Supplementary Methods

RNA Isolation and Quantitative RT-PCR

Total RNA was isolated using Trizol reagent (Invitrogen) and reverse transcribed using random hexamers and superscript II reverse transcriptase (Invitrogen). qRT-PCR was performed in triplicate samples using the SYBR Green master mix (Applied Biosystems) and the BioRad iCycler. Results were normalized to GAPDH. miRNA quantitative PCR was done in triplicate using the TaqMan MicroRNA Assay from Applied Biosystems as per the manufacturer's instructions and normalized to U6. Sequences of primers are listed in Suppl. Table 4.

Transfection of miRNA Mimics, Antisense Oligonucleotides, siRNAs and expression plasmids

HepG2, WI-38 and IMR-90 cells were reverse transfected using Neofx (Ambion, Inc) as per the manufacturer's instructions. K562 cells were transfected using Amaxa nucleofection following the manufacturer's protocol. siRNAs targeting GFP (D-001940-01-05), E2F2 (On-targetplus SMARTpool L-003260-00-005) or MYC (On-targetplus SMARTpool L-003282-00-0005) were purchased from Dharmacon and transfected into K562 cells (1 x 10^6 cells) for 48 hr using Amaxa. In some experiments K562 cells (1 x 10^6 cells) were transfected with miR-24 or cel-miR-67 miRNA mimics (100 nM or indicated concentrations, Dharmacon), with or without a plasmid expressing HA-tagged E2F2 or EGFP (5 μg) for 48 hr using Amaxa. To determine the effect of miR-24 knockdown on E2F2 and MYC expression, K562 cells (1 x 10^6 cells/well) were transfected in triplicate wells with miR-24 or control ASO (100 nM, Ambion) using

Amaxa nucleofection following the manufacturer's protocol and 72 hr later treated with TPA (16 nM) for 6 hr. The cells were then harvested followed by qRT-PCR and Western blot analysis for MYC and E2F2.

Microarray Analysis

HepG2 cells (2.5 x 10^5/well) were reverse transfected in triplicate in six-well plates with either miR-24 mimics or control miRNA mimics (cel-miR-67) at a final concentration of 30 nM using NeoFx (Ambion). Total RNA isolated 48 hr post-transfection (independently for two experiments) was amplified, labeled and hybridized to Illumina arrays (Refseq-8). Raw hybridization intensity data were log-transformed and normalized to yield Z-ratios, which in turn were used to calculate a Z-ratio value for each gene. The Z-ratio was calculated as the difference between the observed gene Z-ratios for the experimental and the control comparisons, divided by the standard deviation associated with the distribution of these differences (Cheadle et al., 2003)**.** Z-ratio absolute values ≥1.5 were chosen as cut-off values, defining increased and decreased expression, respectively. The complete microarray data set is available at http://www.ncbi.nlm.nih.gov/geo.

Analysis of miR-24 target genes by target prediction algorithms and seed analysis

To determine whether a gene is a predicted target of miR-24, the presence of miR-24 binding sites was analyzed using TargetScan 4.2 (http://www.targetscan.org) (Lewis et al., 2003) or rna22 (http://cbcsrv.watson.ibm.com/rna22_targets.html (Miranda et al., 2006).

miR-24 binding sites in the miR-24 down-regulated mRNAs (Z-ratio>1.5) that had a sequence complementary to the miR-24 seed were identified by using PITA (http://132.77.150.113/pubs/mir07/mir07_prediction.html). The miR-24 mature miRNA sequence was obtained from miRBase (www.mirbase.com). The 3' UTR sequences in FASTA format were obtained from the UCSC Genome Browser [1] using RefSeq version (Release 34, [2]). UTR coordinate intervals were filtered through a Perl script to remove redundant UTRs from transcript variants and non-reference genomic sequences yielding a final set of 22,231 sequences (background set) after filtering. Occurrence and frequencies of the target nucleotide sequences (UGAGCC, CUGAGCC, and ACUGAGCC) were established for both the background set as well as the subset of 3'UTR sequences present in the miR-24-target gene set (see Suppl. Table 1). For each target sequence we compared the number of matches in the UTR sequences of both the target and background set to the number of all possible N-mer matches of the same size as the target sequence. The number of matches in the target UTR sequences was contrasted to their background distribution using a chi-square test in the R environment. Of the 249 target genes, 219 genes had an annotated, non-redundant UTR sequence.

