LETTERS

c-Myc-regulated microRNAs modulate E2F1 expression

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MicroRNAs (miRNAs) are 21–23 nucleotide RNA molecules that regulate the stability or translational efficiency of target messenger RNAs[1]. miRNAs have diverse functions, including the regulation of cellular differentiation, proliferation and apoptosis[2]. Although strict tissue- and developmental-stage-specific expression is critical for appropriate miRNA function, mammalian transcription factors that regulate miRNAs have not yet been identified. The proto-oncogene *c-MYC* encodes a transcription factor that regulates cell proliferation, growth and apoptosis[3]. Dysregulated expression or function of c-Myc is one of the most common abnormalities in human malignancy[4]. Here we show that c-Myc activates expression of a cluster of six miRNAs on human chromosome 13. Chromatin immunoprecipitation experiments show that c-Myc binds directly to this locus. The transcription factor E2F1 is an additional target of c-Myc that promotes cell cycle progression[5–7]. We find that expression of E2F1 is negatively regulated by two miRNAs in this cluster, miR-17-5p and miR-20a. These findings expand the known classes of transcripts within the c-Myc target gene network, and reveal a mechanism through which c-Myc simultaneously activates E2F1 transcription and limits its translation, allowing a tightly controlled proliferative signal.

c-Myc is a helix–loop–helix leucine zipper transcription factor that regulates an estimated 10–15% of genes in the human and *Drosophila* genomes[7–9]. Both c-Myc and miRNAs have been shown to influence cell proliferation and death, and select miRNAs are known to have abnormal expression in human malignancies[4,10]. We thus sought to determine whether c-Myc regulates miRNA expression.

A spotted-oligonucleotide array capable of measuring the expression of 235 human, mouse or rat miRNAs was generated and used to analyse a previously described human B-cell line, P493-6, that harbors a tetracycline-repressible *c-MYC* transgene[11] (Fig. 1a, b). miRNA expression profiles were analysed in tetracycline (tet)-treated (low c-Myc) or untreated (high c-Myc) cells. Six upregulated miRNAs were consistently observed in the high c-Myc state: miR-17-5p, miR-18, miR-19, miR-20, miR-92 and miR-106. These miRNAs are encoded by three paralogous clusters located on chromosome 13 (the *mir-17* cluster), the X chromosome (the *mir-106a* cluster) and chromosome 7 (the *mir-106b* cluster, Fig. 2a). As the array did not detect upregulation of miR-25, which is encoded by the *mir-106b* cluster, we focused our analyses on the *mir-17* and *mir-106a* clusters. Northern blotting confirmed that the miRNAs contained within these clusters were upregulated in the high c-Myc state (Fig. 2b). However, miR-17-3p, which has been reported to be

