What we described above can be considered the “standard” mechanism of gene regulation by miRNAs. However, recent experimental studies indicate that miRNAs can translocate into the nucleus, interact with gene promoters and activate expression for given target genes (18). Xiao et al. found that nuclear miR-24-1 can activate gene transcription in a mechanism that involves interaction with enhancers and enhancer RNAs. This indicates that in the future, once we elucidate the mechanisms of interaction between miRNAs, RNA binding proteins, and long non-coding RNAs (lncRNAs), we may see more cases of alternative miRNA target regulation.

## 2 The role of miRNAs in cancer progression, diagnosis, and therapy

In the previous section, we discussed the genetics and molecular mechanisms associated with miRNA biogenesis and function. The large amounts of experimental evidence collected in the last 20 years show that miRNAs participate in the regulation and fine-tuning of crucial processes that drive cellular phenotypes and functions during cell development and repairing and in tissue homeostasis (19, 20). Since cancer cells hijack cell differentiation programs to regain phenotypes that foster their progression, it is not surprising that many miRNAs are associated with the pathogenesis and progression of cancer. In recent years, researchers have investigated the phenomenon to search for new, accurate diagnostic tools based on miRNA expression profiling in cancer patients. Furthermore, there are miRNA-based therapies under development, which promote more targeted and personalized cancer therapies. In the following paragraphs, we discuss the

molecular mechanisms linking miRNAs to cancer pathophysiology and the use of miRNAs in cancer diagnostics and therapy.

### 2.1 Genome-level alterations in miRNAs

As indicated before, miRNAs play a key role in shaping and fine-tuning the gene regulatory circuits controlling tissue development and cell differentiation. Programs controlling these phenotypes are hijacked by cancer cells allowing them to become invasive, metastatic, and therapy-resistant. Hence, one can find mechanisms by which miRNA expression and function are distorted in cancer cells similar to those that cause dysregulation of protein-coding genes.

**Alteration in miRNA gene copy number and gene location.** Comprehensive genome analyses using computational and experimental approaches have identified a large number of miRNA genes that are located in fragile and unstable chromosomal regions linked to cancer. One can find miRNA genes within or close to cancer-associated amplified, deleted, or translocated genes, but also close to chromosomal breakpoints. For example, Soh and co-workers analyzed genomic data from more than 2,000 tumor samples of The Cancer Genome Atlas (TCGA) cohort representing seven prevalent cancer types and found that up to 85% of miRNA genes are located in cancer type-specific genomic regions enriched in somatic copy number alterations (SCNAs) (21). Czubak et al. investigated SCNAs of 14 miRNA genes commonly deregulated cancer and found that most of them displayed copy number alterations in lung cancer (22).

**Alterations in miRNA biogenesis and transcriptional regulation.** Global miRNA depletion caused by epigenetic or genetic alterations in miRNA biogenesis components is oncogenic (23). For example, DICER1 seems to operate as a haploinsufficient tumor suppressor. Kumar et al. showed that deleting a single copy of DICER1 in tumors from animal models reduced survival (24). Others made similar observations in cell lines and mouse models of several aggressive cancers (25). While reduced expression of DICER1 has been associated with various cancers it is not a general pattern and therefore, it is difficult to generalize the role of DICER repression or silencing in cancer (26). This can also be found for other proteins involved in the miRNA biogenesis pathway, which are considered either tumor suppressors or oncogenes for different tumors (27). For example,

DROSHA and DGCR8 both critical components of the miRNA biogenesis pathway, bear recurrent mutations in some cancers (28).

The expression of many miRNAs is also controlled by transcription factors (TFs) and cofactors, some of which are commonly deregulated in cancer. Consequently, miRNAs regulated by those TFs experience cancer-type specific alterations in their expression patterns. The literature contains many studies in which the expression of miRNAs is controlled by TFs that are deregulated or mutated in cancer. To mention a few, there is the tumor suppressor p53 which regulates the expression of the miR-34 family, the translational repressor ZEB1 regulating miR-200 family expression, tumor suppressor p73 regulating miR-205 expression, or the proto-oncogene c-Myc regulating the miR-17~92 cluster. In all these cases, evidence shows that cancer-associated deregulation of these TFs induces abnormal expression of the target miRNAs, which in turn promote post-transcriptional repression of genes linked to key cancer phenotypes, such as cell proliferation, (anti)apoptosis, or migration. In one particular case, the TF p73 promotes the expression of miR-205, a miRNA involved in the repression of several anti-apoptotic members of the BCL2 family. Deregulation of p73 expression in cancer downregulates miR-205, which in turn induces an increased level of anti-apoptotic BCL2 (29, 30). In such a scenario, the cells initiate apoptosis under DNA damage and become resistant to genotoxic drugs.

There are several interesting points here. Firstly, deregulation of these TF can be induced in different ways, e.g., via somatic mutations, overexpression, or alternative splicing. The latter, for example, causes the expression of the anti-apoptotic DNp73 splice isoform instead of wild-type p73. Secondly, some TFs such as p53 or p73 can upregulate the expression of miRNAs, and others can repress miRNA expression like ZEB1 represses the miR-200 family. Finally, in many cases, miRNAs and TFs are part of feedback and feedforward loops becoming deregulated in cancer. For example, miR-205 establishes a negative feedback loop with E2F1 and p73 leading to therapy resistance in malignant melanoma (29). Taken together, the interplay between miRNAs and TFs is a complex multifactorial mechanism, whose features and consequences will be discussed in more detail in **section 3**.