Cell Cycle Analysis

HepG2 cells were reverse transfected with miR-24 mimics or control miRNA mimics as described above and 2 days later, treated with nocodazole (100 ng/ml) to synchronize cells in G2/M phase of the cell cycle. After 16 hr, cells were stained with propidium

iodide and analyzed by flow cytometry using a FACScaliber instrument (Becton Dickinson) and Cellquest Pro software following the manufacturer's protocol. To analyze changes in miR-24 expression with cell cycle progression, K562 cells were arrested in G2/M phase by treatment with nocodazole (100 ng/ml) for 16 hr, and then washed to remove nocodazole and grown in complete medium in the absence of nocodazole. Cells were collected at indicated times and analyzed for cell cycle distribution by propidium iodide staining and flow cytometry using FlowJo software and by qRT-PCR for miR-24 expression.

Luciferase Assay

HepG2 cells were reverse transfected (as above) in triplicate with 30 nM miR-24 mimic or control miRNA mimic. Two days later, cells were transfected using Lipofectamine 2000 (Invitrogen) with psiCHECK2 (Promega) vector (0.5 μg/well) containing a single copy of the predicted MREs or the full-length 3'UTR of indicated genes cloned into the multiple cloning site (Not1 and Xho1) of *Renilla* luciferase or control. After 24 hr luciferase activities were measured using the Dual Luciferase Assay System (Promega) and Top count NXT microplate reader (Perkin Elmer) per manufacturer's instructions. Data were normalized to *Firefly* luciferase. The 3'UTR of miR-24 target genes was PCR amplified using human genomic DNA as template and primers containing the Not1 and Xho1 restriction enzyme sites at the 5'end. The PCR products were digested with Not1 and Xho1 and cloned into the 3'UTR of *Renilla* luciferase of pSICHECK2. Individual wild-type and mutant MREs were cloned into pSICHECK2 by annealing the forward and reverse oligonucleotides containing Not1 and Xho1 sticky ends, followed by

phosphorylation (using T4 polynucleotide kinase (New England Biolabs)) and ligation (quick DNA ligase (New England Biolabs)). The wild-type short fragment in CCNA2 3'UTR (containing WT CCNA2 MRE1) was cloned by PCR and the mutant short CCNA2 3'UTR fragment (containing MT CCNA2 MRE1) was cloned by oligonucleotide annealing as mentioned above. Sequences of primers and oligonucleotides used for cloning are provided in Suppl. Table 5.

Immunoblot

Whole cell lysates, prepared using RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris pH 8.0), were resolved on 10% SDS-PAGE gels and analyzed by immunoblot probed with antibodies to MYC (Santa Cruz Biotechnology), E2F2 (Sigma), Cyclin A (Santa Cruz Biotechnology), HA (Roche), CHEK1, PCNA, BRCA1, AURKB, CDC2 and FEN1 (Cell Signaling Technology). α-Tubulin and HuR (Santa Cruz Biotechnology) served as internal controls. All antibodies were used at a dilution of 1:500. Western blots were quantified by densitometry relative to α-tubulin.

Thymidine Incorporation Assay

To measure the effects of miR-24 on cell proliferation, K562 cells (1 x 10^6 cells/well) were transfected with miR-24 ASO (100 nM) or control ASO using Amaxa nucleofection following the manufacturer's protocol and 36 hr later treated with TPA (16 nM) for 2 hr. The cells in duplicate wells were then incubated with ^{3}H-Thymidine (2 μCi/well) for 2 hr and [^{3}H]-incorporation measured using a liquid scintillation counter (Beckman). The ratio

of [^{3}H]-incorporation in miR-24 ASO-transfected cells relative to that in cells treated with control ASO from 3 independent experiments was compared.

