

## Oncomirs — microRNAs with a role in cancer

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**Abstract** | MicroRNAs (miRNAs) are an abundant class of small non-protein-coding RNAs that function as negative gene regulators. They regulate diverse biological processes, and bioinformatic data indicates that each miRNA can control hundreds of gene targets, underscoring the potential influence of miRNAs on almost every genetic pathway. Recent evidence has shown that miRNA mutations or mis-expression correlate with various human cancers and indicates that miRNAs can function as tumour suppressors and oncogenes. miRNAs have been shown to repress the expression of important cancer-related genes and might prove useful in the diagnosis and treatment of cancer.

Cancer is caused by uncontrolled proliferation and the inappropriate survival of damaged cells, which results in tumour formation. Cells have developed several safeguards to ensure that cell division, differentiation and death occur correctly and in a coordinated fashion, both during development and in the adult body. Many regulatory factors switch on or off genes that direct cellular proliferation and differentiation. Damage to these genes, which are referred to as tumour-suppressor genes and oncogenes, is selected for in cancer. Most tumour-suppressor genes and oncogenes are first transcribed from DNA into RNA, and are then translated into protein to exert their effects. Recent evidence indicates that small non-protein-coding RNA molecules, called microRNAs (miRNAs), might also function as tumour suppressors and oncogenes.

We are just beginning to understand how this novel class of gene regulators is involved in cancer-related processes in humans. Out of hundreds of miRNAs that have recently been identified, only a small number from worm, fly and human genomes have been characterized. They have been shown to control cell growth, differentiation and apoptosis; consequently, impaired miRNA expression has been implicated in tumorigenesis. Continued research into miRNA function might lead to an advanced understanding of the mechanisms that lead to tumorigenesis. In this Review, we discuss the emerging field of 'oncomirs' — miRNAs that are associated with cancer — and how these miRNAs could be used in the diagnosis and treatment of cancer.

### miRNAs are a large family of gene regulators

miRNAs are non-coding, single-stranded RNAs of ~22 nucleotides and constitute a novel class of gene regulators

that are found in both plants and animals<sup>1</sup>. They negatively regulate their targets in one of two ways depending on the degree of complementarity between the miRNA and the target (FIG. 1). First, miRNAs that bind with perfect or nearly perfect complementarity to protein-coding mRNA sequences induce the RNA-mediated interference (RNAi) pathway (see REF. 2 for a review on RNAi). Briefly, mRNA transcripts are cleaved by ribonucleases in the miRNA-associated, multiprotein RNA-induced-silencing complex (miRISC), which results in the degradation of target mRNAs. This mechanism of miRNA-mediated gene silencing is commonly found in plants<sup>3–5</sup>, but miRNA-directed mRNA cleavage has also been shown to occur in mammals<sup>6</sup>. However, most animal miRNAs are thought to use a second mechanism of gene regulation that does not involve the cleavage of their mRNA targets. These miRNAs exert their regulatory effects by binding to imperfect complementary sites within the 3' untranslated regions (UTRs) of their mRNA-targets, and they repress target-gene expression post-transcriptionally, apparently at the level of translation, through a RISC complex that is similar to, or possibly identical with, the one that is used for the RNAi pathway<sup>7–18</sup>. Consistent with translational control, miRNAs that use this mechanism reduce the protein levels of their target genes, but the mRNA levels of these genes are barely affected. However, recent findings indicate that miRNAs that share only partial complementarity with their targets can also induce mRNA degradation, but it is unclear if translational inhibition precedes destabilization of the gene targets in these cases<sup>19,20</sup>.

The biogenesis of miRNAs has only recently been elucidated<sup>1</sup> (FIG. 1). miRNAs, which generally seem to be transcribed by RNA polymerase II, are initially made

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**At a glance**

- MicroRNAs (miRNAs) are an abundant class of negative gene regulators that have been shown to control a wide range of biological functions such as cellular proliferation, differentiation and apoptosis.
- About half of the annotated human miRNAs map within fragile regions of chromosomes, which are areas of the genome that are associated with various human cancers.
- Recent evidence indicates that miRNAs can function as tumour suppressors and oncogenes, and they are therefore referred to as 'oncomirs'. Factors that are required for the biogenesis of miRNAs have also been associated with various cancers and might themselves function as tumour suppressors and oncogenes.
- Expression profiling of miRNAs has been shown to be a more accurate method of classifying cancer subtypes than using the expression profiles of protein-coding genes. The differential expression of certain miRNAs in various tumours might become a powerful tool to aid in the diagnosis and treatment of cancer.
- Gene therapies that use miRNAs might be an effective approach to blocking tumour progression. miRNAs such as *let-7*, which has been shown to negatively regulate the Ras oncogenes, and miR-15 and miR-16, which negatively regulate *BCL2*, are promising candidates for cancer treatment.

as large RNA precursors that are called pri-miRNAs. The pri-miRNAs are processed in the nucleus by the RNase III enzyme, *Drosha*, and the double-stranded-RNA-binding protein, *Pasha* (also known as DGCR8), into ~70-nucleotide pre-miRNAs, which fold into imperfect stem-loop structures<sup>21–28</sup>. The pre-miRNAs are then exported into the cytoplasm by the RAN GTP-dependent transporter *exportin 5* (REFS 29–31) and undergo an additional processing step in which a double-stranded RNA of ~22 nucleotides in length, referred to as the miRNA:miRNA\* duplex, is excised from the pre-miRNA hairpin by another RNase III enzyme, *Dicer*<sup>19,32–34</sup>. Subsequently, the miRNA:miRNA\* duplex is incorporated into the miRISC complex. The mature miRNA strand is preferentially retained in the functional miRISC complex and negatively regulates its target genes (see above).