**Modification and deregulation of miRNA-mRNA interactions**. Not surprisingly, the binding between miRNAs and their target mRNAs can also be distorted in cancer. Binding can be altered through genetic changes in target genes such as somatic point mutations or translocations. A point mutation within the canonical seed-matching sequence of the mRNA 3′ UTR could create a novel miRNA target site but it could also impair an existing target site (31). For example, a point mutation located in the 3′ UTR of the p53 inhibitor MDM4 (rs4245739 SNP, A>C) is associated with an increased risk of prostate cancer. Bioinformatics analysis indicated that this SNP resides within a predicted binding site for miR-191-5p, miR-887, and miR-3669. Stegeman and coworkers investigated these predictions utilizing gene assays and demonstrated that miR-191-5p and miR-887 have a specific affinity for the rs4245739 SNP C-allele in prostate cancer. When targeting MDM4 with miR-191-5p or miR-887 in prostate cancer cell lines they observed decreased cell viability (32). There are other mechanisms altering the molecular structure of miRNA-mRNA binding sites, including chromosomal translocations that eliminate given miRNA binding sites from the 3′ UTR of their mRNA targets (33), or alternative polyadenylation, which can shorten or lengthen a gene's 3′ UTR and thereby erase or add miRNA binding sites, respectively (34).

### 2.2 Oncogenic and tumor-suppressive miRNAs

In the previous sections, we have discussed miRNA biogenesis and target repression mechanisms and we have elucidated mechanisms by which miRNAs can become deregulated in cancer. Now, the actual role that given miRNAs play in cancer will depend largely on two factors: (i) whether they are up-or down-regulated, and (ii) the function of their gene targets.

Oncogenic miRNAs, also known as oncomirs, are miRNAs that repress genes with a known role as a tumor suppressor. For example, miR-125b has a binding site in the 3′ UTR of the tumor suppressor TP53. It has been found that miR-125b overexpression reduces the endogenous levels of TP53 and hampers the activation of apoptosis in several cancers (35). Of note are also miRNAs that repress genes that inhibit or down-regulate oncogenic pathways. This is the case for miR-663, which represses the expression of CDKN1A, a gene that encodes the cell cycle regulator p21. The protein can

induce cell cycle arrest, however, miR-663-mediated suppression of p21 promotes cancer cell proliferation and tumor progression in nasopharyngeal carcinoma and other cancers (36).

Other miRNAs described as tumor suppressors target oncogenes. A well-known example is the role of the let-7 family in melanoma. These miRNAs suppress melanoma proliferation and metastasis by targeting a range of genes including ITGB3, an integrin-linked to the acquisition of invasiveness (37). Such kinds of miRNAs can also target inhibitors of tumor suppressors. A well-known case is miR-34a which represses SIRT1 expression. SIRT1 is an oncogene that would normally repress TP53 activation. It has been shown that miR-34a mediated repression of SIRT1 increases TP53 acetylation and hence the expression of TP53 target genes (such as CDKN1A and PUMA) that regulate cell cycle and apoptosis (38). More recently, it has been shown that miRNAs also play a role in regulating cancer therapy efficiency and resistance to chemotherapy. For example, Alla and coworkers found that DNp73-dependent downregulation of miR-205 induces drug resistance by upregulating anti-apoptotic BCL2 and ABC transporters (30).

Taken together, miRNAs play a crucial role in cancer through the inhibition of tumor suppressors or oncogenes. Interestingly, since miRNAs can have multiple targets, some miRNAs play contradictory roles in different tumor entities or even within same cancer. For example, miR-146a can promote melanoma cell growth by targeting NUMB, a repressor of the NOTCH signaling pathway (39), but can also suppress metastasis formation by downregulating the expression of ITGAV and ROCK1 (40).

### 2.3 miRNAs in cancer diagnostics and therapy

In the last decades, a lot of work has been carried out to find means to use miRNAs for primary or co-adjuvant therapies but also to identify biomarkers to predict disease outcomes or resistance to therapy.

**miRNAs as diagnostic signatures**. Due to the lack of sufficient specificity and sensitivity of classical tumor biomarkers, researchers have been looking for alternative candidates for cancer diagnosis. A good alternative should be minimally invasive and cost-effective. Profiling of circulating miRNAs from liquid biopsies has been found to be a good means to identify tumor-derived molecules secreted into the bloodstream. These miRNAs are

good candidates for biomarkers because they are chemically stable and resistant to RNase activity (41) and are thus a valuable source for the diagnosis and stratification of cancer subtypes (42). A paradigmatic example is a work by van Laar et al. (43). The authors utilized the Nanostring nCounter system to perform extensive profiling and quantification of miRNAs in plasma samples from melanoma patients and healthy controls. After analyzing the data, they identified 38 miRNAs that were differentially expressed between melanoma and healthy plasma samples. Interestingly, most of these miRNAs regulate protein-coding genes linked to angiogenesis, metastasis or therapy resistance, including miR-34a and miR-205 that were discussed in the previous section. To test the prediction accuracy of this miRNA signature, they trained a machine-learning model and validated it using additional independently published datasets. The results indicated a high classification performance (with an area under the ROC curve value of 0.94). Blood profiling of miRNAs is theoretically applicable for any tumor types, and in some specific tumor entities, it is also possible to profile miRNAs in urine (44) or feces (45) for diagnostic purposes.

**miRNA-based therapy.** The increasing knowledge about the roles of miRNAs in the pathogenesis, progression, and dissemination of tumors makes them attractive targets for cancer therapeutic approaches. As indicated above, miRNAs can contribute to cancer progression by acting as either oncogenes or tumor suppressors. This informs the design of miRNA-based therapies of which there are two different approaches:

(i) If the aim is the inhibition of oncogmirs, one can utilize RNA antagonists, such as antisense oligonucleotides, antagomirs, or miRNA sponges. miRNA antagonists designed with sequences complementary to oncomirs prevent them from entering into AGO, thereby avoiding the inhibition of their tumor suppressor gene targets. For example, miR-146a negatively regulates immune activation by repressing STAT1 and the STAT1-dependent secretion of Interferon-γ. miR-146a levels have been found to be increased in the microenvironment of aggressive melanoma. Mastroianni and coworkers combined an anti-mir for miR-146a and anti-PD1 therapy in a melanoma mouse model and found improved survival when comparing with both isotype-control or anti-PD-1 treatment alone (46).

