miR-24 Inhibits Cell Proliferation by Targeting E2F2, MYC, and Other Cell-Cycle Genes via Binding to "Seedless" 3′UTR MicroRNA Recognition Elements

Ashish Lal,[1,*] Francisco Navarro,[1] Christopher A. Maher,[3] Laura E. Maliszewski,[1] Nan Yan,[1] Elizabeth O'Day,[1] Dipanjan Chowdhury,[1,2] Derek M. Dykxhoorn,[1,7] Perry Tsai,[1] Oliver Hofmann,[5] Kevin G. Becker,[4] Myriam Gorospe,[4] Winston Hide,[5,6] and Judy Lieberman[1,*]
[1]Immune Disease Institute, Program in Cellular and Molecular Medicine, Children's Hospital Boston and Department of Pediatrics
[2]Dana Farber Cancer Institute and Department of Radiation Oncology
Harvard Medical School, Boston, MA 02115, USA
[3]Michigan Center for Translational Pathology, University of Michigan, Ann Arbor, MI 48109, USA
[4]Research Resources Branch and Laboratory of Cellular and Molecular Biology, National Institute on Aging-IRP, National Institutes of Health, Baltimore, MD 21224, USA
[5]Department of Biostatistics, Harvard School of Public Health, Boston, MA 02115, USA
[6]South African National Bioinformatics Institute, University of the Western Cape, Bellville 7535, South Africa
[7]The John T. Macdonald Foundation Department of Human Genetics and the Department of Microbiology and Immunology, Miller School of Medicine, University of Miami, Miami, FL 33136, USA
*Correspondence: alal@idi.harvard.edu (A.L.), lieberman@idi.harvard.edu (J.L.)
DOI 10.1016/j.molcel.2009.08.020

SUMMARY

miR-24, upregulated during terminal differentiation of multiple lineages, inhibits cell-cycle progression. Antagonizing miR-24 restores postmitotic cell proliferation and enhances fibroblast proliferation, whereas overexpressing miR-24 increases the G1 compartment. The 248 mRNAs downregulated upon miR-24 overexpression are highly enriched for DNA repair and cell-cycle regulatory genes that form a direct interaction network with prominent nodes at genes that enhance (*MYC*, *E2F2*, *CCNB1*, and *CDC2*) or inhibit (*p27Kip1* and *VHL*) cell-cycle progression. miR-24 directly regulates *MYC* and *E2F2* and some genes that they transactivate. Enhanced proliferation from antagonizing miR-24 is abrogated by knocking down *E2F2*, but not *MYC*, and cell proliferation, inhibited by miR-24 overexpression, is rescued by miR-24-insensitive *E2F2*. Therefore, *E2F2* is a critical miR-24 target. The *E2F2* 3′UTR lacks a predicted miR-24 recognition element. In fact, miR-24 regulates expression of *E2F2*, *MYC*, *AURKB*, *CCNA2*, *CDC2*, *CDK4*, and *FEN1* by recognizing seedless but highly complementary sequences.

INTRODUCTION

MicroRNAs (miRNAs) regulate key steps of cell differentiation and development by suppressing gene expression in a sequence-specific manner (Bartel, 2009). In mammals, the active strand miRNA sequence (typically ∼22 base pairs) is partially complementary to binding sites in the 3′UTR of genes, often with full complementarity to 7 or 8 nucleotides in the "seed region" (residues 2–9) of the miRNA. Gene suppression in mammals is thought to occur primarily by inhibiting translation (Olsen and Ambros, 1999). However, miRNAs in mammals also cause mRNA decay (Chang et al., 2007; Lim et al., 2005; Johnson et al., 2007); recent reports (Baek et al., 2008; Selbach et al., 2008) suggest that reduced protein is frequently associated with decreased mRNA.

miR-24 is consistently upregulated during terminal differentiation of hematopoietic cell lines into a variety of lineages (Lal et al., 2009). miR-24 is also upregulated during thymic development to naive CD8 T cells (Neilson et al., 2007) and during muscle and neuronal cell differentiation (Sun et al., 2008; Fukuda et al., 2005). miR-24 is encoded with miR-23 and miR-27 in two duplicated gene clusters. One cluster (miR-23b, miR-27b, and miR-24-1) is within a chromosome 9 EST, and the other (miR-23a, miR-27a, and miR-24-2) is in a chromosome 19 intergenic region. Both miR-24 genes are processed to the same active strand. Disruption or changes in expression of both sites have been linked to CLL prognosis (Calin et al., 2005). Because miR-24 is upregulated in diverse cell types during terminal differentiation, we sought to identify its function and the target genes that it regulates.

Common approaches to identify miRNA target genes are (1) bioinformatic algorithms that predict potential target genes that contain conserved 3′UTR sequences complementary to a seed region at the 5′ end of the miRNA active strand (Doench and Sharp, 2004; Lewis et al., 2005), (2) analysis of mRNAs that are downregulated when a miRNA is overexpressed (Chang et al., 2007; Johnson et al., 2007; Lim et al., 2005), and (3) identifying mRNAs enriched in coimmunoprecipitates with tagged Argonaute or GW182 proteins in cells overexpressing the miRNA (Easow et al., 2007; Zhang et al., 2007). The bioinformatic approach is hampered by the fact that the existing algorithms have a high margin of error (most predicted genes are not real targets, and some key targets, *such as RAS* for let-7, are not predicted [Johnson et al., 2005]). The utility of the biochemical approach involving Argonaute proteins for genome-wide target

identification of miRNAs is still unclear because Argonaute over-expression globally increases miRNA levels, perhaps obscuring the effect of an individual overexpressed miRNA (Diederichs and Haber, 2007). Because miRNA-mediated mRNA degradation and protein downregulation often occur together (Baek et al., 2008), identifying the mRNAs that decrease when a miRNA is overexpressed might identify many of its targets. Although some bona fide miR-24 targets that are primarily regulated by translation will be missed by this approach and other downregulated genes may not be directly regulated, this strategy has been successfully used to identify targets of some mammalian miRNAs, including miR-124 and miR-1 (Lim et al., 2005), miR-34a (Chang et al., 2007), and let-7 (Johnson et al., 2007). Therefore, we applied this approach to identify the genes regulated by miR-24 in HepG2 cells that express low levels of miR-24 and combined it with bioinformatics to uncover miR-24-regulated pathways. We find that miR-24 regulates a network of genes that control cell-cycle progression and DNA repair (Lal et al., 2009). Overexpressing miR-24 increases the G1 population and reduces DNA replication, whereas antagonizing miR-24 increases cell proliferation, which can be rescued by knocking down *E2F2*, suggesting that *E2F2* is a key miR-24 target gene. *MYC* and other genes important in cell-cycle regulation that are transcriptionally regulated by MYC and E2Fs (*AURKB*, *BRCA1*, *CCNA2*, *CDC2*, *CDK4*, and *FEN1*) are also direct miR-24 targets by luciferase assay. Of note, *E2F2* and most of these genes lack 3′UTR miR-24 seed match sequences. However, miR-24 regulates these genes by base pairing to "seedless" 3′UTR MREs with extensive base pairing elsewhere in the sequence.