Many researchers failed to appreciate the importance of miRNAs when they were initially described more than a decade ago. This is because the first miRNA gene that was identified, *lin-4*, was thought to be unique to the roundworm *Caenorhabditis elegans* and control the timing and progression of the nematode life cycle<sup>8,12,35</sup>. However, the identification of hundreds of miRNAs within worm, fly and mammalian genomes by traditional cloning techniques and bioinformatics<sup>12,17,21,36–50</sup> has now captured the attention of scientists specializing in various different fields. There are estimated to be at least 300 miRNAs (and there might be as many as 1000) in the human genome, comprising ~1–4% of all expressed human genes, which makes miRNAs one of the largest classes of gene regulator<sup>46,51,52</sup>. Most human miRNAs are found within introns of either protein-coding or non-coding mRNA transcripts<sup>53</sup>. The remaining miRNAs are either located far from other transcripts in the genome, within the exons of noncoding mRNA genes or within the 3' UTRs of mRNA genes, or they are clustered with other miRNA genes, including a cluster on chromosome 19 that is comprised of 54 novel miRNAs<sup>1,27,52–54</sup>. miRNAs can be grouped into families on the basis of sequence

homology, which is found primarily at the 5' end of the mature miRNAs, but whether members of the same miRNA family control similar biological events remains to be seen. Many miRNAs are evolutionarily conserved from worms to humans, which implies that these miRNAs direct essential processes both during development and in the adult body.

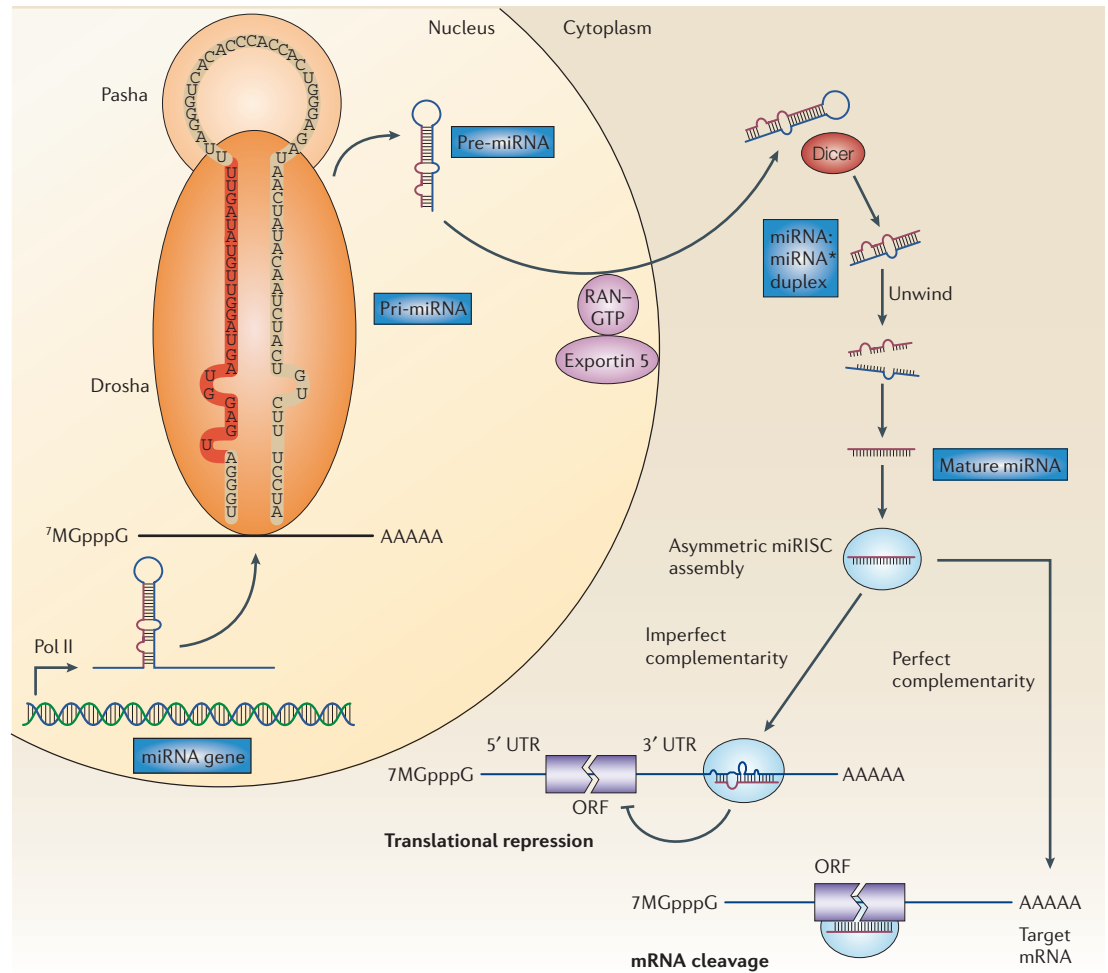
**miRNAs regulate multiple gene targets**

The current challenge is to accurately identify targets that are regulated by miRNAs. Because miRNAs usually bind to their targets with incomplete complementarity, which allows short stretches of mismatched base-pairs and G–U base pairing, the identification of gene targets with a simple BLAST search is impossible. However, current bioinformatic approaches have taken advantage of the fact that miRNAs within families have highest homology at the 5' end of the mature miRNA. Several studies have indicated that the 5' end of the miRNA is crucial for the stability and proper loading of the miRNA into the miRISC complex<sup>55,56</sup>, and this end is also important for biological function<sup>57–59</sup>. Therefore, most bioinformatic algorithms use a 'miRNA seed' that encompasses the first 2–8 bases of the mature miRNA sequence to search for complementarity to sequences in the 3' UTR of all expressed genes. These studies have revealed that a single miRNA might bind to as many as 200 gene targets and that these targets can be diverse in their function; they include transcription factors, secreted factors, receptors and transporters<sup>20,47,60–68</sup>. So, miRNAs potentially control the expression of about one-third of human mRNAs.

However, many targets are missed using the 'miRNA seed' approach as there is evidence that other variables besides the first 2–8 nucleotides of the miRNA sequence dictate miRNA regulation. For example, the complete mature miRNA sequence of *let-7* has been evolutionarily conserved from worms to humans<sup>37</sup>, demonstrating the functional relevance of the 3' end. Furthermore, mutagenesis studies in our laboratory have shown that base pairing at both the 5' and 3' ends of *let-7* is required for downregulation of a target gene<sup>69</sup>. Therefore, the number of known miRNA targets could increase and the challenge will be to determine which predicted targets are biologically relevant. Sites that are complementary to several miRNAs have also been identified within the 3' UTR of a single gene target, indicating that complex patterns of combinatorial regulation by miRNAs exist<sup>12,16,61,70</sup>. Because miRNAs potentially have a broad influence over several diverse genetic pathways, the deletion or misexpression of these small RNAs is likely to be pleiotropic and contribute to disease, including cancer.