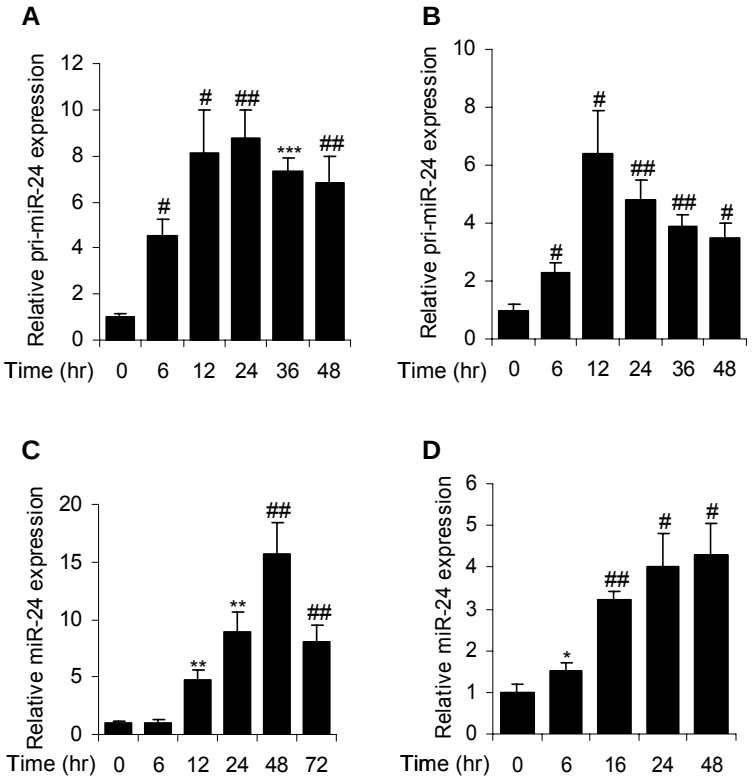
Supplementary references

Cheadle, C., Vawter, M. P., Freed, W. J., and Becker, K. G. (2003). Analysis of microarray data using Z score transformation. J Mol Diagn *5*, 73-81.

Miranda, K. C., Huynh, T., Tay, Y., Ang, Y. S., Tam, W. L., Thomson, A. M., Lim, B., and Rigoutsos, I. (2006). A pattern-based method for the identification of MicroRNA binding sites and their corresponding heteroduplexes. Cell *126*, 1203-1217.

Lewis, B. P., Shih, I. H., Jones-Rhoades, M. W., Bartel, D. P., and Burge, C. B. (2003). Prediction of mammalian microRNA targets. Cell *115*, 787-798.