RESULTS

miR-24 Is Upregulated during Hematopoietic Differentiation

To understand the role of miRNAs during terminal differentiation, we analyzed miRNA expression by microarray in two human leukemia cell lines: K562 cells differentiated to megakaryocytes using 12-O-tetradecanoylphorbol-13-acetate (TPA) or to erythrocytes with hemin and HL60 cells differentiated to macrophages using TPA or to monocytes using vitamin D3. miR-24 was one of only six miRNAs that was consistently upregulated in all four systems of terminal differentiation (Lal et al., 2009). The other uniformly upregulated miRNAs were three other members of the miR-24 clusters (miR-23a, miR-23b, and miR-27a), miR-22, and miR-125a. miR-24 was the most upregulated of these miRNAs. We therefore focused on miR-24, which we hypothesized might regulate terminal differentiation in multiple cell lineages. qRT-PCR confirmed the induction of miR-24 during differentiation of these hematopoietic cells (Figure 1A) with the highest upregulation in K562 cells treated with TPA. The mature miR-24 transcript increased 2- to 8-fold during differentiation into megakaryocytes, erythrocytes, macrophages, monocytes, and granulocytes. Expression of the chromosome 19 miR-24 cluster primary transcript encoding miR-23a, miR-27a, and miR-24 increased in both cell lines within 6 hr of TPA treatment, peaked at ∼12 hr, and remained elevated for at least 2 days (Figures S1A and S1B available online), suggesting that the observed increase in mature miR-24 was due to increased tran-

scription. Upregulation of the Dicer-cleaved mature miRNA was slightly delayed, becoming significant at 12–16 hr (Figures S1C and S1D). Mature miR-24 levels remained elevated for as long as was measured (4 days).

miR-24 Inhibits Cellular Proliferation by Increasing the G1 Compartment

Because cessation of cell proliferation is a hallmark of terminal differentiation, we first examined whether proliferation is altered by either inhibiting or enhancing miR-24 function by transfecting cells with miR-24 2′-OMe antisense oligonucleotide (ASO) or miRNA mimics, respectively. When K562 cells were transfected with miR-24 ASO, miR-24 was dramatically and specifically reduced by qRT-PCR 36 hr later (Figure 1B). DNA replication, measured by thymidine incorporation, doubled in cells transfected with miR-24 ASO compared to cells transfected with control ASO (Figure 1C). When K562 cells were differentiated with TPA for 4 hr, thymidine incorporation declined by 60%. However, in cells transfected with miR-24 ASO and treated with TPA, thymidine uptake was indistinguishable from that of the control ASO-transfected, but TPA-untreated, cells (Figure 1C). Therefore, miR-24 ASO fully restored proliferation to differentiating K562 cells. To examine whether miR-24 also inhibits cell proliferation in nontransformed cells, we next antagonized miR-24 in early passage WI-38 and IMR-90 normal diploid fibroblasts. Antagonizing miR-24 in WI-38 and IMR-90 cells dramatically reduced miR-24 (Figure 1D) and increased thymidine uptake > 2-fold 48 hr after transfection (Figure 1E). Conversely, overexpressing miR-24 in HepG2 cells synchronized with nocodazole, which typically leads to mitotic arrest and only ∼8% of cells in G1, increased G1 cells 3-fold (22%; miR-24 versus cel-miR-67, p < 0.001) (Figure 1F).

We next analyzed how miR-24 expression changes during normal cell-cycle progression using K562 cells released at various times from nocodazole treatment, which synchronized them in G2/M (Bar-Joseph et al., 2008; O'Donnell et al., 2005) (Figures 1G and 1H). Before release, 90% of cells were in G2/M; 8 hr later, 65% were in G1; and 12 hr after removing nocodazole, 45% were in S phase. miR-24 was low in G2/M, increased > 3-fold by 8 hr when most cells were in G1, and then declined by 12 hr as cells progressed into S phase. These results suggest that miR-24 is most highly expressed in G1. Taken together with our finding that cells transduced with miR-24 mimics accumulate in G1, these results suggest that miR-24 regulates cell-cycle progression mostly by blocking or delaying the G1/S transition.

Most Genes Downregulated by miR-24 Contain a miR-24 Seed in Their 3′UTR

We next sought to identify miR-24-regulated targets and cellular pathways by comparing mRNA microarrays of cells transfected with miR-24 or control miRNA (cel-miR-67) mimic. Transfecting HepG2 cells, which have low endogenous miR-24 levels (Figure 2A), with a miR-24 mimic increased miR-24 expression ∼80-fold compared with control cells (Figure 2B). Total RNA, isolated from duplicate miRNA-transfected samples 48 hr later, was amplified, labeled, and hybridized to Illumina mRNA microarrays. 248 mRNAs were downregulated at least 2-fold by miR-24 over-expression (Z ratio > 1.5) (Table S1). We validated the microarray
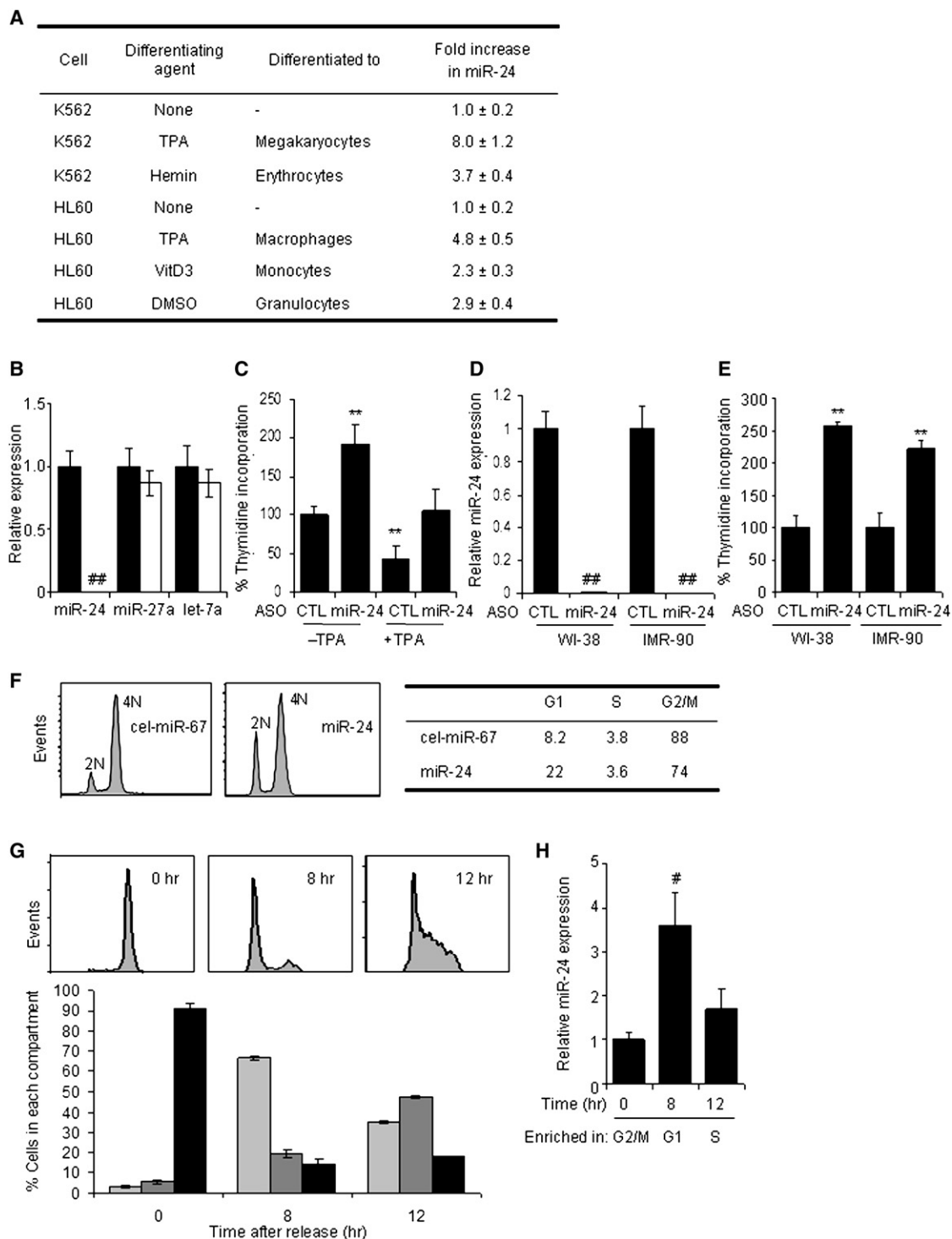
A

Cell	Differentiating agent	Differentiated to	Fold increase in miR-24
K562	None	-	1.0 ± 0.2
K562	TPA	Megakaryocytes	8.0 ± 1.2
K562	Hemin	Erythrocytes	3.7 ± 0.4
HL60	None	-	1.0 ± 0.2
HL60	TPA	Macrophages	4.8 ± 0.5
HL60	VitD3	Monocytes	2.3 ± 0.3
HL60	DMSO	Granulocytes	2.9 ± 0.4

Figure 1. miR-24 Is Upregulated during Hematopoietic Cell Differentiation and Inhibits Cell Proliferation

(A) miR-24 expression, measured using qRT-PCR relative to untreated cells, increases in K562 cells differentiated to megakaryocytes or erythrocytes and HL60 cells differentiated to macrophages, monocytes, and granulocytes. Differentiation in all experiments was verified by cell surface phenotype. Mature miR-24 levels were determined by qRT-PCR and normalized to *U6*.