**The miRNA-associated genes and human cancer**

Components of the miRNA-machinery have been implicated in tumorigenesis. Expression of *Dicer* has been shown to be downregulated in lung cancer<sup>71</sup>. Karube *et al.* examined RNA expression levels of *DICER* and *DROSHA* in 67 non-small-cell lung cancer samples<sup>71</sup>. They found that reduced expression of *DICER* correlates with shortened post-operative survival, which indicates that *Dicer* might be able to prevent the transformation of lung



**Figure 1 | The biogenesis of microRNAs.** MicroRNA (miRNA) genes are generally transcribed by RNA Polymerase II (Pol II) in the nucleus to form large pri-miRNA transcripts, which are capped (7MGpppG) and polyadenylated (AAAAA). These pri-miRNA transcripts are processed by the RNase III enzyme Drosha and its co-factor, Pasha, to release the ~70-nucleotide pre-miRNA precursor product. (Note that the human *let-7a-1* miRNA is shown here as an example of a pre-miRNA hairpin sequence. The mature miRNA sequence is shown in red.) RAN-GTP and exportin 5 transport the pre-miRNA into the cytoplasm. Subsequently, another RNase III enzyme, Dicer, processes the pre-miRNA to generate a transient ~22-nucleotide miRNA:miRNA\* duplex. This duplex is then loaded into the miRNA-associated multiprotein RNA-induced silencing complex (miRISC) (light blue), which includes the Argonaute proteins, and the mature single-stranded miRNA (red) is preferentially retained in this complex. The mature miRNA then binds to complementary sites in the mRNA target to negatively regulate gene expression in one of two ways that depend on the degree of complementarity between the miRNA and its target. miRNAs that bind to mRNA targets with imperfect complementarity block target gene expression at the level of protein translation (lower left). However, recent evidence indicates that miRNAs might also affect mRNA stability (not shown). Complementary sites for miRNAs using this mechanism are generally found in the 3' untranslated regions (3' UTRs) of the target mRNA genes. miRNAs that bind to their mRNA targets with perfect (or nearly perfect) complementarity induce target-mRNA cleavage (lower right). miRNAs using this mechanism bind to miRNA complementary sites that are generally found in the coding sequence or open reading frame (ORF) of the mRNA target.

tissue. As Dicer has been implicated in heterochromatin maintenance and centromeric silencing, reduced protein levels might directly result in genomic instability and lead to tumour formation<sup>71-73</sup>. However, Kanelloupolou *et al.* recently showed that mouse embryonic stem (ES) cells that lack *Dicer* showed no aberrations in chromosome number or structure despite observed heterochromatin deficits, and the injection of these *Dicer*-deficient ES cells into nude mice failed to generate tumours<sup>72</sup>. Therefore, the potential role of Dicer in tumour formation might be indirect and result from the reduction of miRNAs in the

lung that poses tumour-suppressor characteristics. *Dicer* gene-disruption studies in mice have shown that this gene is important during mammalian development and directs proper stem-cell differentiation, T-cell specification and limb morphogenesis, presumably reflecting the role of mature miRNAs in these processes<sup>72,74-77</sup>. Therefore, the association of *Dicer* with lung cancer indicates a further role for this gene, and perhaps miRNAs, during lung differentiation.

The Argonaute proteins, which are crucial components of the RISC complex that direct both short interfering

(siRNA)- and miRNA-mediated gene regulation, have also been associated with various cancers. Three human Argonaute genes — *AGO3* (also known as eukaryotic translation-initiation factor 2 subunit 3 (*EIF2C3*)), *AGO1* (also known as *EIF2C1*) and *AGO4* (also known as *EIF2C4*), which are clustered on chromosome 1 (1p34–35) — are frequently deleted in **Wilms tumours** of the kidney and have also been associated with neuroectodermal tumours<sup>7,78</sup>. *AGO1* is highly expressed in the developing lung and kidney and is notably increased in **renal tumours** that lack the Wilms-tumour suppressor gene, *WT1* (REF. 7). This indicates a role for *AGO1* in the differentiation of these tissues during embryogenesis. An additional Argonaute gene, *HIWI*, which is the human orthologue of the *Drosophila melanogaster* Argonaute gene *piwi*, maps to the genomic region 12q24.33, which is a locus that is associated with **testicular germ-cell cancers**<sup>79</sup>. This testis-specific gene was found to be upregulated in 12 out of 19 testicular seminomas (tumours that are derived from embryonic germ cells). These studies indicate that *HIWI* might have oncogenic activity and might normally function to control the proliferation and maintenance of germ cells. Further insight into the accessory proteins that comprise the machinery mediating miRNA processing and miRNA-directed gene repression will be important when designing therapeutic drugs that use miRNAs to treat disorders such as cancer.

#### miRNAs, growth, differentiation and disease

The biological roles of only a small fraction of identified miRNAs have been elucidated to date. These miRNAs regulate cancer-related processes such as cell growth and tissue differentiation, and therefore might themselves function as oncomirs. For example, the miRNAs that are encoded by *lin-4* and *let-7* control the timing of cell differentiation and proliferation in *C. elegans*. Mutations in these *C. elegans* miRNA genes lead to abnormalities in cell-cycle exit and terminal differentiation, which are characteristics that are found in cancer cells. Interestingly, the mammalian homologues of *lin-4* and *let-7* have been shown to control cell proliferation in human cell lines<sup>80,81</sup> and are also associated with various cancers<sup>81–85</sup> (discussed below).