(ii) If the treatment aims to replace depleted tumor suppressor miRNAs, one can employ miRNA mimics, like miRNA expression vectors and synthetic double-strand miRNAs. miRNA mimics can restore the diminished or lost function of tumor suppressor miRNAs whose downregulation results in the activation of oncogenes or pathways. As discussed earlier, miR-205 is a tumor suppressor and is downregulated in several aggressive tumors including melanoma. Noguchi and coworkers developed a chemically modified synthetic miRNA-205 with the ability to inhibit melanoma growth and progression, which they could demonstrate both *in vitro* and *in vivo*. They found that the synthetic miRNA can downregulate the expression of known miR-205 targets such as E2F1 and VEGF, and repress the anti-apoptotic gene BCL2.

As discussed in the previous section, a miRNA has a multitude of targets, and therefore we have to consider the potential off-target effects caused by any miRNA therapies (47, 48). Furthermore, similar to any other RNA-based therapies, a key challenge for miRNA therapeutics is the development of efficient delivery systems that facilitate a safe and effective application, which is a field under active investigation (49, 50). However, the wide use of mRNA vaccines for protecting us from SARS-CoV-2 has laid the foundation for inventing successful carriers for therapeutic RNAs (51).

## 3 miRNAs in cancer gene regulatory networks

We have discussed the molecular mechanisms for miRNA (de)regulation and function in cancer. We can apprehend the complexity we face when trying to understand and exploit the therapeutic role of miRNAs in cancer. In the following, we introduce and discuss some miRNA-related phenomena that complicate miRNA-gene regulatory circuits in cancer.

### 3.1 miRNA clusters: groups of similarly regulated miRNAs

A miRNA cluster is a group of miRNA genes residing in close proximity in the genome (52). To consider a group of miRNAs as a cluster they have to (a) be transcribed in the same orientation, (b) they are not separated by another transcriptional unit, or (c) a miRNA on the opposite strand. There are approximately 160 miRNA clusters in the human genome. Most of the miRNA clusters are composed of two or three individual miRNAs, though larger ones are possible and often contain miRNA sets with important

regulatory functions. For example, the miR-17-92 cluster contains 6 miRNA genes that reside in an intron of a 7 kb long non-coding RNA known as the MiR-17-92a-1 Cluster Host Gene or *MIR17HG* for short (Figure 5A). Members of this cluster are the miRNAs miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1, and miR-92-1. The cluster can be activated by the TF c-Myc, a well-known oncogene that is often constitutively expressed in cancer and is primarily, but not only, linked to abnormal cell proliferation. Thus, the cluster plays an important role in many cancers. One of its most relevant targets is the tumor suppressor and cell cycle regulator PTEN, which is commonly suppressed in cancer (53).

### 3.2 Target hubs: genes regulated by many miRNAs

miRNA target hubs are genes that are regulated by 10 or more miRNAs, that is, they contain *bona fide* binding sites in their 3′ UTR for ten or more different miRNAs (54). Shalgi and coworkers utilized a computational approach to detect miRNA target hubs in the human genome and found 470 genes potentially regulated by at least 15 different miRNAs (55). Since the number of miRNAs detected has almost duplicated since 2007, one can expect to find many more miRNA target hubs. Since one miRNA alone often induces only mild repression of its gene target, multiple miRNAs with the same target can induce stronger repression when acting in a concerted manner. The first example of a miRNA target hub detected and experimentally investigated is the cell cycle regulator CDKN1A (Figure 5B). This gene can induce cell cycle arrest under normal conditions and in response to DNA damage and is therefore considered a tumor suppressor gene. Interestingly, through bioinformatics analysis researchers detected several hundreds of binding sites for different miRNAs in the CDKN1A 3′ UTR (56). Wu and collaborators proved that at least 28 of these miRNAs can repress the gene *in vitro* (57). Interestingly, eight of these 28 miRNAs originate from the chromosome 19 miRNA cluster, which is known to promote cancer proliferation and is linked to aggressive tumors (58). Lai and coworkers developed a mathematical model of CDKN1A regulation and simulated the concerted inhibition of CDKN1A during the cell cycle, DNA damage, cell cycle arrest, senescence, and apoptosis (56).

### 3.3 miRNA cooperativity: synergistic gene regulation by multiple miRNAs

Two research teams have independently confirmed that miRNAs pairs with binding sites that are in close proximity in a mutual target gene can show cooperative behavior. In other words, the effect of their combined repression is higher than the sum of the individual effects. Sætrom and coworkers experimentally determined the optimal distance between miRNA binding sites facilitating miRNA cooperativity. The optimal range is for the seed sites to be 13-35 nt apart. Based on this criterion Lai and collaborators identified multiple pairs of putatively cooperating miRNAs in the 3′ UTR of the miRNA target hub CDKN1A (59). They then validated the cooperative repression of CDKN1A exerted by miR-572 and miR-93 using a luciferase reporter system as well as immunoblotting. When they extended the computational analysis to the whole human genome, they identified thousands of putatively coopering miRNA pairs and their mutual target genes (60). Since then, other groups have confirmed cooperative miRNA regulation in other genes, some of which are related to cancer (61, 62). The possibility that miRNAs act in a cooperative manner has consequences for miRNA-based therapies. Utilizing this synergistic effect in a miRNA replacement therapy would reduce the overall miRNA concentration required to effectively diminish the target gene expression and thereby reduce off-target effects (48). Lai et al. explored this idea and investigated the therapeutic use of cooperative miR-205-5p and miR-342-3p in the repression of their mutual target E2F1 in the context of cancer chemoresistance (63). Their computational model-driven analysis was confirmed by *in vitro* functional experiments. Most recently, it is shown that the biochemical basis of miRNA cooperativity is regulated by TNRC6. The presence of the AGO-binding region in TNRC6 prevents disassociation of miRISCs from closely spaced target sites on mRNA and therefore improving their binding affinities (64) (Figure 4).