(B) miR-24 knockdown in K562 cells specifically decreases miR-24, assayed by qRT-PCR in cells transfected with miR-24 ASO (white) relative to control ASO (black). Expression relative to *U6* snRNA is normalized to control cells.

(C) miR-24 knockdown with ASO increases K562 cell proliferation measured by thymidine uptake, both in the presence and absence of TPA. The decline in proliferation with TPA is completely restored by antagonizing miR-24.

data by performing qRT-PCR amplification for nine randomly chosen downregulated genes that spanned the range of significantly downregulated genes (Z ratios, 1.6–6.1), of which six (*CNDP2*, *TOP1*, *PER2*, *MBD6*, *H2AFX*, and *STX16*) were predicted miR-24 targets by TargetScan 4.2 and three (*UBD*, *BCL2L12*, and *ZNF317*) were not (Figure 2C). *H2AFX* was previously shown to be directly regulated by miR-24 (Lal et al., 2009). All nine genes were significantly downregulated, and the extent of downregulation correlated well with their reduced expression in the microarray, validating the quality of the microarray data.

To determine what fraction of the downregulated genes are likely direct targets of miR-24, we used two approaches. First, we compared our experimental list of downregulated transcripts with TargetScan predictions. One hundred downregulated genes were also predicted by TargetScan 4.2 (Lewis et al., 2003) (Figure 2D and Table S1). Among these 100 targets, however, only 20 have predicted miR-24 3′UTR miRNA recognition sites (MRE) conserved in human, mouse, rat, and dog. Second, we examined the frequency of a 3′UTR sequence perfectly complementary to the miR-24 seed (hexamer [positions 2–7], heptamer [positions 2–8], and octamer [positions 2–9]). The downregulated genes were highly enriched for miR-24 seed matches (Figure 2E and Table S1). Just more than half of the 219 miR-24-downregulated transcripts that have an annotated 3′UTR contain a 3′UTR complementary hexamer sequence (53%, $p = 2 \times 10^{-16}$ relative to the background frequency of the seed in the known transcriptome); 32% have a heptamer match ($p = 7 \times 10^{-15}$); and 8% have an octamer seed match ($p = 0.0002$). This significant enrichment of predicted miR-24 target genes and the high frequency of genes containing perfect seed matches suggest that a substantial proportion of the downregulated genes may be direct miR-24 targets.

Cell Cycle and DNA Repair Genes Are Regulated by miR-24

We next performed a gene ontology (GO) analysis (GeneGo, Inc.) to identify cellular pathways enriched in the set of genes downregulated after ectopic miR-24 expression. This analysis did not yield a tightly focused set of functions for miR-24 (Table S2). Therefore, we next looked at whether the 100 downregulated genes, which were also predicted miR-24 targets, are enriched for specific biological processes. A functional enrichment and network analysis revealed statistically significant enrichment for 49 processes, many of which are overlapping (Figure 3A and Table S3). The top three most enriched GO processes involve DNA repair (DNA damage checkpoint, double-strand break repair by homologous recombination, and recombinational repair; each enriched with a significance of $p = 0.0001$).

We previously found that miR-24 interferes with the DNA damage response in terminally differentiated hematopoietic cells, predominantly by reducing expression of the histone variant H2AFX, which recruits and retains DNA repair factors at double-strand breaks (Lal et al., 2009). In addition, multiple GO processes involved in cell-cycle regulation were also highly enriched (regulation of cell cycle, $p = 0.0002$; DNA integrity checkpoint, $p = 0.0003$; cell-cycle arrest, $p = 0.0007$; cell cycle, $p = 0.001$; DNA recombination, $p = 0.001$). This was not surprising based on the effect of miR-24 on cell-cycle progression. When networks were developed to identify known directly interacting proteins from these overrepresented biological processes, there was one cluster of six genes centered around *MYC* (c-myc) and three other small clusters involving two or three genes (Figure S2A); the other 87 genes in the data set lack any previously annotated direct interactions. Because the TargetScan algorithm might miss some important miRNA-regulated genes, we also constructed a direct interaction network from the 248 downregulated mRNAs. The direct interaction network constructed from all significantly downregulated mRNAs was a highly interactive set of 68 interacting genes, many of which are important in cell-cycle regulation. The major connected network of miR-24-downregulated genes is shown in Figure 3B; there were also some smaller networks (Figure S2B). Key nodes of the major network are *MYC* (22 interactions), *E2F2* (six interactions), *VHL* (six interactions), *CDC2* (six interactions), *CCNB1* (five interactions), and *CDKN1B* (five interactions). The MYC and E2F2 transcription factors play a central role in regulating G1/S transition and progression through S. They inhibit cell differentiation and apoptosis and promote cellular transformation (Bracken et al., 2004; Lebofsky and Walter, 2007). MYC regulates the transcription of other genes in the network, including *E2F2*, *CDKN1B*, *CCNB1*, *CDC2*, *CDCA7*, and *RRM2*. E2F2 also regulates the transcription of other genes in the network, including *CDC2*, *MYC*, *RRM2*, and the mini-chromosome maintenance proteins MCM4 and MCM10 that are essential for initiating DNA replication. These analyses support our experimental findings that miR-24 regulates cell-cycle progression and DNA repair.

miR-24 Regulates *MYC* by Binding to Its 3′UTR

To determine whether miR-24 directly regulates the downregulated genes, we began with *MYC* because it is a key node of the interaction network, plays an important role in cell-cycle progression, and is a predicted miR-24 target. We transfected HepG2 and K562 cells with miR-24 mimics and, 48 hr later, measured *MYC* mRNA by qRT-PCR. miR-24 overexpression decreased *MYC* mRNA by ~2- to ~4-fold relative to *GAPDH* in

---

(D and E) Knocking down miR-24 in WI-38 or IMR-90 cells also significantly increases proliferation as measured by thymidine incorporation 48 hr posttransfection. miR-24 knockdown by ASO measured by qRT-PCR is normalized to *U6* snRNA.

Error bars in (B–E) represent SD from three experiments. **$p < 0.01$, ##$p < 0.001$.

(F) miR-24 overexpression increases the G1 compartment in HepG2 cells. HepG2 cells transfected with miR-24 or control mimic for 48 hr were stained with propidium iodide and analyzed by flow cytometry. Representative analysis of three independent experiments is shown.

(G) K562 cells, synchronized in G2/M by nocodazole and then released, were analyzed by flow cytometry. A representative experiment is shown in the top panel, and the mean (± SD) percentage of cells in each phase of the cell cycle (from three independent experiments) is shown below (light gray, G1; dark gray, S; black, G2/M).