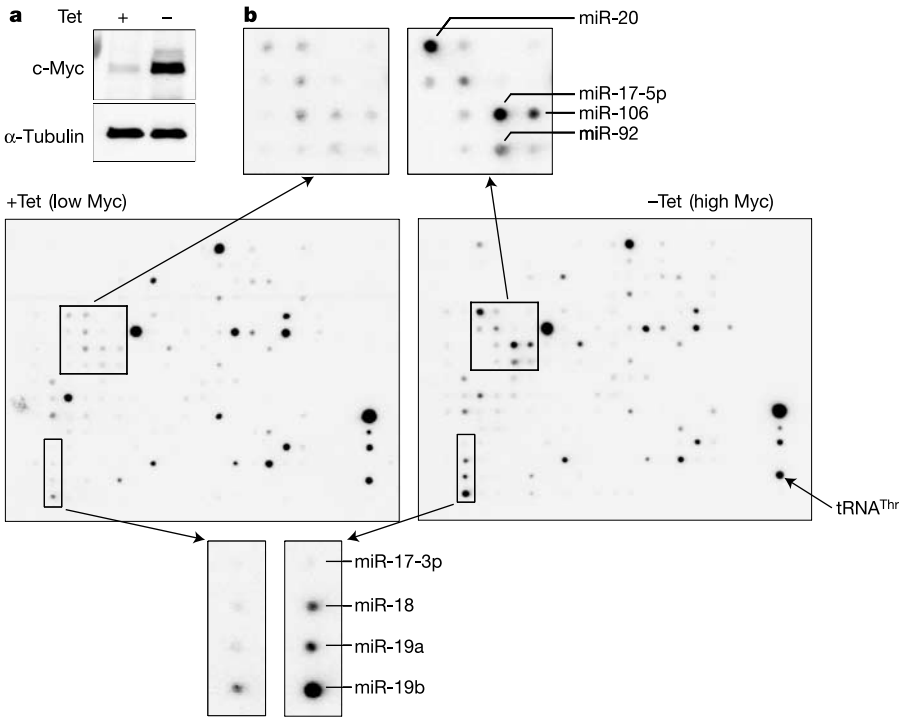

Figure 1 | MicroRNA expression profiling of P493-6 cells with high and low c-Myc expression. **a**, Western blot analysis of c-Myc in untreated cells or in cells treated with tetracycline (Tet). Blots were stripped and reprobed for α-tubulin to demonstrate equal loading of samples. **b**, MicroRNA expression arrays hybridized with RNA from tet-treated (+Tet) or untreated (−Tet) cells. Magnified panels show miRNAs that were consistently upregulated in the high c-Myc state. A probe complementary to threonine tRNA ($tRNA^{Thr}$) served as a control for equal hybridization.

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expressed from the *mir-17* cluster, was not detectable in P493-6 cells, suggesting that it might be a miRNA* sequence (the reverse-complement strand of a miRNA; Fig. 1b and data not shown).

miRNAs are transcribed by RNA polymerase II as long primary transcripts (pri-miRNAs) that undergo sequential processing to produce mature miRNAs[12–16]. Probes for northern blotting were designed to detect pri-miRNA transcripts from the *mir-17* and *mir-106a* clusters. These probes were complementary to unique sequence immediately upstream of the first pre-miRNA hairpin in each cluster. The *mir-17* cluster-specific probe detected three transcripts (approximately 3.2, 1.3 and 0.8 kilobases (kb) in size) that were induced in the high c-Myc state (Fig. 2c). It has been previously reported that the *mir-17* cluster is contained within an alternatively spliced host transcript termed *C13orf25* (ref. 17). The observed transcripts represent alternatively spliced 5′-cleavage products of *C13orf25* that remain following excision of pre-miRNAs (our unpublished observations). A similar probe complementary to sequence immediately upstream of the *mir-106a* cluster did not detect any transcripts in P493-6 cells (data not shown). These data demonstrate that the *mir-17* cluster is upregulated in the high c-Myc state.

In order to confirm that regulation of the *mir-17* cluster by c-Myc was not restricted to P493-6 cells, we examined levels of miR-18 and miR-20 in previously described wild-type rat fibroblasts (TGR), rat fibroblasts containing a homozygous deletion of *c-Myc* (HO15.19), or *c-Myc* null fibroblasts reconstituted with wild-type c-Myc (HO15.19-MYC)[18,19]. miR-18 and miR-20 were expressed at approximately 50% of wild-type levels in the absence of c-Myc, but wild-type expression levels of these miRNAs were restored in the c-Myc-reconstituted null cells (Fig. 2d).

We next performed chromatin immunoprecipitation (ChIP) experiments in P493-6 cells to determine whether human c-Myc binds directly to the *mir-17* cluster genomic locus. First, 10 kb of sequence on chromosome 13 surrounding the *mir-17* cluster was examined for putative c-Myc-binding sites. c-Myc is known to bind to the canonical E-box sequence CACGTG, as well as to non-canonical sequences including CATGTG[20]. We identified seven putative binding sites matching these sequences. Four of these sites were conserved between human and mouse, and located within a 30-base-pair window of at least 65% nucleotide identity between these species (Fig. 3a, labelled in red). Real-time polymerase chain reaction (PCR) amplicons were designed to assay for all putative binding sites (both conserved and non-conserved) in ChIP samples. Background signals were very low at all tested amplicons in negative control samples immunoprecipitated without antibody or with an antibody directed against hepatocyte growth factor (HGF), which is not expressed in these cells (data not shown). We obtained clear evidence for *in vivo* association of c-Myc with a region containing a conserved CATGTG sequence 1,480 nucleotides upstream of *mir-17-5p* (Fig. 3b, amplicon 3). This site is located in intron 1 of *C13orf25*. c-Myc is known to frequently bind to sites in the first intron of its transcriptional target genes[20]. As we were not able to design amplicons between nucleotides −1500 and −3280 (owing to the presence of a CpG island that prevented efficient amplification), we cannot rule out the possibility that c-Myc also binds within this region. Our data demonstrate that c-Myc binds directly to the *mir-17* cluster locus, providing strong evidence that these miRNAs are directly regulated by this transcription factor.