### 3.4 Network motifs: miRNA-enriched feedback and feedforward loops

A network motif is a regulatory structure involving several genes, which recurringly appears in large biochemical networks (65, 66). One can consider miRNA target hubs as a type of network motif, but the term was coined for feedback and feedforward loops. It is known for a long time that some central genes, especially TFs, are integrated into multiple instances of these loops and hence it is not a surprise to find a similar occurrence for

miRNAs, their TFs, and targets. Here, we discuss some examples of network motifs and their role in cancer biology.

**Feedback loops.** In feedback loops, the regulation between molecules forms a closed loop that allows state changing or self-regulating of a system. A **positive feedback loop** often induces signal amplification or sustained system (de)activation. We have found a myriad of feedback loops distorted in cancer, which integrated oncogenes and oncomirs. A well-studied case of a positive feedback loop in cancer is the one established by p53 and miR-34a with the mediation of the oncogene SIRT1 (SIRT1 ⊣ p53 → miR-34a ⊣ SIRT1). SIRT1 is overexpressed in several tumors, including melanoma, and through this circuit, it can impair the p53-mediated DNA damage and anti-proliferative response (67, 68). miR-34a is considered a tumor suppressor and happens to be downregulated in some cancers (69). We also find an abundance of positive feedback loops involving cytokines, their signaling pathways, and downstream TFs, which play a central role in amplifying and (de)regulating the immune response in the tumor microenvironment (70). A special form of a positive feedback loop is called a toggle switch. For example, the mutual repression of a TF and its miRNA target can become a toggle switch. These motifs can display a nonlinear regulation named all-or-nothing. Specifically, the expression of one of the components represses the other in a sustained manner. The well-known and investigated case is the one established by ZEB1 and the miR-200 family (Figure 5C), which plays a pivotal role in the abnormal epithelial to mesenchymal transition in cancer (71). A **negative feedback loop** often induces the quick cessation of signaling like the NF-kB pathway (72, 73). They can also induce homeostasis and hence are employed to fine-tune signaling and gene expression and maintain levels of activity of their components against noise and fluctuation (74, 75). The Ras/Raf/MEK/ERK pathway is a well-known example of a pathway with multiple negative feedback loops which control cell proliferation and can get distorted in cancer (76). Not surprisingly, in recent years several research groups have found miRNAs that regulate the Ras/Raf/MEK/ERK pathway creating negative feedback loops and thereby suppressing cell growth and invasion. However, these miRNAs are often downregulated in cancer (77, 78). There are other more sophisticated means of distortion in miRNA-mediated negative feedback loops. This is the case for the E2F1-p73/DNp73-miR-205 circuit. miR-205 represses E2F1 and

is simultaneously activated by p73 a target of E2F1, therefore forming a negative feedback loop (Figure 5D) (29). However, in some cancers, there is a shift towards an alternative splice isoform of p73 named DNp73, which represses miR-205 and amplifies E2F1 expression. This is often observed in aggressive tumors, such as malignant melanoma and lung adenocarcinoma, and can lead to the development of metastasis (29)(37).

**Feedforward loops.** Another frequently observed network motif involving miRNAs, TFs, and their mutual targets are feedforward loops (79). In such motifs, the TF regulates both the target gene and the miRNA, while the miRNA inhibits the mutual target. In this way, the TF regulates the target via two or more branches, i.e. directly via transcriptional regulation and indirectly via miRNA translational repression. While the notion of feedback loops is firmly imprinted in the experimentalist's way of thinking, feedforward loops have received little attention until recently (80). We can distinguish two types of these loops: (i) a coherent feedforward loop, when the TF regulation is consistent through the two branches, (ii) an incoherent feedforward loop when the TF regulation is inconsistent. **Coherent feedforward loops** can act as a safeguard mechanism, i.e. the effect on a downstream target is triggered only if both branches of the loop are active at the same time. This is the case for the feedforward loop established by E2F1, p73 and its apoptosis-related targets, which is mediated by miR-205 (29). Triggering apoptosis requires the expression of the pro-apoptotic targets of E2F1 and the coordinated repression of the anti-apoptotic protein BCL2 by miR-205 (Figure 5E). Since both processes must occur simultaneously, this provides a window of opportunity to the cell to either confirm or prevent the irreversible activation of apoptosis. In the case of the E2F1-p73/DNp73-miR-205 circuit, miR-205 expression is inhibited via the oncogenic DNp73 splice isoform of p73 preventing apoptosis of some aggressive tumors. **Incoherent feedforward loops** can also induce sophisticated regulatory patterns. In recent publications (81, 82), researchers detected a plethora of feedforward loops linked to cell differentiation. Many of these motifs involve interactions between miRNAs and the SOX family, whose deregulation is critical in melanoma pathogenesis (83). One of the loops detected by Reiprich and his coworkers involves the TFs SOX10 and SOX9, the SOX9-repressing miR-338 and miR-335, and mutual targets of the TFs that promote cell migration. The

authors hypothesized that the incoherent feedforward loop established (SOX10 → target → cell migration; SOX10 → miR-338/335 ⊣ SOX9 → target → cell migration) can generate a time window during differentiation, in which cell migration is possible because of the concerted activation of SOX10 and SOX9 (Figure 5F). Beyond this period, SOX10-mediated activation of the two miRNAs represses SOX9 and the cells lose their migratory capacity**.** Sustained migratory capacity can be an advantage for metastatic tumors like melanoma and therefore the repression of this type of loop could be advantageous. Interestingly, miR-335 is repressed in metastatic melanoma (84), while SOX10 is overexpressed.