(H) qRT-PCR analysis of miR-24 (normalized to *U6* snRNA) from the partially synchronized K562 cells in (G) shows that miR-24 is most highly expressed in G1 and declines as cells progress to S and G2/M phase. #$p < 0.005$.
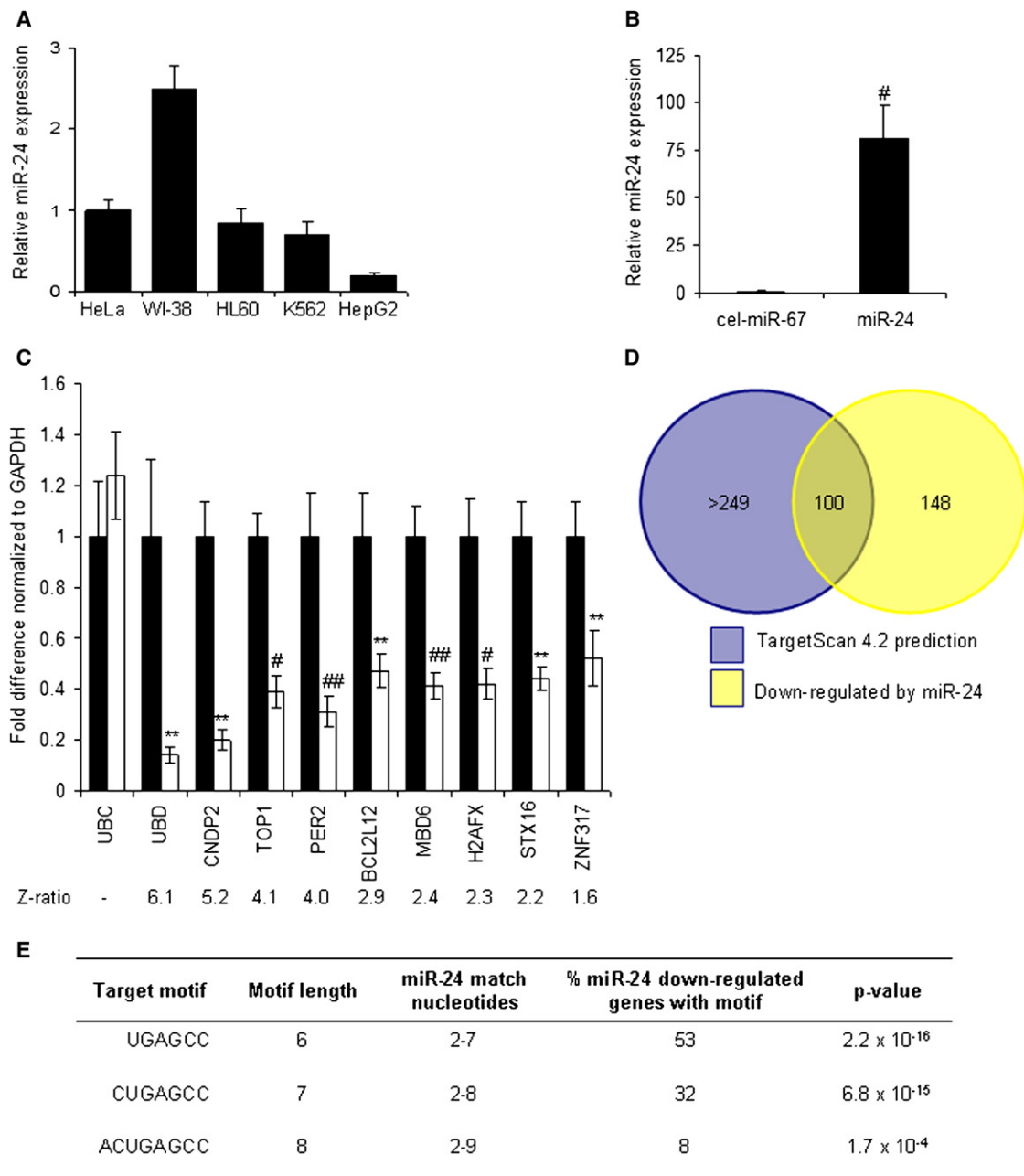
Figure 2. mRNA Downregulation after miR-24 Overexpression

(A) HepG2 cells express low levels of miR-24, assayed by qRT-PCR analysis normalized to *U6*, compared to HeLa, WI-38, HL60, and K562 cells.

(B) Effective increase in miR-24 in HepG2 cells 48 hr after transfection with miR-24 mimic compared to cel-miR-67-transfected control cells. $^{\#}p < 0.005$.

(C) Genes identified by microarray as downregulated by miR-24 overexpression were confirmed to be downregulated by qRT-PCR normalized to *GAPDH*. *UBC* is a housekeeping gene. Cells were transfected with cel-miR-67 (black) or miR-24 mimic (white). $^{**}p < 0.01$, $^{\#}p < 0.005$, and $^{\#\#}p < 0.001$. The downregulated genes are graphed in order of their downregulation on the microarray; the Z ratio of the microarray analysis is shown below. Error bars represent SD from three independent experiments (A, B, and C).

(D) Venn diagram of genes downregulated by miR-24 in HepG2 cells and genes predicted to be regulated by miR-24 using TargetScan 4.2. Of the 100 predicted genes, whose mRNA is also significantly downregulated, only 20 have conserved predicted miR-24 recognition sites. TargetScan 4.2 predicts 349 conserved miR-24 targets and many more that are not conserved.

(E) Sites complementary to the miR-24 seed are enriched in the 3′UTR of downregulated transcripts. The table shows the frequency of perfect hexamer (positions 2–7), heptamer (positions 2–8), and octamer (positions 2–9) miR-24 3′UTR seeds in the downregulated genes.

HepG2 and K562 cells (Figures 4A and 4B). *UBC* mRNA, a control transcript, did not change significantly. MYC protein also decreased substantially (85%) (Figure 4C). To investigate whether reduced MYC expression was directly mediated by miR-24, we measured changes after miR-24 cotransfection in luciferase activity from a *MYC* 3′UTR reporter. miR-24 reduced luciferase activity 2.2-fold (Figure 4D). We next sought to identify miR-24 MREs in the *MYC* 3′UTR. TargetScan 4.2 predicts a single MRE containing a poorly conserved 7-mer exact seed match at positions 462–468 (although the recent TargetScan 5.0 algorithm does not list *MYC* as a miR-24 target), whereas rna22, an algorithm that does not require a seed match (Miranda et al., 2006), identifies six potential miR-24 MREs in the 488 nt *MYC* 3′UTR, including the TargetScan 4.2-predicted MRE (MRE6) (Figure 4E). miR-24 overexpression specifically and significantly reduced luciferase activity by 1.9- and 3.9-fold for MRE3 and MRE6, respectively, but the other MREs had no significant effect on luciferase activity (Figure 4F). MRE3 has no seed but has extensive complementarity with the 3′ end of miR-24. Point mutations of MRE3 and MRE6 that disrupted miR-24 binding restored luciferase activity (Figures 4G and 4H). These findings suggest that miR-24 binds to two partially complementary sites (MRE3 and MRE6) in the *MYC* 3′UTR.

miR-24 Downmodulates *E2F2*

We next examined the effect of miR-24 on *E2F2* because *E2F2* is downregulated by miR-24 overexpression by microarray, is a key node in the gene interaction network (Figure 3B), and plays a crucial role in regulating progression through G1, where miR-24-overexpressing cells pile up (Polager and Ginsberg, 2008). qRT-PCR analysis confirmed that *E2F2* mRNA was significantly downregulated by overexpressing miR-24 (Figure 5A). In addition, the related E2F family members, *E2F1* and *E2F3*, were also significantly decreased, although these two genes were not identified by the less sensitive microarray analysis. *E2F1* and *E2F3* downregulation may be secondary to *E2F2* downregulation because the E2F family of transcription factors regulates each other (Bracken et al., 2004; Vernell et al., 2003) or may be mediated by MYC, given that MYC and E2F1 have been shown to transactivate each other (Fernandez et al., 2003). As expected, E2F2 protein (Figure 5B) was also substantially reduced (9-fold).