Seven putative binding sites in the vicinity of the *mir-106a* cluster were also identified and assayed for c-Myc binding. No ChIP signals were observed, consistent with the northern blot data showing an absence of detectable transcripts produced from this locus in P493-6 cells (data not shown).

The behaviour of the *mir-17* cluster was also examined during serum stimulation in primary human fibroblasts. Serum deprivation followed by serum stimulation of fibroblasts results in a transient induction of c-Myc[21] (Fig. 3c). Real-time PCR analysis demonstrates that under these conditions, expression of the *mir-17* host transcript is induced with similar kinetics (Fig. 3d). Consistent with the behaviour of other known c-Myc target genes, expression levels remain elevated after c-Myc levels decrease[22]. Furthermore, ChIP analysis demonstrates that association of c-Myc with the *mir-17* genomic locus mirrors c-Myc expression and coincides with induction of miRNA cluster expression (Fig. 3e). These results provide further evidence that the *mir-17* cluster is directly regulated by c-Myc, and show that induction of these miRNAs is a physiologic response to growth stimuli.

To study the functional consequences of induction of the *mir-17* cluster by c-Myc, we examined mRNAs that are predicted targets of these miRNAs. The transcription factor E2F1, which is predicted to be regulated by miR-17-5p and miR-20a (ref. 23), was initially chosen for further analysis. E2F1 expression promotes G1-to-S phase progression in mammalian cells by activating genes involved in DNA replication and cell cycle control[5]. Expression of the *E2F1* gene is known to be induced by c-Myc[6,7]. c-Myc expression is also induced by E2F1, revealing a putative positive feedback circuit[21]. We hypothesized that negative regulation of *E2F1* translation by miR-17-5p and miR-20a provides a mechanism to dampen this reciprocal activation, promoting tightly controlled expression of c-Myc and E2F1 gene products.