## 4 Bioinformatics and systems approaches as the "lifeline" to navigate miRNA networks

As a single miRNA can have hundreds of targets, the effective regulation of its target genes may depend on other interacting molecules like lncRNAs and RNA binding proteins. Often, multiple miRNAs target the same central cancer gene or genes belonging to the same cancer pathway. The expression of each miRNA is regulated by different TFs, and miRNAs are entangled with their TFs and targets in feedback and feedforward loops. As a final point, these regulatory events do not happen in isolation, but they form large, densely connected regulatory networks of miRNAs, TFs, lncRNAs, and gene targets. The only way to navigate this level of complexity, gain insights into oncogenesis, and design personalized therapies is to develop and apply a systematic approach. Specifically, high throughput molecular data (e.g., transcriptomes and proteomes) are analyzed and integrated utilizing bioinformatics algorithms and computational models. Bioinformatics algorithms that focus on the genome can be used to identify novel miRNA genes (85), miRNA targets (86), and mutations within miRNA binding sites (87) or to detect miRNA clusters (88) and miRNA regulatory hubs (89). Network-based analysis of omics data can be utilized to detect deregulated miRNA-mediated feedback and feedforward loops and to obtain core regulatory subnetworks important for the regulation of tumor initiation, progression, and therapy resistance (90). Finally, when considering the spatiotemporal dynamics of these circuits, one can utilize computational modeling (29, 66). Ultimately,

all these tools can be employed to obtain predictive gene signatures for cancer progression or stratification (91) or to detect therapeutic miRNA targets (48).

**Acknowledgements**

Our work in cancer and immunotherapy modeling is supported by the German Ministry of Education and Research (BMBF) through the e:Med initiatives: MelAutim [01ZX1905A] and KI-VesD [031L0244A]. We also acknowledge the funding of our research activities in melanoma from the Manfred Roth-Stiftung, the Staedtler-Stiftung, the Trunk-Stiftung, the Matthias-Lackas-Stiftung, and the Hiege Stiftung. Financial support was also provided by the National Health & Medical Research Council Investigator Grant (#1196405) and by the Cancer Council NSW (RG20-12).

**Author contribution**

Conceptualization: XL, US, JV; Visualization: XL; Writing-original draft: XL, JV; Writing-review & editing: XL, US, JV.

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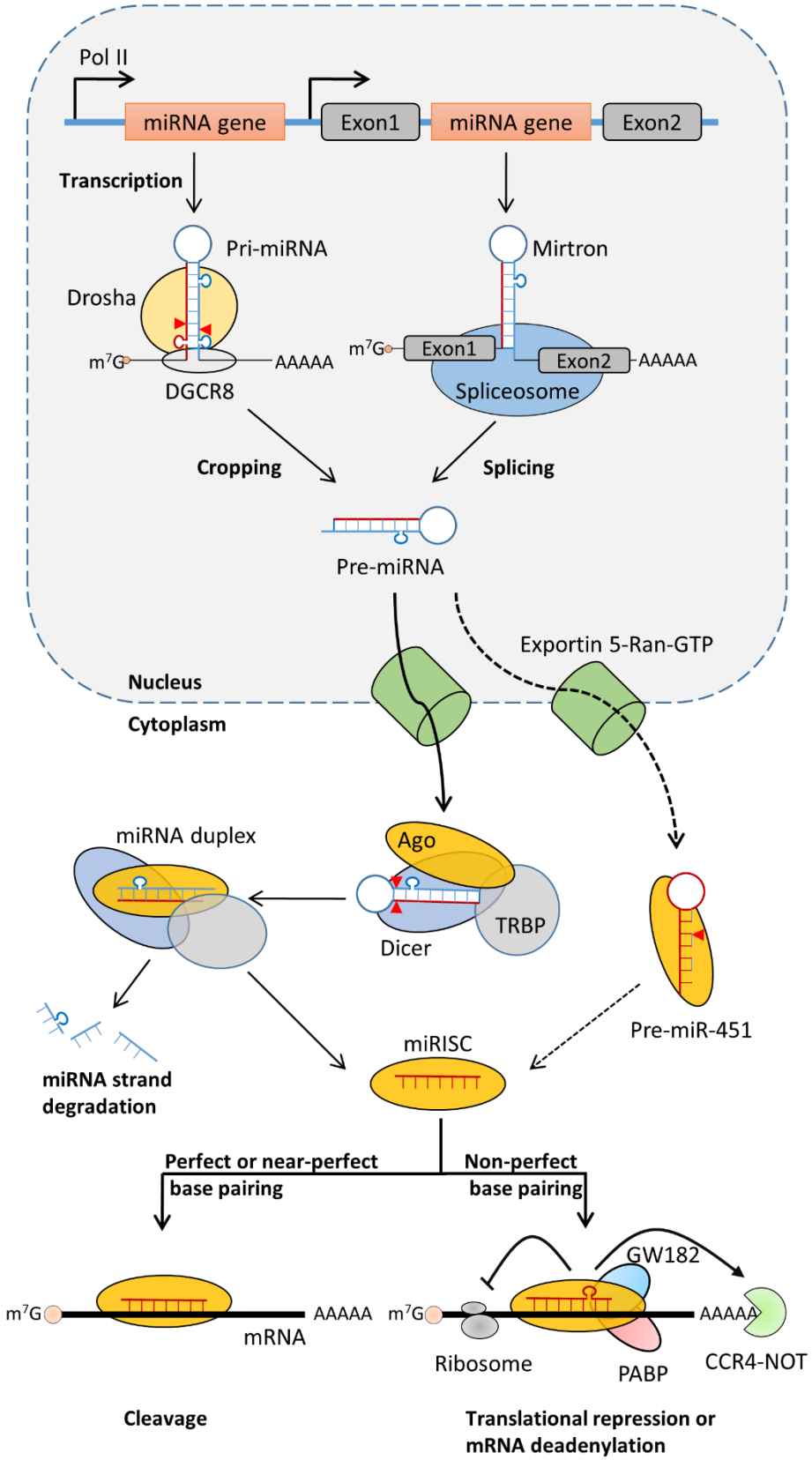
## Figures


Pol II
miRNA gene
Exon1
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Transcription
Pri-miRNA
Drosha
m7G
AAAAA
DGCR8
Mirtron
m7G
Exon1
Exon2
AAAAA
Spliceosome
Cropping
Splicing
Pre-miRNA
Exportin 5-Ran-GTP
Nucleus
Cytoplasm
miRNA duplex
Ago
TRBP
Dicer
Pre-miR-451
miRNA strand degradation
miRISC
Perfect or near-perfect base pairing
Non-perfect base pairing
GW182
m7G
AAAAA
mRNA
m7G
AAAAA
Ribosome
PABP
CCR4-NOT
Cleavage
Translational repression or mRNA deadenylation