miR-24 Downregulates Multiple *E2F*- and *MYC*-Regulated Genes

The E2F transcription factors activate the transcription of many genes essential for DNA replication, cell-cycle progression, and DNA repair. If miR-24 overexpression downregulates *E2F2*, *E2F2* target gene mRNAs would also be expected to decline after ectopic miR-24 expression. The effect of ectopic miR-24 expression in HepG2 cells on transcripts of 10 E2F targets that are important for cell-cycle progression and DNA repair (*AURKB*, *BRCA1*, *CCNA2*, *CDC2*, *CHEK1*, *FEN1*, *PCNA*, *RRM2*, *MCM4*, and *MCM10*) was analyzed 48 hr later. Eight of the ten transcripts, with the exception of *BRCA1* and *PCNA*, were significantly reduced (>40%) (Figure 5A). A subset of these genes (*CDC2*, *MCM4*, *MCM10*, *RRM2*, and *FEN1*) was also significantly downregulated by miR-24 overexpression by microarray (Table S1). mRNA microarray may not be sensitive enough to identify
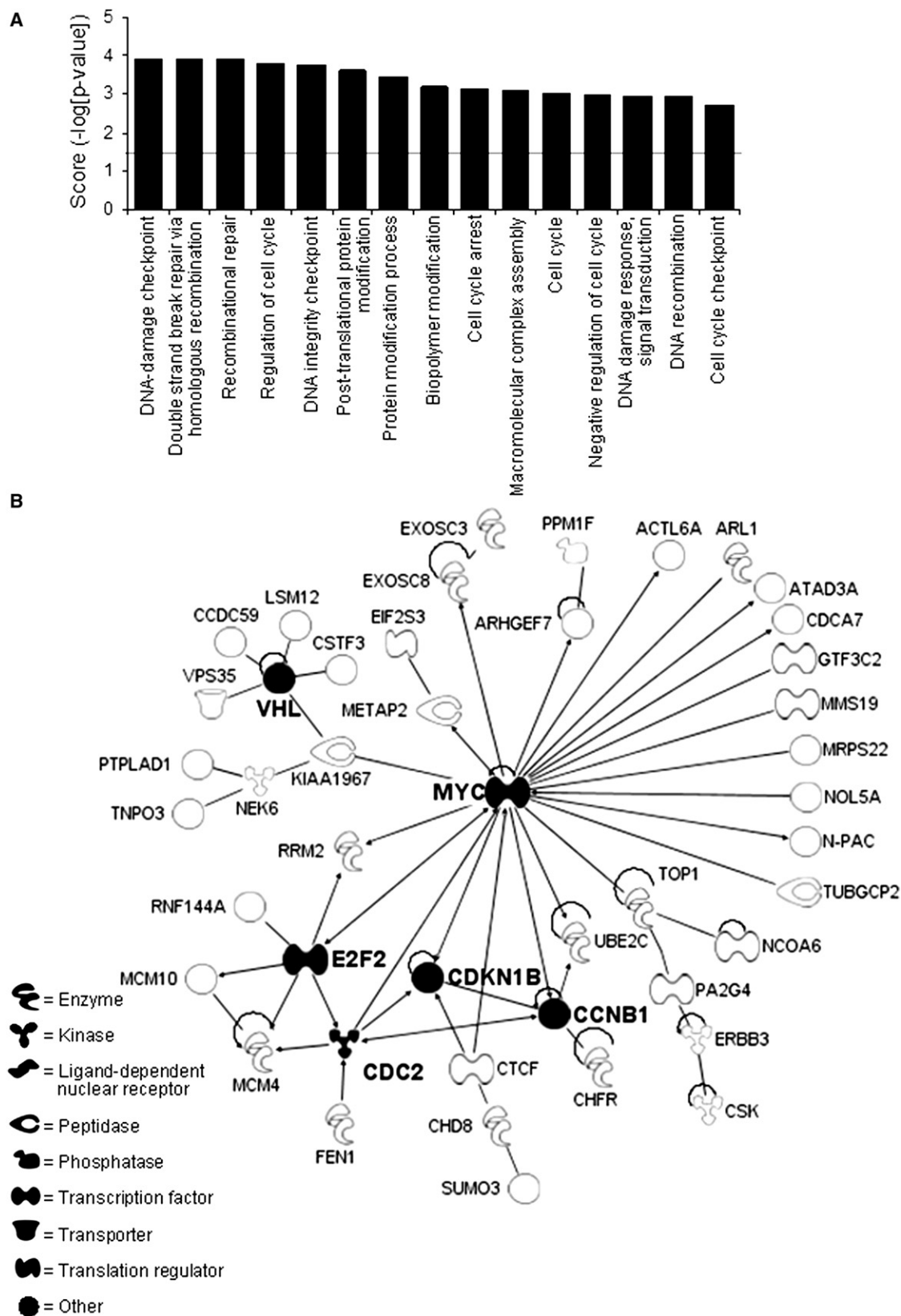
some genes whose expression is suppressed, either directly or indirectly, by a miRNA. Protein levels of E2F2 and all seven miR-24 target genes examined (AURKB, BRCA1, CCNA2, CDC2, CHEK1, FEN1, and PCNA), quantified by densitometry of immunoblots, decreased by at least 2-fold (Figure 5B). A possible explanation for the lack of correlation between the mRNA and protein levels of BRCA1 and PCNA is that mRNA levels were measured 48 hr after transfection of one cell type (HepG2), whereas protein levels were assayed 72 hr posttransfection in K562 cells. In fact, *BRCA1*, but not *PCNA*, mRNA was reduced by ∼2-fold when K562 cells were transfected with miR-24 for 3 days (Figure S5).

Because miR-24 overexpression reduced MYC protein by 85% (Figure 4C), MYC target genes should also be downregulated. Consistent with this hypothesis, 11 known MYC-regulated genes (*ATAD3A*, *ACTL6A*, *ARHGEF7*, *CCNB1*, *CDCA7*, *EXOSC8*, *E2F2*, *METAP2*, *N-PAC*, *RRM2*, and *UBE2C*) were significantly downregulated in miR-24-overexpressing HepG2 cells by mRNA microarray (Table S1). Among MYC-regulated genes, CDK4 is an important mediator of MYC's effects on cellular proliferation (Hermeking et al., 2000). Although *CDK4* mRNA was not significantly altered by microarray, *CDK4* mRNA declined ∼4-fold after ectopic expression of miR-24 in HepG2 cells by more sensitive qRT-PCR assay (Figure 5A), and CDK4 protein became undetectable (Figure 5B). Therefore, miR-24 overexpression decreases the levels of many genes that are important in cell-cycle progression.

In these experiments, we overexpressed miR-24 ∼80-fold above the level in undifferentiated K562 cells, whereas the physiological increase after TPA treatment of K562 cells is only 8-fold. To determine whether these genes are regulated by a physiological increase in miR-24, these experiments were repeated by transfecting K562 cells with varying miR-24 mimic concentrations (2–50 nM). Transfection of 2 nM miR-24 did not significantly alter miR-24, whereas 10 and 50 nM miR-24 increased miR-24 levels by 4- and 28-fold, respectively (Figure 5C). *E2F2*, *MYC*, and three of four other E2F2-regulated mRNAs (*AURKB*, *CCNA2*, and *H2AX*, but not *PCNA*) (Figure S5) and protein levels of all seven genes tested (*E2F2*, *AURKB*, *CHEK1*, *CCNA2*, *CDK4*, *MYC*, and *PCNA*) were all significantly reduced by a 4-fold increase in miR-24 (Figure 5D). Therefore, the genes that were identified by mRNA microarray as downregulated after ectopic miR-24 expression are likely physiologically relevant direct and/or indirect miR-24 targets.

E2F2 and Some E2F Target Genes Are Directly Regulated by Seedless 3′UTR MREs

None of the three *E2F* paralogs (*E2F1*, *E2F2*, and *E2F3*) are a predicted target of miR-24, and their 3′UTRs do not contain a miR-24 seed match sequence. We nonetheless tested whether the *E2F* 3′UTRs might be directly regulated by miR-24 by luciferase assay. The *E2F2*, but not *E2F1* or *E2F3*, 3′UTR significantly repressed luciferase activity in a miR-24-dependent manner (Figure 6A), suggesting that *E2F2* is a direct miR-24 target. rna22 identified five candidate *E2F2* 3′UTR miR-24 MREs (Figures 6B and S3). miR-24 significantly suppressed luciferase activity of a reporter gene containing the *E2F2* MRE1. *E2F2* MRE1 does not have a seed match in the 3′UTR, even if G:U

A

B

= Enzyme

= Kinase

= Ligand-dependent nuclear receptor

= Peptidase

= Phosphatase

= Transcription factor

= Transporter

= Translation regulator

= Other

wobbles are allowed, but has extensive complementarity to miR-24 elsewhere. Point mutations that disrupt base pairing between miR-24 and *E2F2* MRE1 rescued luciferase expression, verifying that miR-24 specifically recognizes the *E2F2* MRE1.

To verify further that *MYC* and *E2F2* are direct targets of miR-24, we also looked at changes in expression of luciferase reporter genes 24 hr after transduction of HepG2 cells with miR-24 mimic. At this early time, thymidine uptake of HepG2 cells does not significantly change (Figure S4A), but ectopic miR-24 still suppresses luciferase reporters encoding *MYC* MRE3 or MRE6 or *E2F2* MRE1 (Figure S4B). The identification of seedless *E2F2* and *MYC* MREs confirms previous studies showing that MREs lacking a seed with good downstream complementarity can contribute to miRNA gene regulation (Didiano and Hobert, 2006, 2008; Vella et al., 2004).