To determine whether *E2F1* is a target of miR-17-5p and miR-20a,

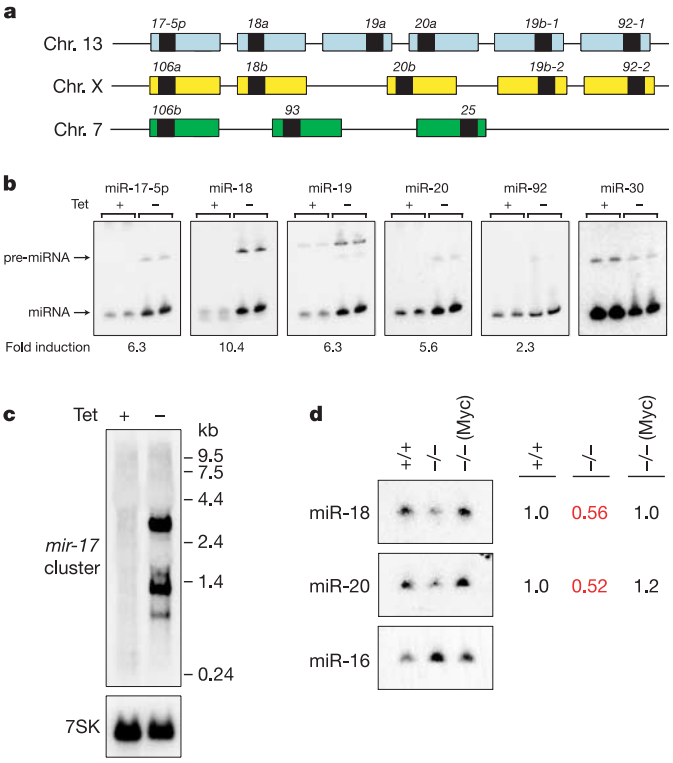

Figure 2 | c-Myc induces expression of the miR-17 cluster. a, Schematic representation of the *mir-17*, *mir-106a* and *mir-106b* clusters. *mir-18b* and *mir-20b* are predicted on the basis of homology to *mir-18a* and *mir-20a*, respectively[30]. **b**, Northern blot analysis of miRNAs in P493-6 cells. Duplicate samples are shown, and miR-30 served as a loading control. Blots were also probed for miR-16 and miR-29 as loading controls, and similar results were obtained (data not shown). **c**, Northern blot analysis of total RNA from P493-6 cells with a probe specific for the *mir-17* cluster. 7SK RNA served as a loading control. **d**, Northern blot analysis of miRNAs in wild-type rat fibroblasts (+/+), rat fibroblasts with a homozygous deletion of *c-Myc* (−/−), or knockout fibroblasts reconstituted with wild-type c-Myc (−/−(Myc)). Quantification of radioactive signal intensity is shown on the right.

we used HeLa cells, which naturally express the *mir-17* cluster. 2′-*O*-methyl antisense oligoribonucleotides, which have been shown to block miRNA function[24,25], were designed to inhibit miR-17-5p and miR-20a. To monitor the degree of miRNA inhibition, we generated sensor constructs with sites perfectly complementary to miR-17-5p or miR-20a in the 3′-untranslated region (UTR) of firefly luciferase. When introduced into HeLa cells, these constructs showed an 80–90% reduction in luciferase activity compared with control constructs containing the reverse-complement sequence of the miRNA-binding sites; this demonstrates efficient downregulation by endogenous miR-17-5p and miR-20a. Co-transfection of these plasmids with miR-17-5p or miR-20a antisense oligonucleotides, but not scrambled oligonucleotides, enhanced expression of the sensor constructs, indicating inhibition of these miRNAs (Fig. 4a). Because of nucleotide similarity between miR-17-5p and miR-20a, both were inhibited by antisense oligonucleotides directed against either miRNA. Transfection with miR-17-5p and miR-20a antisense oligonucleotides, but not scrambled oligonucleotides, resulted in an approximately fourfold increase in E2F1 protein levels without altering *E2F1* mRNA abundance (Fig. 4b, c).

We also determined the consequence of overexpressing the *mir-17* cluster on E2F1 expression. The entire *mir-17* cluster and approximately 150 nucleotides of flanking sequence were cloned into a mammalian expression vector, under the control of a cytomegalovirus (CMV) promoter. When transfected into HeLa cells, this construct (CMV-*mir-17* cluster) produces the appropriately processed miRNAs, as assessed by northern blotting (Fig. 4d and not shown). Transient overexpression of these miRNAs resulted in a 50% decrease in E2F1 protein levels (Fig. 4e) without affecting *E2F1* mRNA abundance (Fig. 4f).

To demonstrate that miR-17-5p and miR-20a directly regulate E2F1 expression, luciferase reporter constructs containing a portion of the *E2F1* 3′-UTR were generated and mutations were introduced into the predicted miRNA-binding sites (see Supplementary Fig. 1a, b). The mutant construct yielded approximately threefold higher luciferase expression compared with the wild-type construct when transfected into HeLa cells, providing evidence that the endogenously expressed miRNAs decrease E2F1 expression by recognizing these sites (see Supplementary Fig. 1c).