**Figure 1. miRNA biogenesis.** A miRNA can be processed either from a pri-miRNA or a mirtron. The pri-miRNA, containing a 5' terminal $m^7G$ cap and a 3' poly(A) tail (AAAAA), is transcribed from miRNA genes by Pol II and is subsequently cleaved (red arrowheads) by Drosha with the cofactor DGCR8 and becomes a pre-miRNA. The mirtron situated between two exons is spliced and becomes a pre-miRNA without the requirement of Drosha-DGCR8 complex. The pre-miRNA is transported from the nucleus to the cytoplasm by exportin 5 with Ran-GTP. In the cytoplasm, most pre-miRNAs are processed into double-stranded miRNA duplexes with the help of Dicer and TRBP. One strand of the duplexes is loaded into the AGO containing miRISCs, whereas the other strand is degraded. When a miRNA is perfectly or near-perfectly pairing to its target mRNA, it can result in the cleavage of the mRNA. Otherwise, non-perfect base pairing between a miRNA and its target mRNA leads to translation repression or target mRNA deadenylation. Both processes are implemented through the interaction of miRISCs with GW182 and PABP.

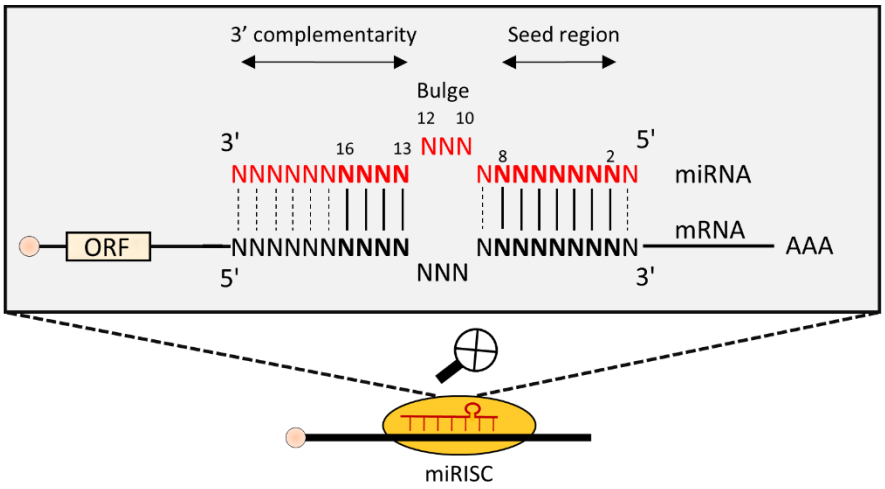


**Figure 2. Illustration of base pairing between miRNAs and their target mRNAs**. In the seed region, continuous Watson-Crick pairing (vertical solid lines) is crucial for efficient duplex formation and miRNA-mediated repression. When a mismatch (vertical dashed lines) or a bulge appears in the seed region, Watson-Crick pairing centering on miRNA nucleotides 13-16 of the 3' complementarity region can compensate and thereby construct a functional miRNA binding site.