The *D. melanogaster* miRNA that is encoded by *bantam* induces tissue growth by both stimulating cell proliferation and inhibiting apoptosis<sup>17,86</sup>, which are features that are shared with *bona fide* oncogenes. However, there are no close homologues of *bantam* in humans. Another *D. melanogaster* miRNA, **miR-14**, is a strong suppressor of apoptosis, which is also an important oncogenic characteristic<sup>49</sup>. In addition, miR-14 also seems to have unrelated functions in the *D. melanogaster* stress-response pathway and in regulating fat metabolism<sup>49</sup>. Other characterized miRNAs have essential functions during development and direct the proper differentiation of cells into various tissues. Examples include **miR-273** and the miRNA that is encoded by *lys-6*, which are involved in patterning the *C. elegans* nervous system<sup>43,50</sup>; miR-430 in *Danio rerio* brain development<sup>87</sup>; **miR-181** in the differentiation of mammalian haematopoietic cells towards the B-cell lineage<sup>88</sup>; **miR-375** in mammalian pancreatic islet-cell

development and the regulation of insulin secretion<sup>89</sup>; **miR-143** during mammalian adipocyte differentiation<sup>90</sup>; **miR-196** in mammalian limb patterning<sup>91</sup>; and the miR-1 genes during mammalian heart development<sup>92</sup>. Further characterization of novel miRNAs might reveal other gene regulators that coordinate proper organ formation, embryonic patterning and body growth, and might also provide insight into the mechanisms of human diseases such as cancer.

#### miRNAs that are implicated in cancer

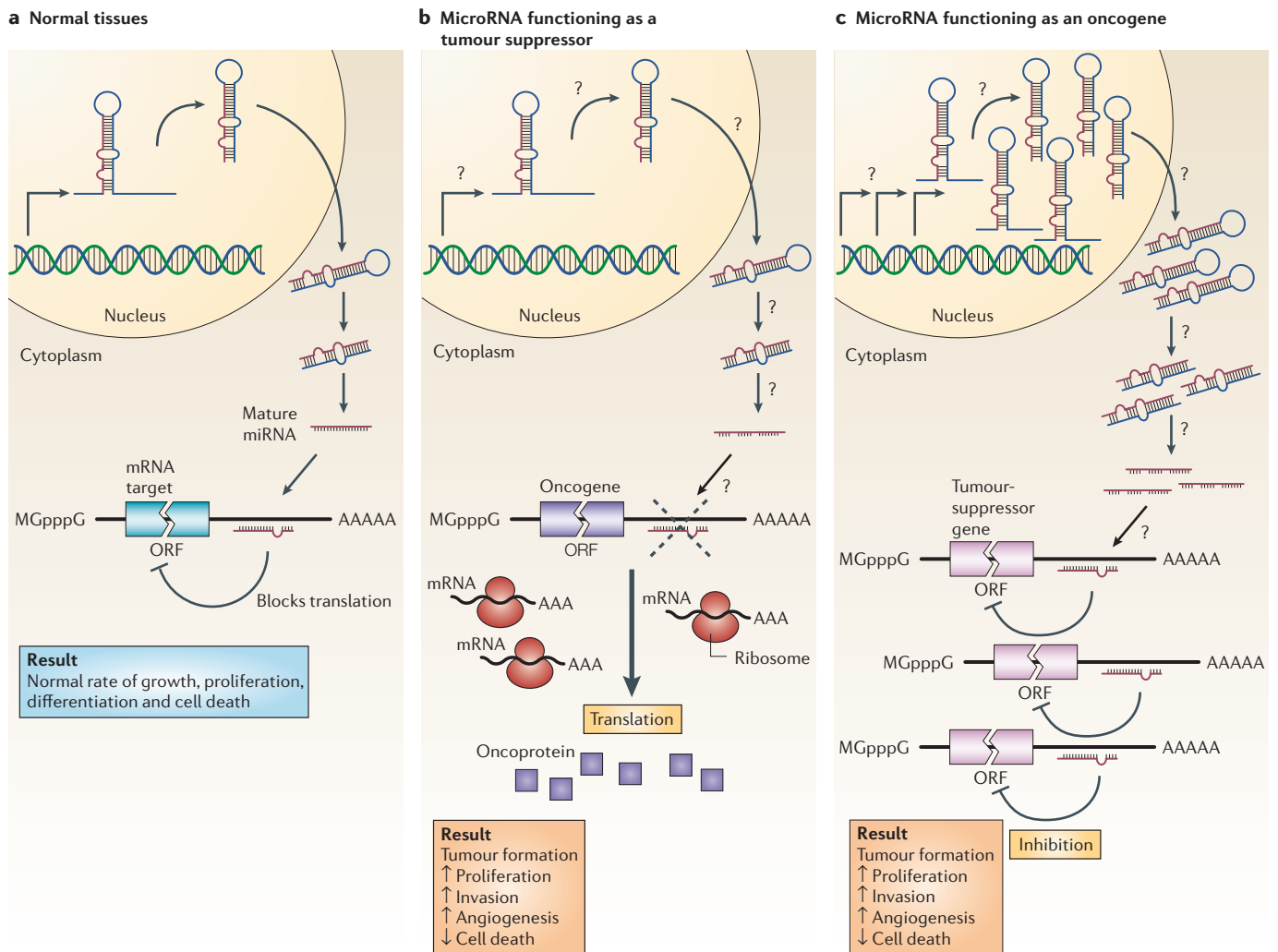
miRNA expression correlates with various cancers, and these genes are thought to function as both tumour suppressors and oncogenes (FIG. 2; TABLE 1). A recent study showed that about 50% of annotated human miRNAs are located in areas of the genome, known as fragile sites, that are associated with cancer. This indicates that miRNAs might have a crucial function in cancer progression<sup>83</sup>. For example, *mir-125b-1*, the homologue of *C. elegans lin-4*, is located in a fragile site on chromosome 11q24, which is deleted in a subset of patients with **breast, lung, ovarian and cervical cancers**<sup>83</sup>. A report by Sonoki *et al.* also linked this gene with **leukaemia**, and described a patient with precursor-B-cell acute lympho-blastic leukaemia who carried an insertion of pre-miRNA into the immunoglobulin heavy-chain locus<sup>85</sup>. Although the investigators could not determine how *mir-125b-1* expression<sup>85</sup> was modulated in the tumour cells, this study supports a role for this gene as an oncomir.