Last, we examined *E2F1* mRNA and protein levels in P493-6 cells with high and low c-Myc expression (leading to high and low expression of the *mir-17* cluster, respectively). Consistent with previously reported data[6,7], c-Myc potently induces *E2F1* mRNA (Fig. 4g). Remarkably, E2F1 protein levels were only modestly induced under these conditions, suggesting a greatly reduced translational yield (Fig. 4h). Taken together with the results from HeLa cells, these data support a model in which miR-17-5p and miR-20a limit c-Myc-mediated induction of E2F1 expression, preventing uncontrolled reciprocal activation of these gene products. As E2F1 protein is known to accumulate late in G1, and c-Myc (and consequently the *mir-17* cluster) are activated early in G1 (refs 5, 21), we speculate that E2F1 translational efficiency is decreased, but not completely inhibited, by these miRNAs during normal cell-cycle progression. Consistent with a dampened translational efficiency, E2F1 protein accumulation is delayed relative to *E2F1* mRNA induction during a time course of serum stimulation in primary fibroblasts. In contrast, c-Myc protein levels closely mirror mRNA levels under these conditions (see Supplementary Fig. 2). As several other documented c-Myc target genes are also predicted targets of the *mir-17* cluster (for example, *RPS6KA5, BCL11B, PTEN* and *HCFC2*)[20,23], a widespread mechanism may exist through which c-Myc and other transcription factors precisely control expression of target genes by simultaneously activating their transcription and limiting their translation.

These results identify miRNAs as targets of c-Myc, expanding the known classes of transcripts within the c-Myc target gene network. Furthermore, they suggest that the *mir-17* cluster, by decreasing E2F1 expression, tightly regulates c-Myc-mediated cellular proliferation.

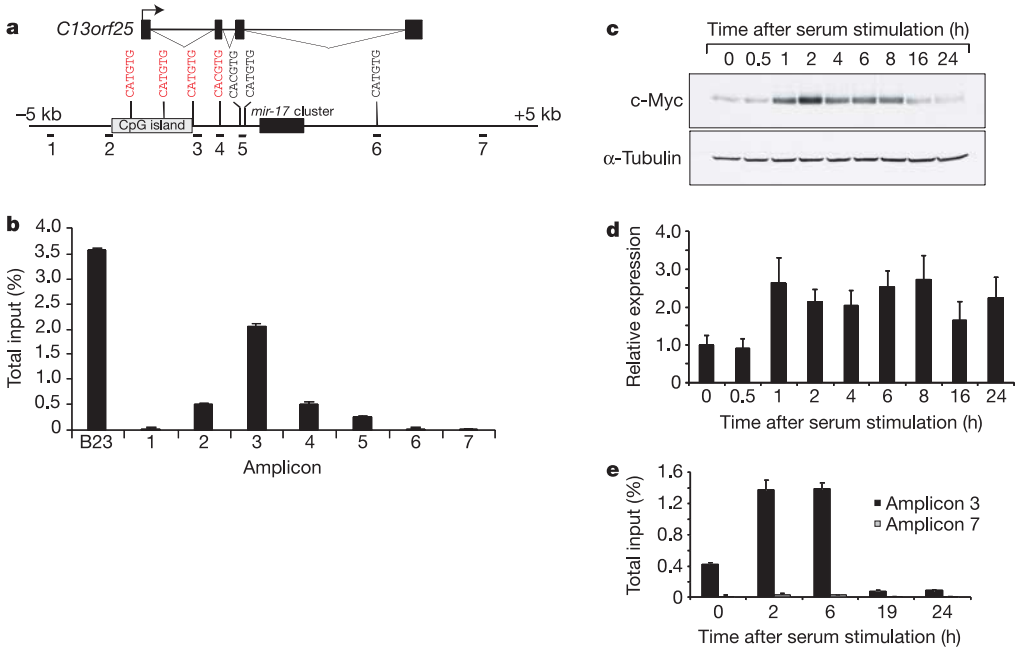

Figure 3 | c-Myc binds directly to the *mir-17* cluster genomic locus. **a**, Schematic representation of the genomic interval encompassing the *mir-17* cluster. Putative c-Myc binding sites are indicated (CACGTG or CATGTG); those in red are conserved between human and mouse. The location and structure of *C13orf25* is indicated. Real-time PCR amplicons are represented by numbered lines. **b**, Real-time PCR analysis of c-Myc chromatin immunoprecipitates. Amplification of a validated c-Myc-binding site in intron 1 of the B23 gene served as a positive control[22]. **c**, Western blot analysis of c-Myc protein levels following serum stimulation of primary human fibroblasts. **d**, **e**, Real-time PCR analysis of *mir-17* cluster expression (**d**) and c-Myc chromatin immunoprecipitates in serum-stimulated fibroblasts (**e**). Error bars for all panels represent standard deviations derived from at least three independent measurements.