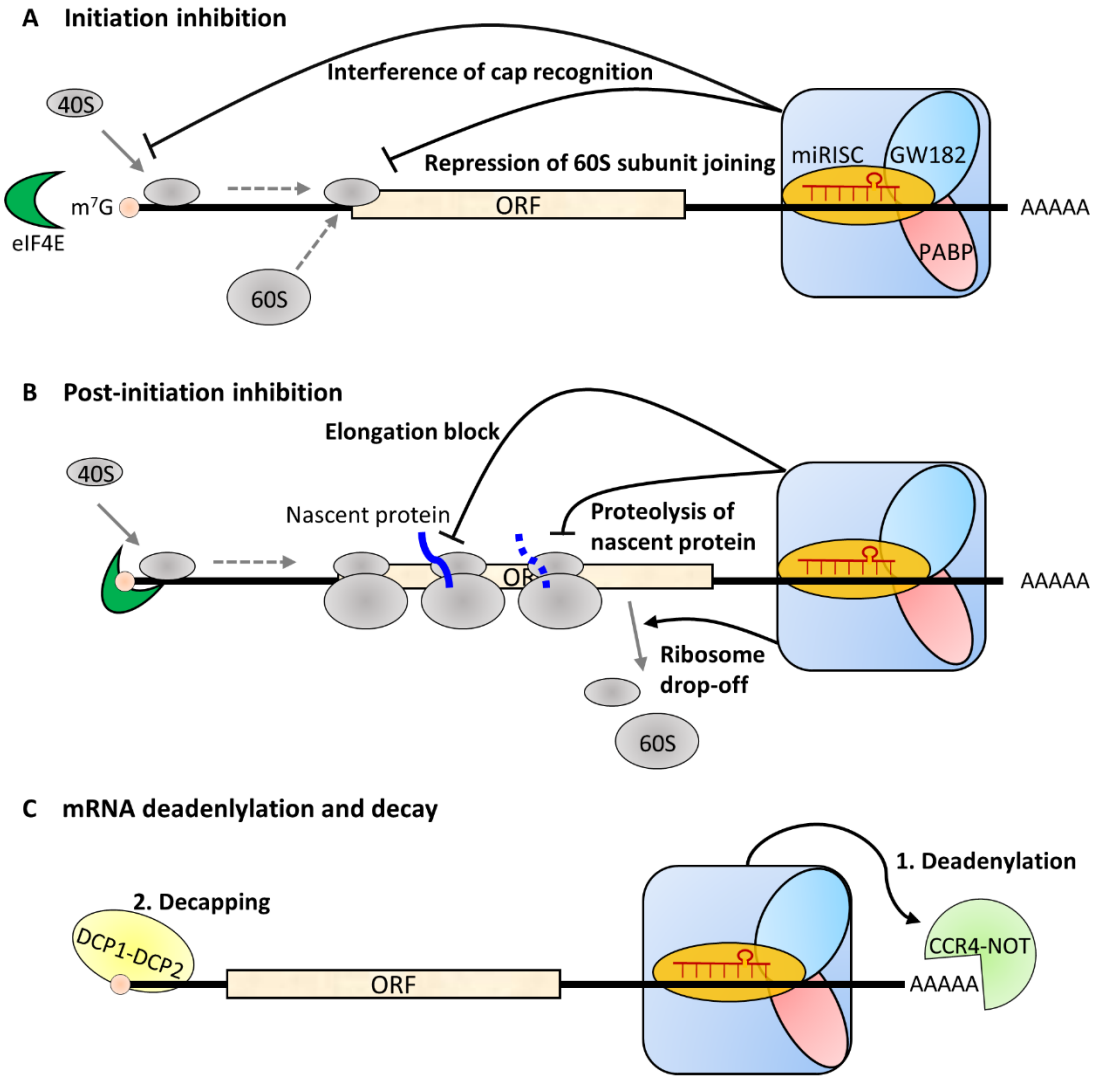


**Figure 3. miRNA-mediated translation repression mechanisms.** With the help of GW182 and PABP, miRISCs can repress translation at the initiation and post-initiation stage, or induce the deadenylation and decay of target mRNAs. (**A**) At the initial stage, binding of the miRISC complexed with GW182 and PABP to the target mRNA can repress translation by either interfering with the cap recognition or by repressing the 60S subunit joining. (**B**) The miRISC can inhibit translation at the post-initiation step by blocking translation elongation, causing ribosome drop-off or proteolytic cleavage of the nascent polypeptides. (**C**) Deadenylation of the target mRNA is facilitated by the interaction of the miRISC with CCR4-NOT. Subsequently, the decay of the target mRNA happens after the removal of the 5'-terminal m7G cap by the decapping DCP1-DCP2 complex.

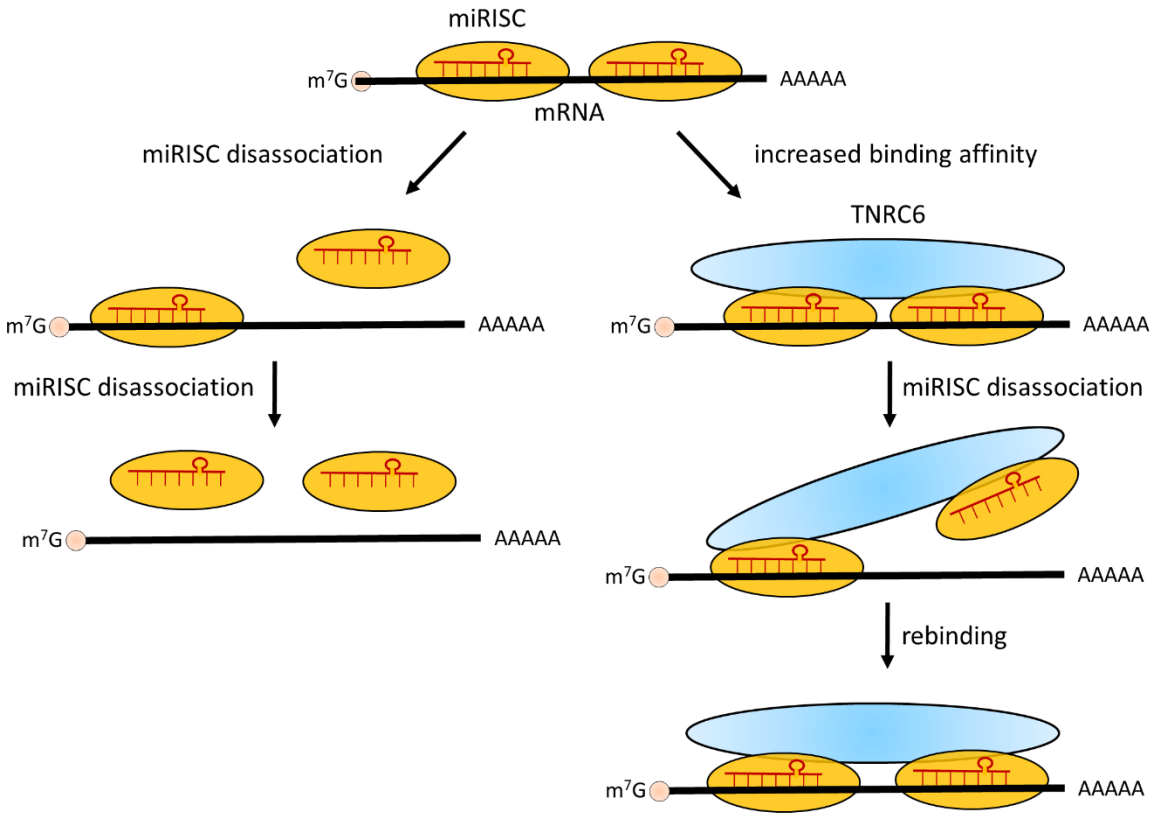


**Figure 4. Model of miRNA cooperativity with or without the participant of TNRC6.** Proximate miRNA binding sites on the target miRNA can result in cooperative gene repression by two miRNAs. The participant of TNRC6 can decrease the disassociation rate of miRISC (formed by a miRNA and AGO) from the mRNA. Because TNRC6 simultaneously binds to two miRISCs, when one miRISC disassociates from the mRNA, TNRC6 could prevent the disassociation of the other miRISC, therefore allowing for rebinding of the disassociated miRISCs.