Because seedless MREs contributed to the regulation of *E2F2* and *MYC* by miR-24, we next investigated whether some of the *E2F2*- and *MYC*-regulated genes, whose transcripts declined in response to miR-24, might also be direct miR-24 targets even though they might lack a predicted MRE. We selected eight genes (*AURKB*, *BRCA1*, *CCNA2*, *CHEK1*, *CDC2*, *CDK4*, *FEN1*, and *PCNA*) that play important roles in cell-cycle progression and cloned their entire 3′UTRs into the luciferase reporter. The 3′UTR of six out of eight genes (*AURKB*, *BRCA1*, *CCNA2*, *CDC2*, *CDK4*, and *FEN1*, but not *CHEK1* or *PCNA*) was significantly repressed by miR-24, suggesting that these genes may be direct targets (Figure 6C). To confirm that these genes are direct miR-24 targets, we next sought to identify the miR-24 MREs that regulate their expression. Among the six genes whose 3′UTR was specifically repressed by miR-24, *BRCA1* is the only gene that is predicted by TargetScan. The *BRCA1* 3′UTR contains a nonconserved perfect 7-mer seed match sequence that functions as a miR-24 MRE by luciferase assay (Figures 6D and 6E). To identify potential miR-24 MREs in these *E2F2*- and *MYC*-regulated genes, we used the rna22 or PITA algorithms, which allow G:U wobbles or seed mismatches. These algorithms identified one candidate MRE for *AURKB*; five for *BRCA1* (which included the TargetScan *BRCA1* site); and three sites each for *CCNA2*, *CDC2*, *CDK4*, and *FEN1* (Figure S6). miR-24 significantly repressed luciferase activity of one MRE for five out of six of these reporter genes (*AURKB* MRE1, *BRCA1* MRE5, *CDC2* MRE1, *CDK4* MRE1, and *FEN1* MRE1) (Figures 6D, 6E, and S7). Although *CCNA2* MRE1 appeared to be inactive, a longer fragment (181 nucleotides) from the *CCNA2* 3′UTR (that included only *CCNA2* MRE1) significantly repressed luciferase expression in a miR-24-dependent manner when cloned into the luciferase vector 3′UTR (Figure 6F). Point mutations that disrupt base pairing between miR-24 and the five minimal MREs and the *CCNA2* MRE within the extended sequence rescued luciferase expression, verifying that these

MREs are regulated by miR-24 (Figures 6D–6F). Therefore, we have identified and verified by mutation seven seedless miR-24 MREs in genes important in cell-cycle progression.

E2F2 Downregulation Is Key to miR-24's Inhibition of Cell Proliferation

Because both MYC and E2F2 are important cell-cycle progression regulators, we next examined their contributions to the increased cellular proliferation from antagonizing miR-24 by knocking down *MYC* and/or *E2F2* in K562 cells cotransfected with miR-24 ASO (Figures 7A and S8). Introducing miR-24 ASO into K562 cells doubled thymidine incorporation (as in Figure 1C). *E2F2* knockdown completely abrogated the proliferative effect of miR-24 ASO, but *MYC* knockdown had no significant effect. Moreover, *E2F2* downregulation by miR-24 is physiologically relevant. When K562 cells were terminally differentiated to megakaryocytes with TPA, the decrease in *E2F2* mRNA and protein was completely blocked by inhibiting miR-24 (Figures 7B and 7C). Conversely, ectopic expression of miR-24-insensitive *E2F2* lacking the 3′UTR restored proliferation to miR-24-treated K562 cells (Figures 7D and 7E). Therefore, the miR-24 antiproliferative effect is largely mediated by its downregulation of *E2F2*.

Antagonizing miR-24 elevated MYC protein levels in untreated K562 cells, and the downregulation of *MYC* mRNA in TPA-treated K562 cells could be partially rescued by antagonizing miR-24 (Figures S9A and S9B). However, antagonizing miR-24 did not restore MYC protein to differentiating cells, suggesting that, although miR-24 suppresses *MYC* expression, downregulation of MYC protein during postmitotic differentiation is also controlled by miR-24-independent changes in protein stability. This may help to explain why *MYC* siRNAs had no significant effect on proliferation of cells transduced with miR-24 ASO (Figure 7A).

DISCUSSION

miR-24 and its clustered miRNAs are among only a handful of miRNAs consistently upregulated during hematopoietic cell terminal differentiation. Here, we show that miR-24 suppresses expression of several key genes that regulate cell-cycle progression. Overexpressing miR-24 increases the percentage of cells in the G1 phase, whereas antagonizing it causes differentiating cells to keep proliferating. The antiproliferative effect of miR-24 is not restricted to tumor cells (HepG2 and K562 cells) but also occurs in human diploid fibroblasts.

miRNAs can regulate expression of hundreds of genes. Genome-wide analysis of miRNA target genes has been assessed following miRNA overexpression or knockdown for only a handful of miRNAs (Chang et al., 2007; Johnson et al., 2007; Lim et al., 2005). Using this approach for miR-24 enabled us to identify

Figure 3. Bioinformatic Analysis of miR-24-Downregulated Genes Suggests that miR-24 Regulates Cell-Cycle Progression and DNA Repair
(A) Top 15 overrepresented cellular processes for the 100 overlapping genes in Figure 2D. Overrepresented processes are sorted by score (−log [p value]). A highly positive score suggests that the subnetwork is saturated with genes identified experimentally and doesn't have many nodes not experimentally identified. The complete list of 100 genes in the overlap and further statistical analysis are provided in Tables S1 and S3. The dotted line represents the statistically significant limit.
(B) Major direct interaction network of the 248 genes significantly downregulated after transfection of HepG2 cells with miR-24 mimics. Nodes with at least five interactions (including autoregulation) are highlighted.
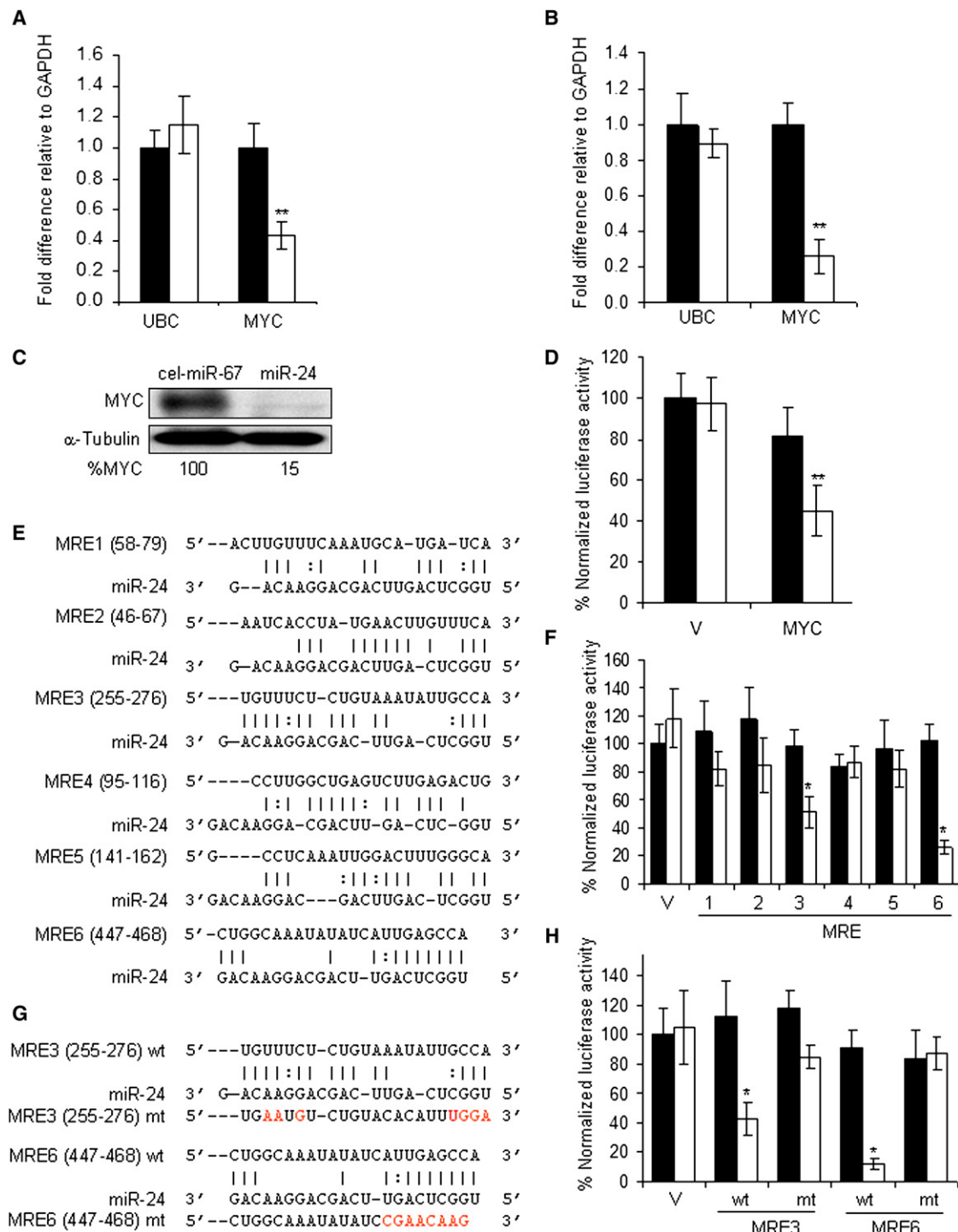
Figure 4. miR-24 Regulates MYC Expression

(A and B) miR-24 overexpression in HepG2 (A) or K562 cells (B) decreases *MYC* mRNA, analyzed by qRT-PCR and normalized to *GAPDH* (black, cel-miR-67; white, miR-24).