In this context, these miRNAs might have tumour suppressor activity. Accordingly, loss-of-heterozygosity of the chromosomal region encompassing the *mir-17* cluster (13q31) has been observed in human malignancies[26]. However, amplification of this region and overexpression of *C13orf25*, the host transcript of the *mir-17* cluster, has been described in diffuse large B-cell lymphoma[17], and miR-19a and miR-92-1 have been shown to be upregulated in B-cell chronic lymphocytic leukaemia[27]. These observations suggest that miRNAs might also possess oncogenic activity. It is thus likely that these miRNAs influence cell proliferation and tumorigenesis in a cell-type-specific manner, depending on the milieu of target mRNAs that are expressed. The results described here provide an experimental framework for further functional dissection of this miRNA cluster, in order to fully delineate its role in normal cellular physiology and malignancy.

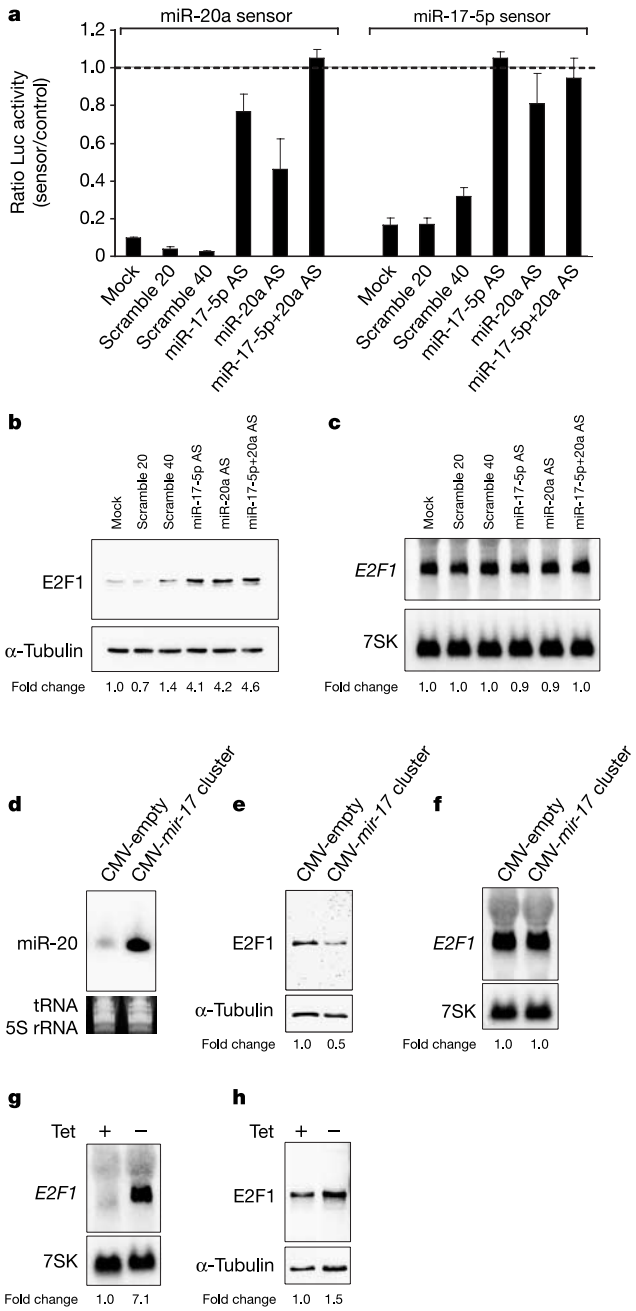

Figure 4 | miR-17-5p and miR-20a regulate E2F1 translational yield. **a**, Inhibition of miR-17-5p and miR-20a by 2′-*O*-methyl-oligoribonucleotides. Sensor or control luciferase constructs were transfected into HeLa cells alone (mock) or with the following oligonucleotides: scrambled nucleotide at 20 or 40 pmol, or 20 pmol of miR-17-5p or miR-20a antisense (AS), either individually (miR-17-5p AS or miR-20a AS) or pooled (miR-17-5p + 20a AS). The ratio of normalized sensor to control luciferase activity is shown. Error bars represent standard deviations. **b**, **c**, Western blot (**b**) and northern blot (**c**) analysis of E2F1 in antisense-treated HeLa cells. **d**, Northern blot analysis of miR-20 in transfected HeLa cells. **e**, **f**, Western blot (**e**) northern blot (**f**) analysis of E2F1 in transfected HeLa cells. **g**, **h**, Northern blot (**g**) and western blot (**h**) analysis of E2F1 in P493-6 cells. Fold changes shown are mean values derived from three experiments.

METHODS