The first indication that miRNAs could function as tumour suppressors came from a report by Calin *et al.* that showed that patients who were diagnosed with a common form of adult leukaemia, B-cell chronic lymphocytic leukemia (CLL), often have deletions or downregulation of two clustered miRNA genes, *mir-15a* and *mir-16-1* (REF. 93). Deletions within the 13q14 locus occur in more than 65% of CLL cases, as well as in 50% of mantle cell lymphomas, 16–40% of **multiple myelomas** and 60% of **prostate cancers**. Therefore, it was predicted that a tumour-suppressor gene must reside in this 30-kb region. Interestingly, *mir-15a* and *mir-16-1* map within the intron of a non-protein-coding RNA gene of unknown function, which is called *LEU2*. Clinicians had previously noted that CLL patients with 13q14 deletions have a more favourable prognosis than those with abnormal karyotypes or deletions at other loci such as 11q23 or 17p13. This might be due to the fact that homologues of *mir-15a* and *mir-16-1* that are clustered on chromosome 3 (*mir-15b* and *mir-16-2*, respectively) continue to be expressed at low levels in patients with CLL; therefore, 13q14 deletions do not lead to the complete elimination of these miRNA families.

A recent report by Cimmino *et al.* showed that miR-15a and miR-16-1 negatively regulate *BCL2*, which is an anti-apoptotic gene that is often overexpressed in many types of human cancers, including leukaemias and lymphomas<sup>94</sup>. Therefore, it is thought that the deletion or downregulation of *mir-15a* and *mir-16-1* results in increased expression of *BCL2*, promoting leukaemogenesis and lymphomagenesis in haematopoietic cells. The tumour-suppressor role

#### Fragile site

A site in a chromosome that is susceptible to chromosome breakage, amplification and fusion with other chromosomes. This can be induced by exposure to inhibitors of DNA replication, such as aphidicolin, and there are more than 80 commonly described sites.



**Figure 2 | MicroRNAs can function as tumour suppressors and oncogenes. a** | In normal tissues, proper microRNA (miRNA) transcription, processing and binding to complementary sequences on the target mRNA results in the repression of target-gene expression through a block in protein translation or altered mRNA stability (not shown). The overall result is normal rates of cellular growth, proliferation, differentiation and cell death. **b** | The reduction or deletion of a miRNA that functions as a tumour suppressor leads to tumour formation. A reduction in or elimination of mature miRNA levels can occur because of defects at any stage of miRNA biogenesis (indicated by question marks) and ultimately leads to the inappropriate expression of the miRNA-target oncoprotein (purple squares). The overall outcome might involve increased proliferation, invasiveness or angiogenesis, decreased levels of apoptosis, or undifferentiated or de-differentiated tissue, ultimately leading to tumour formation. **c** | The amplification or overexpression of a miRNA that has an oncogenic role would also result in tumour formation. In this situation, increased amounts of a miRNA, which might be produced at inappropriate times or in the wrong tissues, would eliminate the expression of a miRNA-target tumour-suppressor gene (pink) and lead to cancer progression. Increased levels of mature miRNA might occur because of amplification of the miRNA gene, a constitutively active promoter, increased efficiency in miRNA processing or increased stability of the miRNA (indicated by question marks). ORF, open reading frame.

of miR-16 is further supported by the finding that two patients with CLL carried a germline C to T mutation 7 base-pairs downstream of the *mir-16-1* precursor, resulting in decreased miR-16-1 expression levels<sup>95</sup>. Although further characterization of the biological functions of miR-15 and miR-16-1 have not been completed, evidence indicates that *mir-16-1* expression is preferentially downregulated in various leukaemias but not in cancers that originate from other tissues. This supports a role for this miRNA in the immune system and B-cell differentiation<sup>93,96-100</sup>.

Additional studies have shown a strong correlation between abrogated expression of miRNAs and oncogenesis. For example, mature miRNA levels of miR-143 and miR-145 are significantly reduced in colorectal tumours<sup>97</sup>. Interestingly, the hairpin precursor transcripts of these miRNAs were detected in similar quantities in normal and tumour tissues, indicating that the processing of *mir-143* and *mir-145* might be disrupted in these cancer samples. However, the tumour-suppressor roles of miR-143 and miR-145 might not be specific to colorectal tissue as their

Table 1 | **MicroRNAs that are associated with human cancers**

miRNA	Gene loci	Cancer association	Function	References
miR-15a, miR-16-1	Chromosome 13q14	Frequently deleted or downregulated in B-cell chronic lymphocytic leukemia; negatively regulates the anti-apoptotic gene <i>BCL2</i>	TS	93,94
miR-143, miR-145	Chromosome 5q32–33	Decreased abundance in colorectal cancer; downregulated in breast, prostate, cervical and lymphoid cancer cell lines; miR-145 is decreased in breast cancer	TS	84,97
miR-21	Chromosome 17q23.2	Anti-apoptotic factor; upregulated in glioblastomas and breast cancer	OG	84,100,101
<i>let-7</i> family members	Multiple loci	Negatively regulate the Ras oncogenes; direct cell proliferation and differentiation; decreased abundance in lung cancer	TS	81,82
miR-142	Chromosome 17q22	A t(8;17) translocation that places the <i>MYC</i> oncogene downstream of the <i>mir-142</i> hairpin, resulting in an aggressive B-cell leukemia that is due to <i>MYC</i> overexpression	N/A	41
BIC/miR-155	Chromosome 21q21	Upregulated in paediatric Burkitt, Hodgkin, primary mediastinal and diffuse large-B-cell lymphomas; upregulated in human breast cancer	OG	84,105–108
miR-17–19b cluster	Chromosome 13q31–32	Upregulated by <i>MYC</i> ; negatively modulates the <i>E2F1</i> oncogene; loss of heterozygosity of this cluster is found in hepatocellular carcinoma; overexpressed in B-cell lymphomas	TS/OG	109,110

N/A, not applicable; OG, oncogene; TS, tumour suppressor.

expression is also downregulated in breast, prostate, cervical and lymphoid cancer cell lines<sup>84,97</sup>. Another report demonstrated that **miR-21** is upregulated in **glioblastoma**<sup>101</sup>. This gene was found to be expressed at a 5–100-fold higher rate in gliomas than in normal tissue. Antisense studies of miR-21 in glioblastoma cell lines showed that this miRNA controls cell growth by inhibiting apoptosis but does not affect cell proliferation, which implies an oncogenic role for this miRNA. An independent study that used microarray analysis to compare the expression of 245 miRNAs in glioblastoma versus normal tissues also identified miR-21 levels as being increased in glioblastoma tumours<sup>100</sup>. However, as *mir-21* is not a brain-specific gene<sup>100</sup> and miR-21 expression is increased in human breast cancer samples<sup>84</sup>, this miRNA might have a wider function in tumour progression. More studies need to be done

to show whether these miRNAs have a direct function in cancer progression or are simply differentially modulated in tumours.