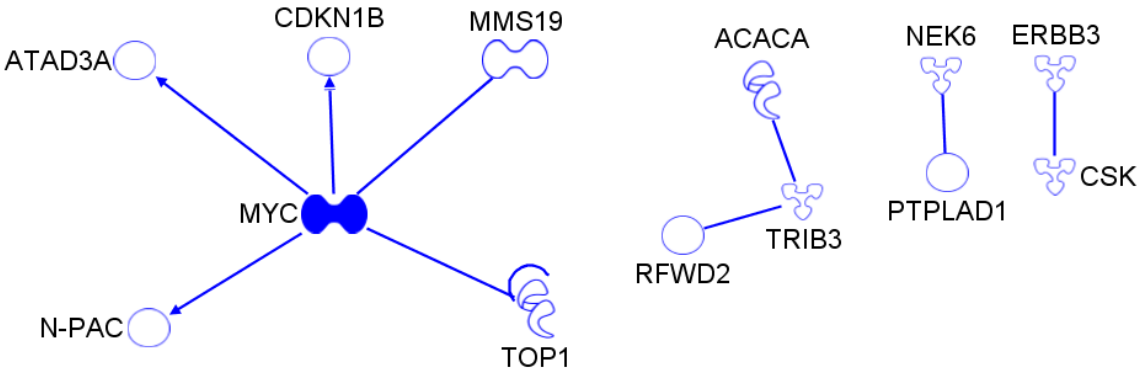
Supplementary Figure 1



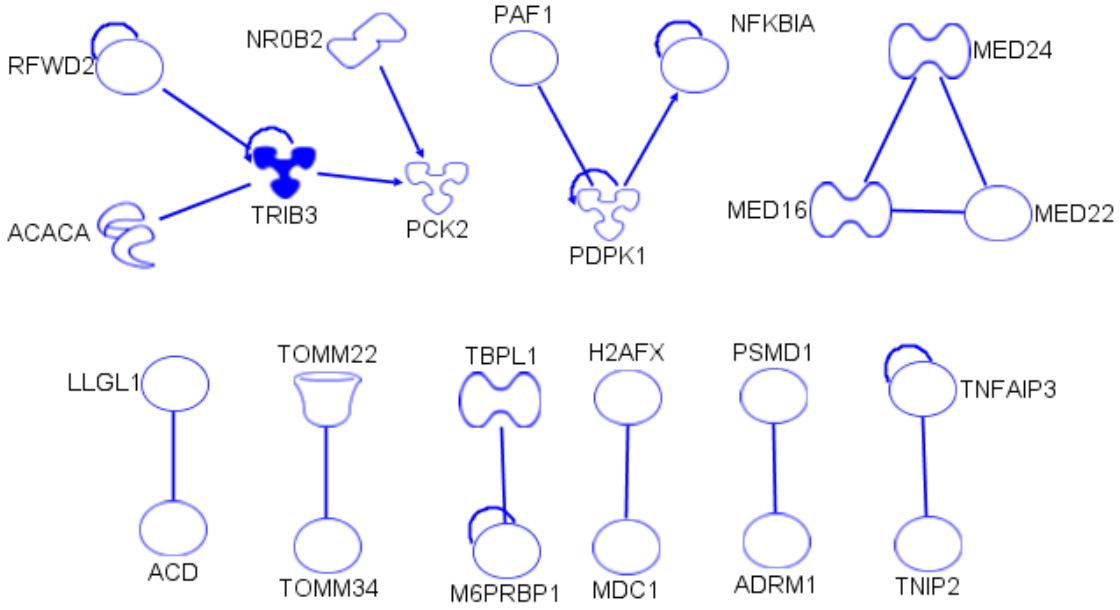
Supplementary Figure 1. Both the primary transcript corresponding to the chromosome 19 miR-24 cluster (A, K562; B, HL60) and mature miR-24 (C, K562; D, HL60) increase rapidly and remain elevated when cells are differentiated with TPA. Mature miR-24 expression was normalized to U6 whereas GAPDH was used as an internal control to measure changes in levels of pri-miR-24. Error bars in (A-D) represent standard deviation from 3 independent experiments (*, $p<0.05$; **, $p<0.01$; #, $p<0.005$; ##, $p<0.001$; ***, $p<0.0001$).

Supplementary Figure 2

A



B



Supplementary Figure 2. (A) Direct interaction network of over-represented subnetworks built from the 100 genes down-regulated by miR-24 and also predicted to be miR-24 targets by TargetScan 4.2. (B) Direct gene interaction small subnetworks built from the 248 mRNAs that are down-regulated in miR-24 over-expressing cells. The dominant highly connected network for the 248 miR-24 target mRNAs is shown in Fig. 3B (Ingenuity symbols: transcription factor, ; enzyme, ; kinase, ; ligand-dependant nuclear receptor ; transporter, ; other, ●).

Supplementary Figure 3

A

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  E2F2 MRE1  5′ GUGGGUGCU-CUGGGCUGAACCA   3′
(1401-1422)     ||   | || |||::|||| |||
     miR-24  3′ GACA-AGGACGACUUGACUCGGU   5′

  E2F2 MRE2  5′  –GCUCCUGUGGAAACAGGAGCCA  3′
  (754-775)       | |||||: |||    ||||||
     miR-24  3′ GACAAGGACGACUUGA—-CUCGGU  5′

  E2F2 MRE3  5′ -UGGCUCCUGAGCUGA-CUGA-CUG 3′
(1581-1602)      ||  ||||  ||||| |||| |
     miR-24  3′ GACA-AGGA—-CGACUUGACUCGGU 5′

  E2F2 MRE4  5′ AC—UCCUGACCUCAAGUGAUCCA   3′
(2568-2589)       |||||  || || ||| |||
     miR-24  3′GACAAGGAC—-GACUUGACUCGGU   5′