Tissue culture. P493-6 cells (from D. Eick) were cultured in RPMI 1640 media supplemented with 10% fetal bovine serum (FBS), penicillin and streptomycin. To repress *c-MYC* expression, cells were grown in the presence of 0.1 μg ml^{-1} tetracycline (Sigma) for 72 h. TGR-1 cells (wild-type rat fibroblasts) and HO15.19 cells (*c-Myc*$^{-/-}$ rat fibroblasts) were a gift from J. Sedivy. Rat cells, HeLa cells, and primary human fibroblasts (obtained from ATCC) were grown in Dulbecco's Modified Eagle's Medium (DMEM) with 10% FBS, penicillin and streptomycin. For serum stimulation experiments, primary fibroblasts were grown in DMEM with 0.1% FBS for 48 h. DMEM containing 10% FBS was then added and cells were collected at the indicated time points.

miRNA expression profiling. miRNA expression arrays were generated and probed essentially as described[28], with the following modifications. Oligonucleotide probes (each a concatemerized triple repeat of sequence antisense to a mature miRNA) were synthesized and spotted at a concentration of 10 μM on GeneScreen Plus membranes (Perkin Elmer) with a 96-pin spotter (V&P Scientific). In addition to 235 miRNA probes, 25 probes each containing three mismatches were spotted to assess hybridization specificity. These probes produced significantly less signal intensity (in general, an approximately tenfold decrease) compared with wild-type probes. A probe complementary to tRNA^{Thr} was used to control for hybridization efficiency. All probe sequences are provided in Supplementary Table 1. Total RNA (10 μg) was isolated with Trizol Reagent (Invitrogen) and samples were enriched for low molecular weight RNA using a Microcon YM-100 column (Amicon). The resulting RNA was end-labelled with γ-[^{32}P]-ATP using the KinaseMax kit (Ambion). After removing unincorporated radionucleotide using Microspin G-25 columns (Amersham), labelled RNA was hybridized to membranes using MicroHyb buffer (Invitrogen). Signals were quantified using a Personal FX phosphoimager (Bio-Rad).

Northern blot analysis. For miRNA northern blots, 20 μg of total RNA was separated on 15% denaturing polyacrylamide gels, electrotransferred to GeneScreen Plus membranes, and hybridized using UltraHyb-Oligo buffer (Ambion). Oligonucleotides complementary to mature miRNAs, and end-labelled with T4 Kinase (Invitrogen), were used as probes. Probe sequences were as follows: miR-17-5p, 5′-ACTACCTGCACTGTAAGCACTTTG-3′; miR-18a, 5′-TATCTGCACTAGATGCACCTTA-3′; miR-19a, 5′-TCAGTTTTG-CATAGATTTGCACA-3′; miR-20a, 5′-CTACCTGCACTATAAGCACTTTA-3′; miR-92, 5′-ACAGGCCGGGACAAGTGCAATA-3′, miR-30c, 5′-GCTGAGAG TGTAGGATGTTTACA-3′; miR-16, 5′-CGCCAATATTTACGTGCTGCTA-3′.