### The *let-7* family negatively regulates Ras

The miRNAs that are encoded by the *let-7* family were the first group of oncomirs shown to regulate the expression of an oncogene, specifically the Ras genes. Ras proteins are membrane-associated GTPase signalling proteins that regulate cellular growth and differentiation. About 15–30% of human tumours possess mutations in Ras genes, and activating mutations that result in the increased expression of Ras cause cellular transformation. Therefore, a miRNA that regulates the expression level of these potentially oncogenic proteins is predicted to keep the rate of cellular proliferation in check. The miRNAs that are encoded by the *let-7* family, which include 12 human homologues, were implicated as tumour suppressors because they map to fragile sites associated with lung, breast, **urothelial** and cervical cancers<sup>83</sup>. More direct evidence, presented by Takamizawa *et al.*, showed that transcripts of certain *let-7* homologues were significantly downregulated in human lung cancer and that this correlated with a poor prognosis<sup>81</sup>. These studies further indicate that *let-7* could be used diagnostically, as patients with non-small-cell lung carcinomas who expressed lower levels of *let-7* had a poorer prognosis and shortened post-operative survival. *In vitro* tissue-culture experiments with a human lung adenoma cell line that showed that transient delivery of *let-7* can inhibit cellular proliferation also indicates that *let-7* probably is a tumour-suppressor gene in lung tissue. Therefore, *let-7* could be used therapeutically to treat lung cancer<sup>81</sup>.

Clues to how the *let-7* family controls cellular proliferation came from studies in *C. elegans* in our laboratory. We showed that certain members of the *let-7* family genetically interact with oncogenic Ras and that the *let-7* family and Ras are reciprocally expressed. Moreover, the 3' UTR of Ras genes contains multiple complementary sites for the *let-7* family<sup>60,82</sup>. We also found that *let-7* negatively regulates Ras in human cells. Overexpression of *let-7* in human cancer cell lines results in decreased levels of Ras compared with untreated cells. Conversely, the depletion of RNA levels of *let-7* in human cancer cell lines that normally express high levels leads to a marked increase in Ras protein expression<sup>82</sup>. We also provided evidence that *let-7* directly controls Ras expression through 3'-UTR-mediated repression in human cells. Reporter constructs that contain Ras 3' UTRs were downregulated at the translational level by *let-7*, and *let-7* inhibitors could reverse this repression. Furthermore, when comparing lung tumours to normal adjacent cells taken from patients with squamous-cell carcinoma of the lung, *let-7* and Ras genes were reciprocally expressed: *let-7* homologues were downregulated in the tumours, whereas Ras was highly expressed.

Taken together, these studies indicate that *let-7* is a promising therapeutic agent to treat lung cancers caused by activating mutations in Ras genes. Interestingly, repression of *let-7* might be a unique characteristic of

lung cancer progression. Analysis of the expression of *let-7* in 21 different cancer patients, 12 of whom suffered from lung cancer, revealed that *let-7* was markedly reduced in all lung cancer samples but was only sporadically decreased in other tumour types. In support of this finding, microarray analysis of the expression profile of 167 miRNAs in lung tumours showed that most miRNA genes were not dramatically modulated in lung cancer<sup>82</sup>.

### miRNAs associated with the MYC oncogene

The *MYC* oncogene, which encodes a basic helix-loop-helix transcription factor, is often mutated or amplified in human cancers and has been shown to function as an important regulator of cell growth owing to its ability to induce both cell proliferation and apoptosis<sup>102</sup>. Interestingly, there seems to be a close relationship between miRNAs and the increased expression of MYC, and this leads to the development of B-cell malignancies. For example, an aggressive B-cell leukaemia occurs when *MYC* is translocated into the *mir-142* locus. This t(8;17) translocation places *MYC* just downstream of the miRNA hairpin sequences and under the control of the miRNA promoter<sup>41</sup>. Loss of a conserved region of about 20 nucleotides, which is downstream of the *mir-142* precursor, on *MYC* translocation is thought to disrupt miRNA processing, leading to the increased expression of MYC and the transformation of B cells.

Another miRNA, *miR-155*, has also been linked with MYC overexpression and B-cell cancers. This miRNA is encoded by nucleotides 241–262 of *BIC*. This gene was initially identified as a common integration site for the avian leukosis virus and was found to be overexpressed in B-cell lymphomas<sup>103,104</sup>. For years, researchers were puzzled as to how an RNA that was presumably not protein-coding, and was poorly conserved between avian, mouse and human genomes, could promote lymphomas in cells that also overexpressed MYC. Metzler *et al.* found that the only phylogenetically conserved region within *BIC* spans 138 nucleotides and encodes the hairpin region of *mir-155* (REF. 105). This group then showed that *miR-155* expression is upregulated 100-fold in paediatric **Burkitt lymphoma**, and other studies showed increased *miR-155* levels in **Hodgkin lymphoma** and in primary mediastinal and diffuse large-B-cell lymphomas with activated rather than germinal-centre phenotypes<sup>106–108</sup>. Therefore, it seems that *mir-155* can function as an oncogene in cooperation with *MYC*, whereas it normally functions during B-cell selection and possibly targets genes that antagonize the MYC pathway.

*miR-155* has also been reported to be upregulated in breast carcinomas, which indicates that there are further roles for this gene outside of the haematopoietic system<sup>84</sup>. Furthermore, the organization of the *mir-155/BIC* gene is reminiscent of the *mir-15a* and *mir-16-1* genes, which are encoded within the intron of the non-protein-coding *LEU2* gene, as well as the *C13orf25* gene, which contains the *mir-17-92* cluster that is described below. However, the significance of a non-protein-coding RNA harbouring miRNAs within a transcript and being linked to oncogenesis is unclear.