  E2F2 MRE5  5′ -CACAUCUCCAGCUGAGCU—-GCCG 3′
  (661-682)           ||| |||||:||  |||:
     miR-24  3′  GACA-—AGGACGACUUGACUCGGU 5′
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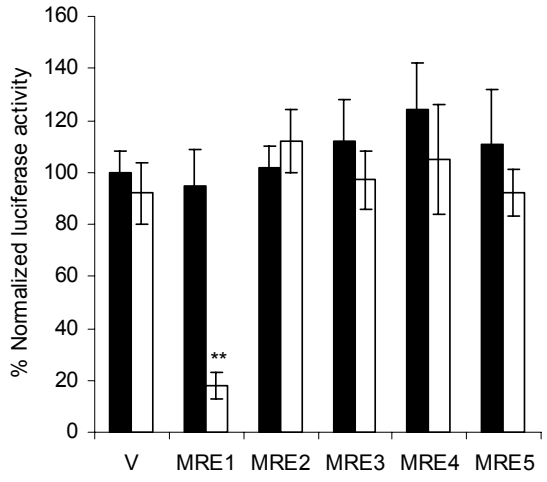
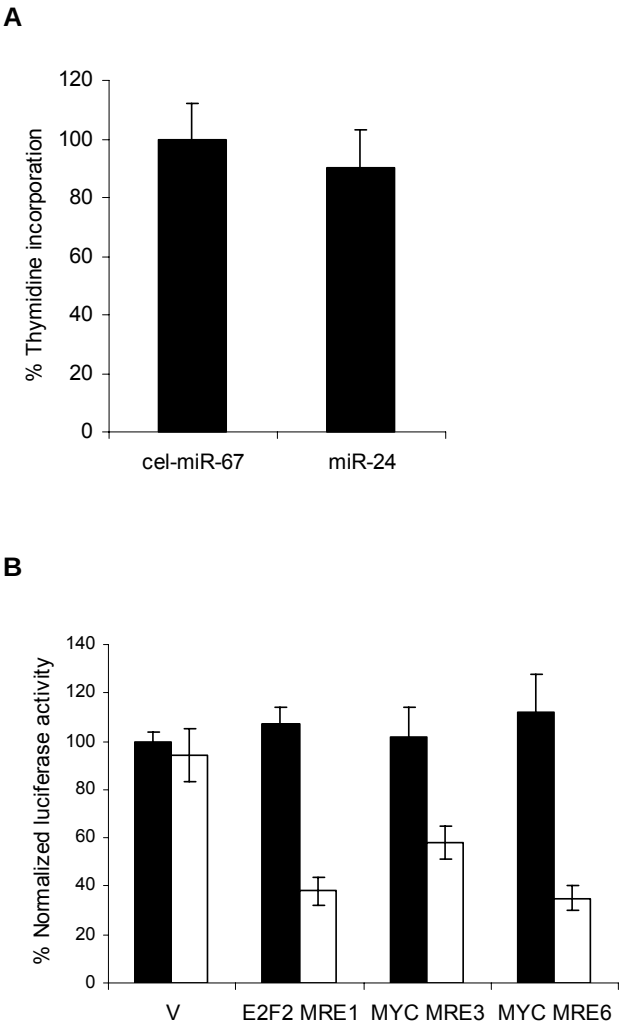
B



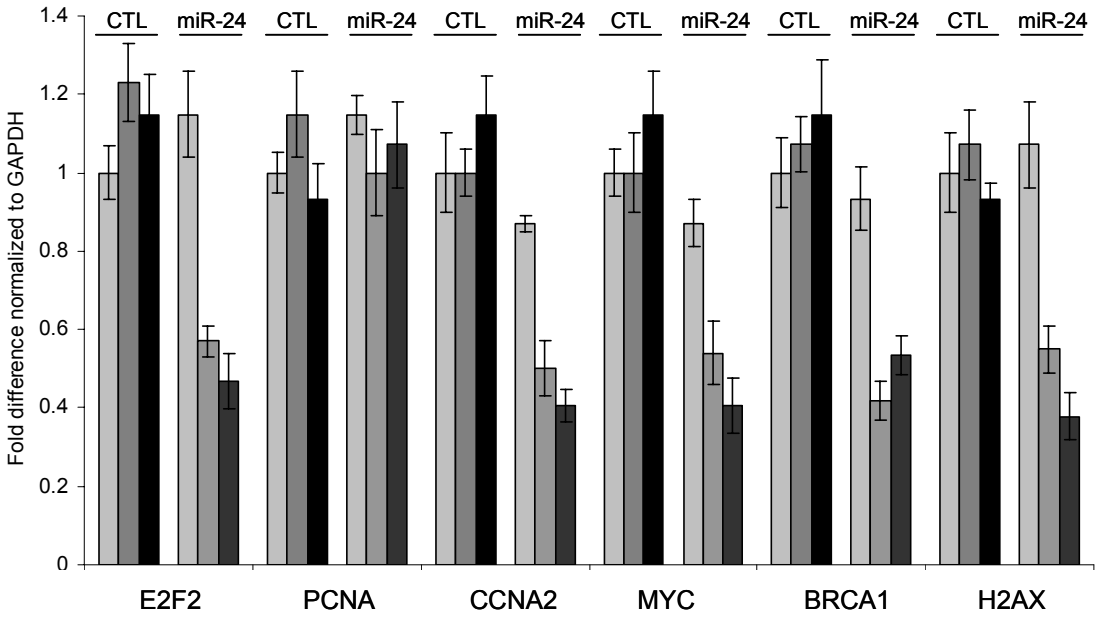
Supplementary Figure 3. (A) Candidate miR-24 microRNA recognition elements (MRE) in the 3'UTR of E2F2 mRNA predicted by rna22. Numbers in parenthesis represent the location in the E2F2 3'UTR**.** (B) Only E2F2 MRE1 was found to be repressed by miR-24 by luciferase assay. Inserting the other E2F2 MREs (MRE2-5) in the 3'UTR of a luciferase gene had no significant effect on luciferase expression in HepG2 cells after over-expressing miR-24. Data are an average of two independent experiments. ** $p < 0.01$