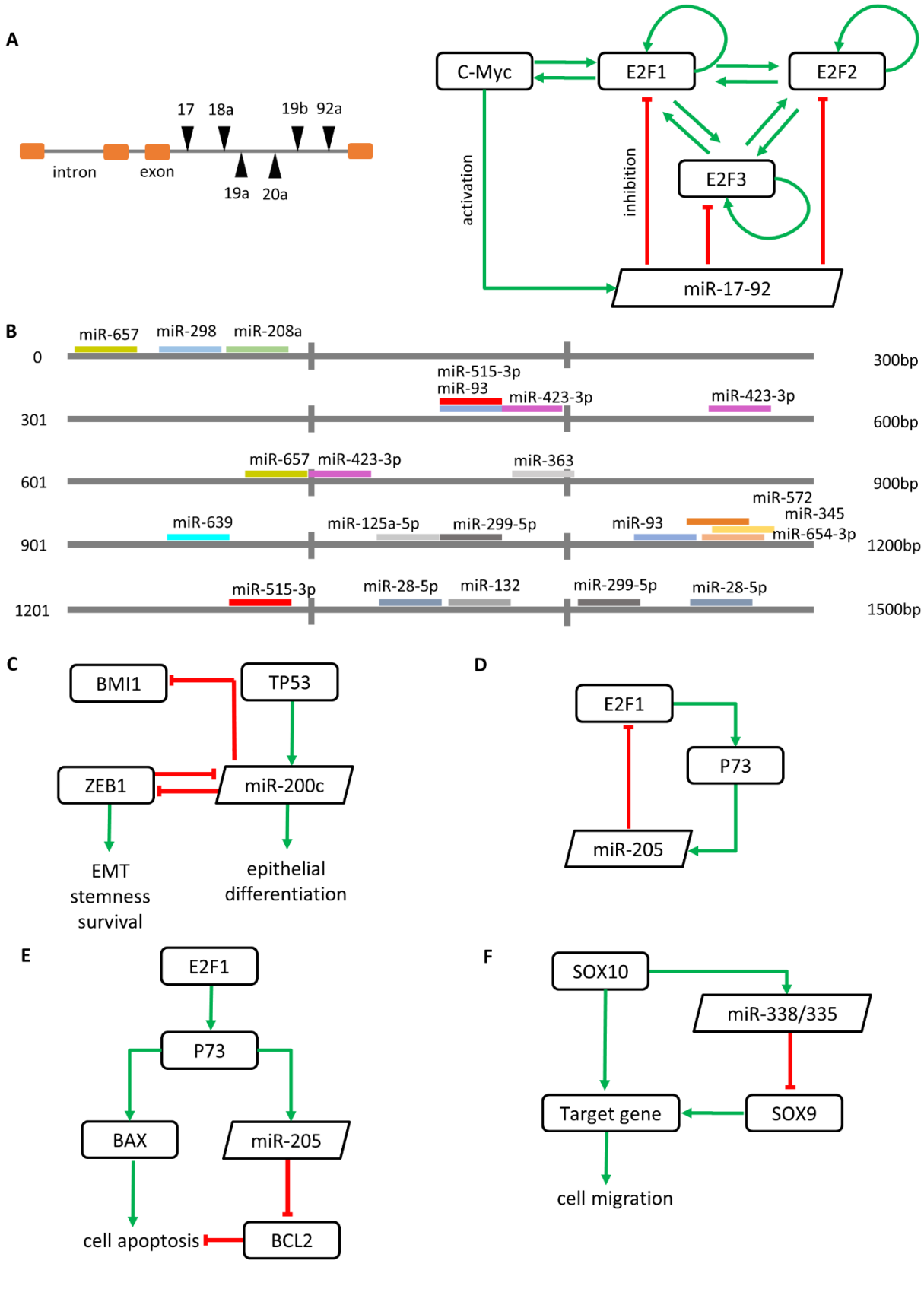
A
17
18a
19b
92a
intron
exon
19a
20a
C-Myc
E2F1
E2F2
E2F3
activation
inhibition
miR-17-92
B
miR-657
miR-298
miR-208a
0
300bp
miR-515-3p
miR-93
miR-423-3p
miR-423-3p
301
600bp
miR-657
miR-423-3p
miR-363
601
900bp
miR-572
miR-345
miR-654-3p
miR-639
miR-125a-5p
miR-299-5p
miR-93
901
1200bp
miR-515-3p
miR-28-5p
miR-132
miR-299-5p
miR-28-5p
1201
1500bp
C
BMI1
TP53
ZEB1
miR-200c
EMT
stemness
survival
epithelial
differentiation
D
E2F1
P73
miR-205
E
E2F1
P73
BAX
miR-205
cell apoptosis
BCL2
F
SOX10
miR-338/335
Target gene
SOX9
cell migration

**Figure 5. A zoo of miRNA-mediated network motifs**. (**A**) Example of a miRNA cluster. The miR-17-92 cluster composed of 6 miRNAs (indicated by arrows) is located in an intron of the miR-17-92 cluster host gene (MIR17HG). miR-17-92 cluster expression is regulated by the TF c-Myc. miR-17-92 cluster members repress transcripts of the E2F1 gene family. (**B**) An example of a miRNA target hub gene. The gene CDKN1A is a miRNA target hub with at least 22 predicted miRNA binding sites in its 3′ UTR. (**C**) An example of a positive feedback loop. ZEB1 and miR-200c repress the expression of one another, thereby forming a positive feedback loop. In addition, p53 activates transcription of miR-200c and miR-200c inhibits translation of stem cell factors, such as BMI1. p53 stimulates expression of miR-200c, thereby driving epithelial differentiation and counteracting epithelial-mesenchymal transition (EMT) and stemness. (**D**) An example of a negative feedback loop. The TF E2F1 promotes the transcription of P73, which can upregulate the expression of miR-205. In turn, miR-205 represses E2F1, thereby forming a negative feedback loop. (**E**) An example of a coherent feedforward loop. Two signaling pathways can lead to upregulation of E2F1-related cell apoptosis - one via pro-apoptotic gene BAX and the other via anti-apoptotic gene BCL2 targeted by miR-205. (**F**) An example of an incoherent feedforward loop. SXO10 can regulate cell migration in an inconsistent manner – one pathway promotes it and the other suppresses it via miR-338 and miR-335.