Two recent papers from He *et al.* and O'Donnell *et al.* describe a more direct relationship between miRNAs, MYC and cancer<sup>109,110</sup>. The 13q31 locus is preferentially amplified in cancers such as diffuse large-B-cell lymphoma, follicular lymphoma, mantle cell lymphoma and primary cutaneous B-cell lymphoma. However, the only gene that has been found to be upregulated within this amplicon is a non-protein-coding RNA, *C13orf25* (REF. 11). This transcript was shown to encode the *mir-17-92* cluster, which includes seven miRNAs: *miR-17-5p*, *miR-17-3p*, *miR-18a*, *miR-19a*, *miR-20a*, *miR-19b-1* and *miR-92-1*. He and colleagues analysed cell lines carrying an amplification of 13q31 for the expression of 191 miRNAs and found that 6 miRNAs had increased expression, which corresponded to more copies of *C13orf25*, and 5 of these belonged to the *mir-17-92* cluster<sup>109</sup>. This group then showed that the pri-miRNA for the *mir-17-92* cluster was upregulated in 65% of B-cell lymphoma samples that were tested. They therefore postulated that increased expression of this cluster contributes to cancer formation.

To test this directly, the researchers overexpressed the *mir-17-19b-1* cluster (the vertebrate-specific portion of the *mir-17-92* cluster) using a retroviral system in haematopoietic stem cells (HSCs) that had been isolated from mice carrying the *Myc* transgene. Wild-type mice were given a sublethal dose of radiation to wipe out the bone marrow and were then transplanted with the retrovirally transduced HSCs. Irradiated animals that received HSCs overexpressing both *Myc* and the *mir-17-19b-1* cluster developed malignant lymphomas faster (~51 days) than those animals that received HSCs expressing *Myc* alone (3–6 months). Interestingly, similar experiments that used HSCs overexpressing *Myc* in combination with either 96 unrelated, single miRNAs or any individual miRNA of the *mir-17-19b-1* cluster did not show accelerated onset of disease. Lymphomas that resulted from the increased expression of both MYC and miRNAs from the *mir-17-19b-1* cluster had increased cell proliferation and lower levels of cell death in comparison with latent malignancies that arose from increased MYC expression levels alone, which underwent extensive apoptosis.

These experiments indicate that miRNAs within the truncated *mir-17-19b-1* cluster function cooperatively as oncogenes, possibly by targeting apoptotic factors that are activated in response to MYC overexpression. When the brakes of the apoptotic pathway are removed, MYC can induce cells to proliferate uncontrollably, which results in cancer. The oncogenic function of the *mir-17-92* cluster is further validated by the fact that *miR-19a* and *miR-92-1* expression levels are increased in cells from patients with B-cell CLL, that miRNAs from the *mir-17-92* cluster are overexpressed in lung cancers and that *C13orf25* is frequently amplified in alveolar rhabdomyosarcoma and liposarcoma<sup>111–116</sup>.

O'Donnell *et al.* independently identified the *mir-17-92* cluster as a group of potentially cancer-related genes when they carried out a microarray screen of 235 human, mouse and rat miRNAs to identify miRNAs that were modulated in a human B-cell line, P493-6, that overexpresses MYC<sup>110</sup>. This study showed that MYC induces

the expression of the *mir-17-92* cluster, and chromatin immunoprecipitation experiments revealed that MYC binds within the first intron of *C13orf25*, which implies that MYC directly regulates the *mir-17-92* pri-miRNA transcript. The next step was to identify gene targets of the MYC-regulated *mir-17-92* cluster. Previous bioinformatic studies had identified the transcription factor **E2F1** as a target of two miRNAs within this cluster — miR-17-5p and miR-20a. E2F1 controls the transition from G1 to S phase of the cell cycle by regulating genes that are involved in DNA replication, cell division and apoptosis<sup>117</sup>.

Interestingly, E2F1 is known to function in a reciprocal positive-feedback loop with MYC. O'Donnell *et al.* found that inhibition of *mir-17-5p* and *mir-20a* in a cervical cancer cell line markedly increased E2F1 expression levels without affecting mRNA abundance, which is a hallmark of miRNA-mediated gene repression. Mutations of sites in the *E2F1* 3' UTR that are complementary to miR-17-5p and miR-20a resulted in increased reporter activity compared with those that result from wild-type constructs. Taken together, these studies show that MYC induces the transcription of both the *mir-17-92* cluster and *E2F1*, and, in turn, these miRNAs negatively regulate the translation of E2F1. Therefore, a model is proposed in which MYC-mediated cell growth is tightly regulated by the *mir-17-92* cluster. In the presence of MYC, miRNAs of the *mir-17-92* cluster limit the activity of E2F1 and dampen the cell-proliferative effects of MYC by breaking the positive-feedback loop between MYC and E2F1. In this context the *mir-17-92* cluster would function as a tumour suppressor, which is in contrast to the findings of He *et al.* that are discussed above. Consistent with this model, which was proposed by O'Donnell and colleagues, loss of heterozygosity at the 13q31 loci that encodes the *mir-17-92* cluster has been reported in **hepatocellular carcinomas**<sup>118</sup>. However, it has also been reported that although E2F1 drives cellular proliferation, when E2F1 expression levels cross a certain threshold, excessive levels of this protein induce apoptosis<sup>117</sup>. In this context, the negative regulation of E2F1 by miRNAs of the *mir-17-92* cluster might function to block the apoptotic activity of E2F1 and stimulate MYC-mediated cellular proliferation, so supporting the model that was proposed by He *et al.*

The tumour-suppressing and oncogenic nature of the *mir-17-92* cluster emphasizes the complexities of cancer progression as well as the intricacies of miRNA-mediated gene regulation. These results might also reflect the fact that a single miRNA can control many unrelated gene targets, resulting in the control of opposing activities such as cellular proliferation and differentiation. Perhaps the ability of the *mir-17-92* cluster to function as either tumour suppressors or oncogenes depends on which cell types these miRNAs are expressed in and what tissue-specific target mRNAs are present to be regulated. Future studies might reveal that miRNAs function as key regulators of many cancer-related genes as well as *BCL2*, Ras and *E2F1*. In fact, a recent report by Felli *et al.* described the ability of **miR-221** and **miR-222** to down-regulate the *KIT* oncogene to modulate erythropoiesis

of CD34<sup>+</sup> HPCs and inhibit cell growth of an erythro-leukaemic TF-1 cell line in a *KIT*-dependent manner<sup>119</sup>. In support of the regulatory role of the *KIT* oncogene by miRNAs, He *et al.* found that patients with papillary thyroid carcinomas showed decreased *KIT* transcript and protein levels and reciprocally increased levels of miR-221, miR-222, and miR-146 in the tumours, implicating that these miRNAs act as oncogenes in this setting<sup>132</sup>. The study also showed that five out of ten papillary thyroid carcinomas showed point mutations in the *KIT* loci specifically within the complimentary binding regions for miR-221, miR-222, and miR-146 and therefore altered the miRNA–target interactions in these tumours. More work will need to be done in order to understand how these miRNAs contribute to cancer progression by down-regulating the *KIT* oncogene. Therefore, miRNAs might be powerful drug targets that could be used in a broad range of cancer therapies.