Supplementary Figure 4



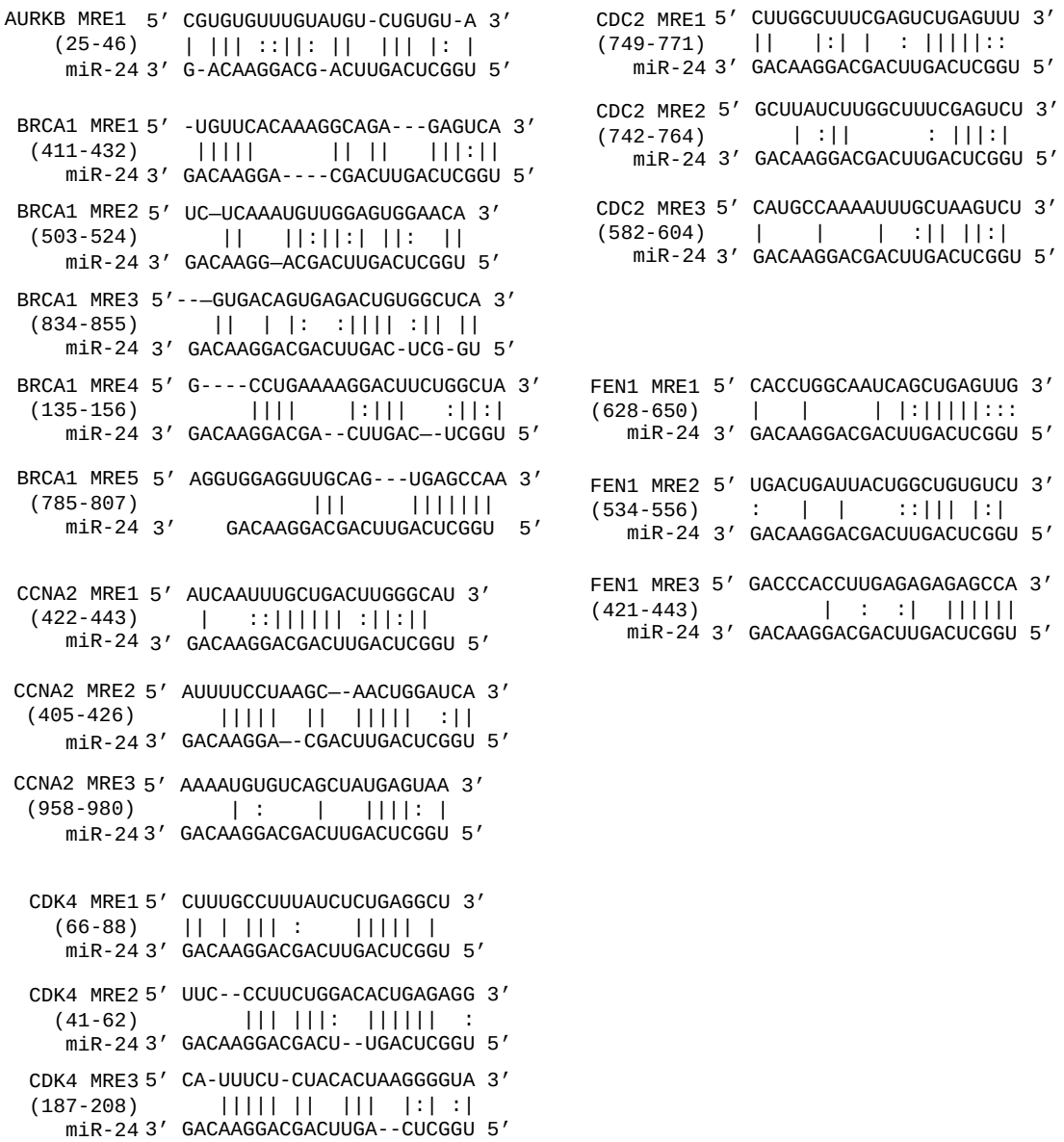
Supplementary Figure 4 miR-24 down-regulates luciferase expression from a reporter gene containing the MYC and E2F2 MREs, before its effect on cell proliferation can be detected. (A) 24 hr after ectopic introduction of miR-24 mimic into HepG2 cells there is no significant change in cellular proliferation compared to control mimic transduced cells as measured by thymidine uptake. (B) Luciferase assays performed 24 hr post transfection of HepG2 with miR-24 (white) or cel-miR-67 (black) show significant decreases in reporter expression from reporters encoding E2F2 MRE1 or MYC MRE3 or MRE6 in miR-24 over-expressing cells (white). Luciferase activity was normalized. The mean and S.D. of 3 independent experiments is shown.