For conventional northern blotting, 20 μg of total RNA was separated on 1.2% formaldehyde-agarose gels, transferred to GeneScreen Plus, and hybridized with randomly primed labelled probes using Ultrahyb buffer. Probes were generated by PCR using the following primers: *mir-17* cluster probe, sense 5′-ACATGGAC TAAATTGCCTTTAAATG-3′, antisense 5′-AATCTTCAGTTTTACAAGGTG ATG-3′; *mir-106a* cluster probe, sense 5′-CATCCTGGGTTTTACATGCTCC-3′, antisense 5′-CAAAATTTTAAGTCTTCCAGGAGC-3′; 7SK RNA probe, sense 5′-GACATCTGTCACCCCATTGATC-3′, antisense 5′-TCTGCAGTC TTGGAAGCTTGAC-3′; *E2F1* probe, sense 5′-TGTGTGCATGAGTCCAT GTGTG-3′, antisense 5′-GCAAATCAAAGTGCAGATTGGAG-3′.

Western blot analysis. Antibodies for immunoblotting were as follows: anti-c-Myc mouse monoclonal (clone 9E10; Zymed), anti-E2F1 mouse monoclonal (clones KH20 and KH95; Upstate) and anti-α-tubulin mouse monoclonal (Calbiochem). Scanned images were quantified using Quantity One software (Bio-Rad).

Chromatin immunoprecipitation and real-time PCR. Cells were crosslinked with formaldehyde, and chromatin was immunoprecipitated as previously described[29]. Rabbit polyclonal c-Myc (sc-764, Santa Cruz Biotechnology) and human HGF antibodies (sc-7949, Santa Cruz) were used to immunoprecipitate chromatin fragments. Real-time PCR was performed using an ABI 7700 Sequence Detection System with the SYBR Green PCR core reagent kit (Perkin Elmer Applied Biosystems). Sequences of primers used to amplify ChIP samples

are provided in Supplementary Table 2. For quantification of the *C13orf25* transcript by real-time PCR, primers for amplicon 5 (Supplementary Table 2) were used. Reactions lacking reverse transcriptase were performed in parallel, to ensure that amplified fragments were derived from complementary DNA.

Oligoribonucleotides, sensor plasmids and luciferase assays. 2′-O-methyl oligoribonucleotides (scramble, 5′-AAAACCUUUUGACCGAGCGUGUU-3′; miR-17-5p antisense, 5′-ACUACCUGCACUGUAAGCACUUUG-3′; miR-20a antisense, 5′-CUACCUGCACUAUAAGCACUUUA-3′) were synthesized by Integrated DNA Technologies. Sensor and control luciferase constructs were made by ligating oligonucleotides containing two sites with perfect complementarity to miR-17-5p or miR-20a into the *Xba*I site of the pGL3-control vector (Promega). Twenty-four hours before transfection, HeLa cells were plated at 150,000 cells per well in a 24-well plate. Sensor or control plasmid (200 ng) plus 80 ng phRL-SV40 (Promega) were transfected alone or in combination with 20 or 40 pmol of 2′-*O*-methyl oligoribonucleotides, using Lipofectamine 2000 (Invitrogen). Luciferase assays were performed 24 h after transfection using the Dual Luciferase Reporter Assay System (Promega). Firefly luciferase activity was normalized to *renilla* luciferase activity for each transfected well. Two independent plasmid preparations were each transfected at least three times (on different days). Each transfected well was assayed in triplicate.

For analysis of E2F1 mRNA and protein levels, 200 pmol 2′-*O*-methyl oligoribonucleotides were transfected into HeLa cells growing in 6-well dishes (plated at 170,000 cells per well 24 h before transfection) using oligofectamine (Invitrogen). RNA and protein were collected 72 h after transfection.

Overexpression of the *mir-17* cluster. The *mir-17* cluster was amplified from genomic DNA and cloned into pcDNA3.1/V5-His-TOPO (Invitrogen). The following primers were used: sense 5′-CTAAATGGACCTCATATCTTTGAG-3′; antisense 5′-GAAAACAAGACAAGATGTATTTACAC-3′. The correct sequence of the amplified product was confirmed by sequencing. The expression plasmid was transfected into HeLa cells using HeLa Monster (Mirus).

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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