### miRNA profiling might aid cancer diagnosis

Northern-blot analyses and miRNA microarrays have been useful in determining tissue-specific 'signatures' of miRNA genes in humans<sup>37,44,46,96,120–128</sup>. The distinct miRNA-expression profiles that have been observed in certain organs underscore the importance of miRNAs in stem-cell maintenance and in directing the differentiation of certain cell types during development. Researchers are now using miRNA-expression signatures to classify cancers and to define miRNA markers that might predict favourable prognosis<sup>81,84,95,98,100,101,109,110,112</sup>.

A recent report from Lu *et al.* found that the expression profiles of relatively few miRNAs (~200 genes), were required to accurately classify human cancers<sup>98</sup>. This group designed a novel method, which is based on bead-based flow cytometry, to study the expression of miRNAs in both normal and cancerous tissues. Tumours that originated from various tissues were clustered solely on the basis of their miRNA-expression profiles. This resulted in tumours being grouped according to their embryonic lineage. For example, tumours of endothelial origin such as colon, liver, **pancreas** and **stomach** were clustered together, and tumours of haematopoietic origin also clustered together. Interestingly, when the expression profiles of about 16,000 protein-coding mRNA genes were analysed in the same tumour dataset, hierarchical clustering was unable to group tumours in a coherent manner — for example, gastrointestinal-derived tumours could not be grouped together. Therefore, miRNA signatures within tumours reflect their developmental history, a hypothesis that is compatible with the evidence that miRNAs direct tissue-specific developmental functions.

These researchers also compared the miRNA-expression profiles of tumour samples versus normal tissue and found that 129 out of 217 miRNAs had a lower level of expression in tumours regardless of the tissue of origin. Therefore, in a global sense, miRNAs might function to drive cells into a more differentiated state, and the expression profiles of miRNAs in tumours compared with normal tissues might represent the degree of differentiation in those cells. These studies establish miRNAs as 'oncomirs' and imply that

#### Bead-based flow cytometry

A technique that is used to profile miRNA gene expression and miRNA abundance. This method couples oligonucleotides that are complementary to the miRNAs of interest onto polystyrene beads that are impregnated with a variable mixture of two fluorescent dyes (yielding 100 colours to differentiate up to 100 individual miRNAs). Purified miRNAs from tissue samples are hybridized to the oligo-coupled beads and the fluorescent intensity is measured using a flow cytometer.



### Anti-miRNA oligonucleotides

Oligonucleotides of about 22 nucleotides that are complementary to a given miRNA sequence and can specifically inhibit miRNA activity. RNA modifications that are made to the ribose group and target the 2' position (such as in 2'-O-methyl oligos or those with a 2'-O, 4'-C-methylene bridge) increase the stability, efficacy and affinity of the oligonucleotide.

abnormalities in miRNA expression might directly result in the de-differentiation of cells, allowing tumour formation to occur.

An established library of miRNA signatures or miRNA-expression profiles, assigned for each class of tumour, might help both the diagnosis and treatment of cancer. These libraries could easily be established as miRNAs can be isolated from formalin-fixed paraffin-embedded archival tissues<sup>120</sup>. The differential expression of certain miRNAs has already been shown to be an accurate predictor of a patient's overall prognosis. From a therapeutic standpoint, miRNA-expression profiling might prove to be a powerful tool for clinicians when defining a treatment regimen for their patients. As proof of principle, Lu *et al.* found that miRNA signatures could successfully categorize poorly differentiated tumours that were considered histologically non-diagnostic into their appropriate tissue-specific lineages<sup>98</sup>.

### Future approaches using miRNA therapies

The emergence of miRNAs as important cancer-prevention genes is likely to have a large effect on gene therapies that are designed to block tumour progression. Large-scale expression screens, similar to those described above that compare miRNA levels in tumours versus normal tissues, will be useful in identifying novel miRNAs that are involved in cancer. Also, functional screens that are designed to select miRNA genes that specifically control cancer-related processes such as cell proliferation and apoptosis, similar to those that were conducted by Cheng *et al.*<sup>99</sup>, will also help in this search. In the future, the administration of synthetic anti-sense oligonucleotides that encode sequences that are complementary to mature oncogenic miRNAs

— termed anti-miRNA oligonucleotides (AMOs) — might effectively inactivate miRNAs in tumours and slow their growth. In the clinic, miRNA inactivation could be accomplished through frequent or continuous delivery of 2'-O-methyl or locked-nucleic-acid antisense oligonucleotides, which are specifically designed to be more stable and less toxic than other cancer treatments in order to target transforming miRNAs such as *mir-155*.

The use of antagomirs, which are AMOs that are conjugated with cholesterol, has recently emerged as an effective approach to inhibit miRNA activity in various organs when injected into mice, and might be promising therapeutic agents<sup>129</sup>. Conversely, techniques to overexpress miRNAs that function as tumour suppressors, such as those that are encoded by the *let-7* family, could be used to treat specific tumour types. Transient expression systems that use viral or liposomal delivery might be useful approaches for administering large quantities of miRNAs. These techniques would involve expressing the pre-miRNA hairpin and flanking sequences, potentially under the control of tissue-specific promoters, to stimulate the endogenous miRNA machinery to direct proper miRNA processing and gene-targeted repression. However, employing similar methods that use siRNAs as cancer gene therapies have shown that the immune response can limit the effectiveness of RNA delivery<sup>130,131</sup>. More development of these methods is needed before miRNA treatments can move from the laboratory bench to the bedside. Whether miRNAs become the 'magic bullet' of the future remains to be seen, but research in this area will undoubtedly provide insight into the underlying mechanisms of oncogenesis.

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#### Competing interests statement

The authors declare no competing financial interests.

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