Supplementary Figure 5



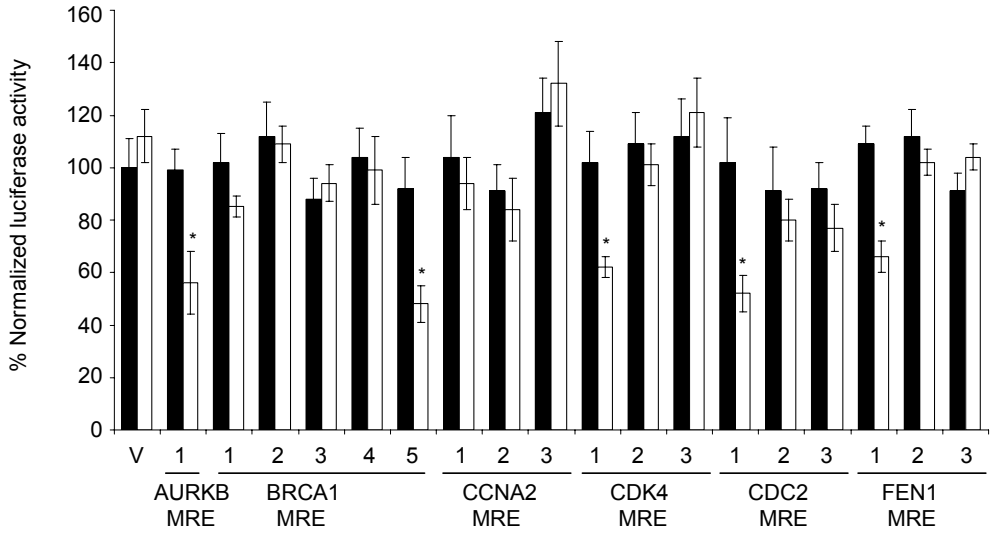
Supplementary Figure 5. miR-24 down-regulates target mRNAs when transfected at physiological levels. K562 cells were transfected with miR-24 or cel-miR-67 (CTL) mimics at 2 nM, 10 nM and 50 nM. miR-24 levels significantly increased when cells were transfected with 10 nM or 50 nM miR-24 (Fig. 5C). Target gene mRNA was assessed by qRT-PCR 72 hr later. E2F2, CCNA2, MYC, AURKB and H2AX mRNAs were down-regulated in cells transfected with 10 nM or 50 nM miR-24 mimic. miR-24 over-expression had no effect on PCNA mRNA levels as shown before (Fig. 5A). Light grey, dark grey and black bars correspond to miRNA concentrations of 2 nM, 10 nM and 50 nM, respectively. Expression is normalized to mRNA level in cells transfected with 2 nM control miRNA. Representative experiments are shown; each experiment was done twice with similar results.