(C) MYC protein is decreased in K562 cells upon miR-24 overexpression. Densitometry was used to quantify protein; α-tubulin served as loading control.

(D) miR-24 targets the *MYC* 3′UTR in a luciferase reporter assay. HepG2 cells were transfected with control miRNA (black) or miR-24 (white) mimic for 48 hr and then with *MYC* 3′UTR-luciferase reporter (MYC) or vector (V) for 24 hr.

(E) Predicted binding sites in the *MYC* 3′UTR for miR-24 (MRE1–6) by rna22. The numbers in parentheses correspond to the position in the *MYC* 3′UTR. Perfect matches are indicated by a line; G:U pairs, by a colon.
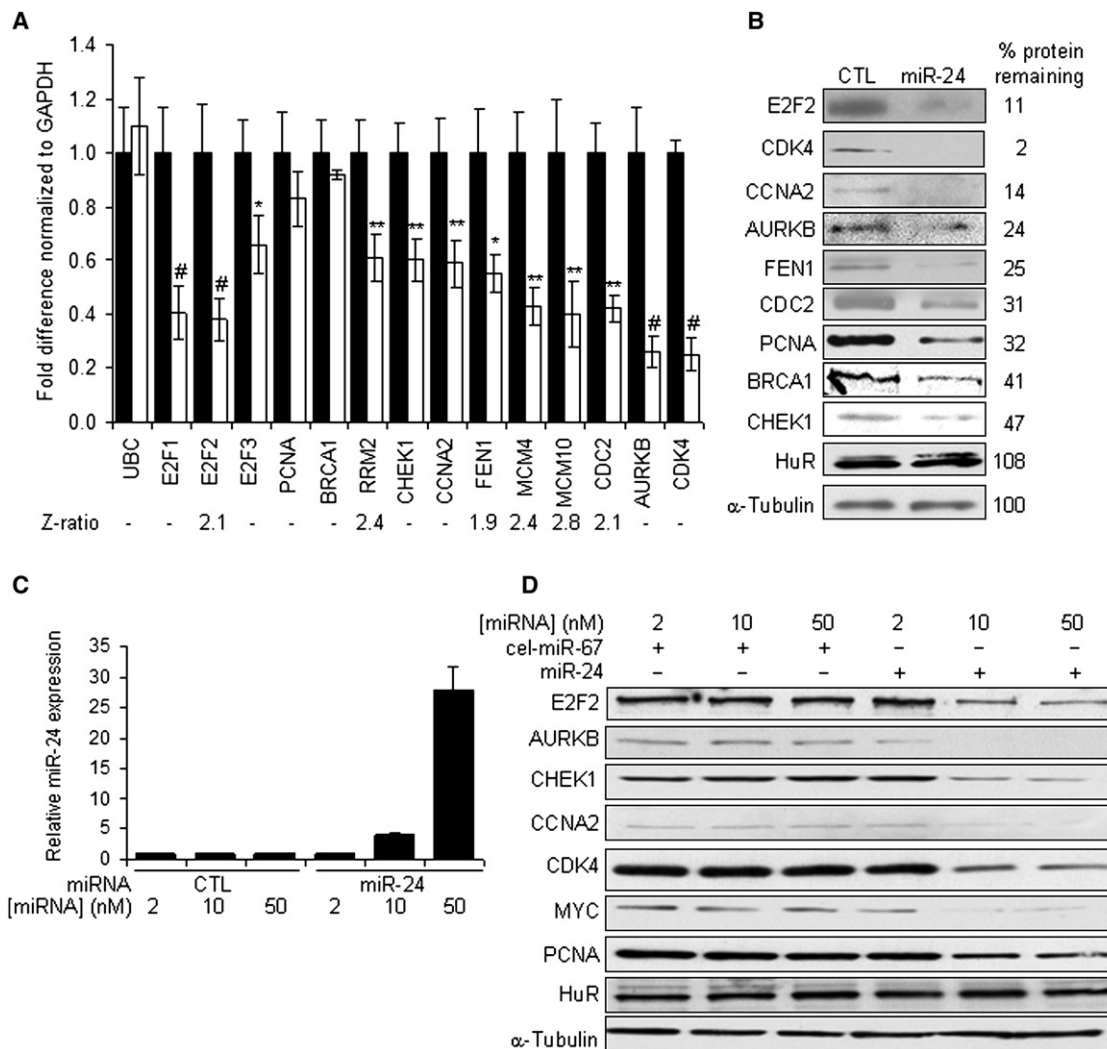
Figure 5. miR-24 Overexpression Downregulates *E2F*s and Some E2F-Regulated Genes

(A) miR-24 significantly reduces mRNA levels of *E2F1*, *E2F2*, and *E2F3* and of some E2F target genes (*RRM2*, *CHEK1*, *CCNA2*, *FEN1*, *MCM4*, *MCM10*, *CDC2*, and *AURKB*), but not *BRCA1* and *PCNA*. *CDK4*, a key MYC target gene, is also downregulated. HepG2 cells were transfected with miR-24 (white) or control mimics (black) for 48 hr, and *E2F* and their target mRNAs were measured by qRT-PCR. Data normalized to *GAPDH* are expressed relative to control mimic-transfected cells. *UBC* is a control housekeeping gene. The Z ratios refer to the significantly downregulated mRNAs in the mRNA microarray in miR-24-overexpressing cells.

(B) Protein expression of miR-24 target genes is substantially reduced in miR-24 mimic-transfected K562 cells 72 hr after transfection, relative to control miRNA-transfected cells. Densitometry was used to quantify protein relative to α-tubulin. HuR and α-tubulin are loading controls.

(C) miR-24 levels significantly increased when K562 cells were transfected with 10 or 50 nM miR-24 mimics as measured by qRT-PCR analysis. Expression relative to *U6* snRNA is depicted normalized to control cells.

(D) A 4-fold increase in miR-24, obtained by transfecting 10 nM miR-24 mimic, reduces target proteins. Cell lysates of K562 cells, obtained 72 hr after transfection with indicated concentrations of control or miR-24 mimics, were analyzed by immunoblot. α-tubulin and HuR are loading controls.

Error bars represent mean ± SD from three independent experiments. *$p < 0.05$, **$p < 0.01$, and #$p < 0.005$.

248 candidate genes that might be either directly or indirectly regulated by miR-24. Of these downregulated genes, 40% are predicted miR-24-regulated genes by TargetScan, and 53% have a 3′UTR hexamer sequence complementary to the miR-24 seed, suggesting that a large proportion of miR-24-downregulated genes may be direct targets.

To make sense of the set of 248 genes downregulated by miR-24 overexpression, we used bioinformatics to identify

(F–H) miR-24 regulates MRE3 and MRE6 by luciferase reporter assay. HepG2 cells were cotransfected with cel-miR-67 (black) or miR-24 (white) mimics and luciferase reporters containing the wild-type (wt) MRE1-6 in (E) and (F) or mutated (mt) MRE3 and MRE6 in (G) and (H) or vector (V). Luciferase activity was measured 48 hr after transfection. In (G), red letters denote point mutations that disrupt base pairing.