Supplementary Figure 6



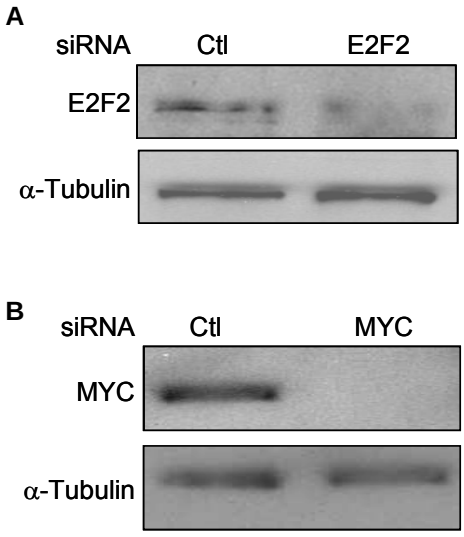
Supplementary Figure 6. Candidate miR-24 microRNA recognition elements (MRE) in the 3'UTR of target mRNAs (AURKB, BRCA1, CCNA2, CDK4, CDC2 and FEN1) predicted by rna22 or PITA. Only BRCA1 MRE 5 is predicted by TargetScan 4.2. Numbers in parenthesis represent the location in the 3'UTR of the target gene**.** The effect of inserting these MREs on luciferase expression is shown in Suppl. Fig. 7.

Supplementary Figure 7



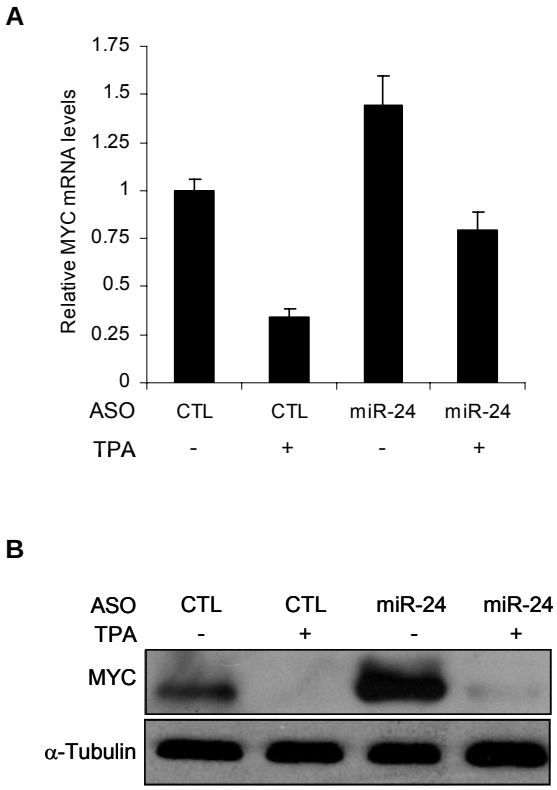
Supplementary Figure 7. Inserting the miR-24 recognition elements present in the 3'UTR of AURKB (MRE1), BRCA1 (MRE5), CDK4 (MRE1), CDC2 (MRE1) and FEN1 (MRE1) in a luciferase gene significantly reduces luciferase expression in HepG2 cells transfected with miR-24 mimics (white) and not the control mimics (black). Sequences of these MREs are provided in Suppl. Fig. 6. Data are an average of 3 experiments. Error bars represent standard deviation. *p< 0.05

Supplementary Figure 8



Supplementary Figure 8. siRNAs knockdown MYC and E2F2 expression. K562 cells were transfected with siRNAs targeting E2F2 or MYC or GFP (Ctl) for 48 hr before immunoblot analysis for E2F2 (A) or (B) MYC. α-Tubulin was probed as a loading control.

Supplementary Figure 9



Supplementary Figure 9. (A) Inhibiting miR-24 partially rescues MYC mRNA expression in K562 cells treated with TPA. K562 cells were transfected with miR-24 ASO or a control (CTL) ASO for 72 hr and then treated with TPA for 6 hr. MYC mRNA levels were measured by qRT-PCR analysis after normalizing to GAPDH mRNA. (B) Immunoblot analysis shows that antagonizing miR-24 in K562 cells increase MYC protein levels in untreated cells. However, MYC protein expression is still down-regulated after TPA treatment in cells transfected with miR-24 ASO (miR-24).