Mean ± SD, normalized to vector control, of three independent experiments is shown. *$p < 0.05$, **$p < 0.01$.
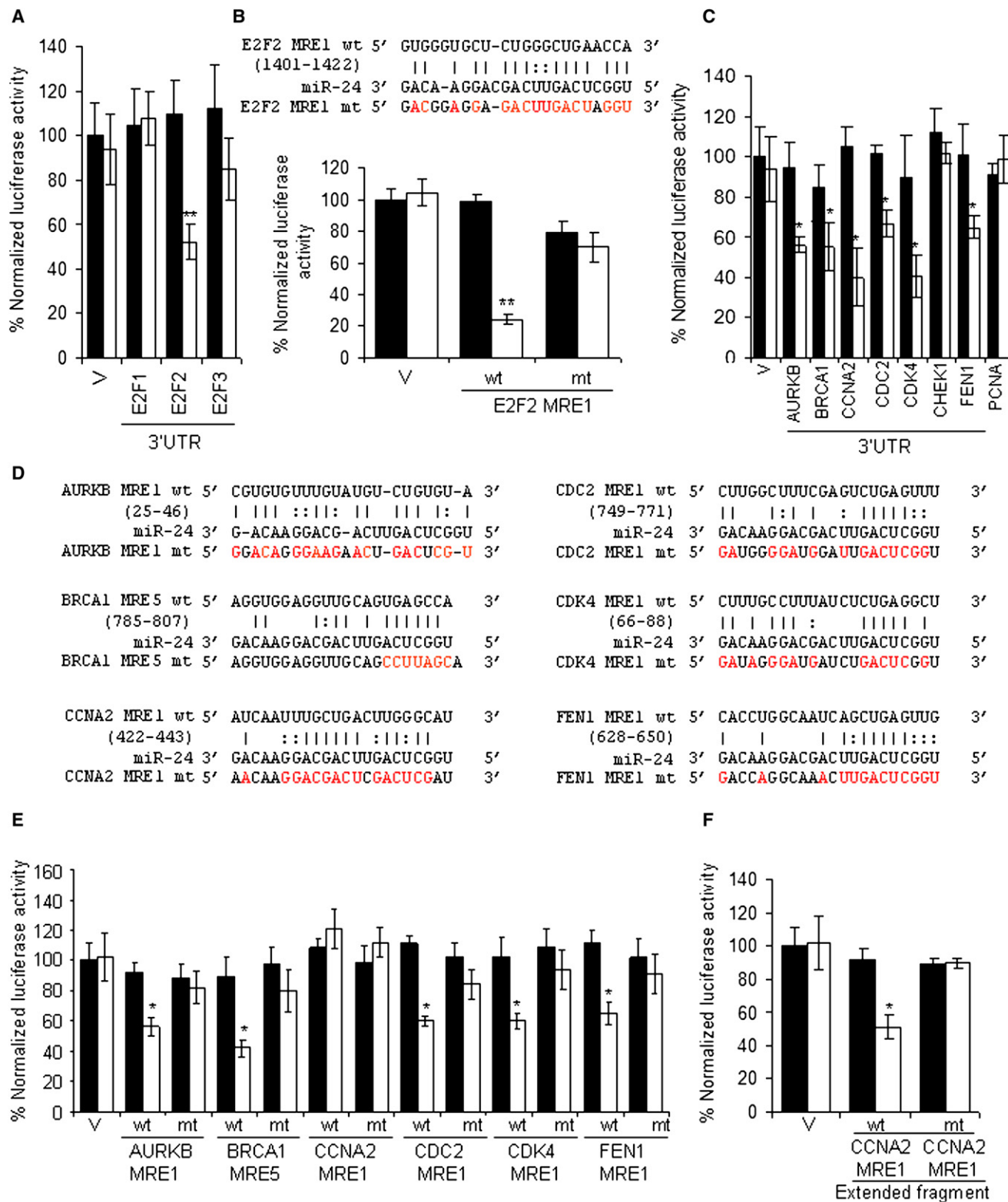
Figure 6. *E2F2* and Multiple E2F Target Genes Are Direct Targets of miR-24, Recognized by Seedless MREs

(A) miR-24 silences the expression of luciferase genes engineered with the 3′UTR of *E2F2*, but not with *E2F1* or *E2F3* 3′UTRs, suggesting that *E2F2* is a direct miR-24 target but *E2F1* and *E2F3* are downregulated indirectly. Luciferase assays were performed in HepG2 cells overexpressing miR-24 (white) or control mimics (black).

overrepresented processes and direct interacting protein networks within this gene set. This type of analysis, which surprisingly does not seem to have been previously applied to understanding miRNA regulation, led to the hypothesis that miR-24 might regulate cell-cycle progression during postmitotic differentiation by targeting *MYC* and/or *E2F2*, given that they constituted nodes of the major interaction network of the down-regulated gene set. Both *MYC* and *E2F2* are directly regulated by miR-24, but neither of these genes is a predicted miR-24 target. *MYC*, which has a 3'UTR hexamer seed sequence, is regulated both by a seed-containing MRE and a noncanonical seedless MRE. *E2F2* lacks any miR-24 seed match. However, *E2F2* turned out to be the key gene for miR-24 inhibition of the cell cycle because overexpressing miR-24-insensitive *E2F2* completely restored proliferation.

The GO analysis of miR-24-downregulated genes also suggested that miR-24 might regulate DNA repair. We recently verified this prediction by showing that overexpression of miR-24 enhances sensitivity to DNA damage (Lal et al., 2009). The key miR-24 target for this biological effect is *H2AFX*, which has two seed-bearing predicted MREs.

An unbiased analysis, which did not filter out genes whose 3'UTR lack seed binding sites, was critical for enabling us to identify *E2F2* as the key miR-24 target gene for cell-cycle regulation. In addition to *MYC* and *E2F2*, we found five other miR-24-downregulated genes whose 3'UTR was inhibited by miR-24 through seedless MREs. These genes (*AURKB*, *CCNA2*, *CDK4*, *CDC2*, and *FEN1*) are also transcriptionally regulated by E2Fs or MYC and play crucial roles in cell-cycle progression. Our results suggest that, in addition to genes containing miR-24 perfect seed matches, seedless MREs are also important. Indeed, seedless MREs are critical for miR-24 function because the antiproliferative effect of miR-24 can be recapitulated by silencing or obliterated by overexpressing the seedless *E2F2* gene. However, the importance of recognition of seedless versus seed-bearing MREs could vary between miRNAs. An assessment of this question could be determined by experimental testing of a large set of randomly chosen genes, whose protein or mRNA is downregulated by miRNA overexpression or increased by miRNA inhibition. In addition to seedless 3'UTR MREs, we previously identified a coding region miR-24 MRE in *p16INK4A* (Lal et al., 2008). Other recent studies also identified coding region MREs (Duursma et al., 2008; Tay et al., 2008). Taken together, these results suggest that target gene identification might be improved by not disregarding noncanonical MREs.

miR-24 directly regulates not only critical nodes of the interactome of cell-cycle regulatory genes, but also genes downstream of these nodes. This multitiered gene regulation may guarantee that cell-cycle arrest is not easily evaded. In fact, we have preliminary data suggesting that miR-24 may directly regulate many additional periodic genes, including others that lack a canonical seed-bearing MRE. Because expression of many of these genes is suppressed in nondividing cells, we were careful to show that miR-24-mediated gene suppression occurs before miR-24-transduced cells have stopped dividing (Figure S4), so their downregulation is a cause, not consequence, of cell-cycle arrest.

MYC and E2F regulate progression through G1. Regulating the transition to S phase may be the major site of miR-24 action because miR-24-treated cells accumulate in G1. Because MYC and E2F2 promote each other's transcription, miR-24 may prevent the reciprocal activation of these genes by regulating both of them. The dramatic downregulation of both proteins in miR-24-overexpressing cells could, therefore, be a combined effect of posttranscriptional and transcriptional regulation. Other miR-24 targets that are also transcriptionally regulated by MYC or E2F2 are implicated in controlling progression through G1, the G1/S checkpoint, S, and G2/M. For example, overexpressing miR-24 downregulated the mRNA and protein levels of the E2F-regulated genes, *CCNA2* and *CDC2*, which act together to promote G1/S and G2/M transitions. CCNA2 binds to and activates CDC2, thereby promoting G1/S and G2/M transition. Although the mRNA for *CDK6*, which regulates G1-to-S transition, was not significantly changed in our microarrays, we previously showed that *CDK6* is directly regulated by miR-24 (Lal et al., 2008). *CDK6* may be an example of a target gene regulated primarily by translational inhibition. *p16INK4A* is another direct target of miR-24 that is translationally regulated by miR-24 and, therefore, not downregulated in the microarrays (Lal et al., 2008). In addition to genes, which act at the G1/S transition, cells transfected with miR-24 mimics also have decreased expression of genes that principally act at other phases of the cell cycle. Important genes required for DNA replication in S phase were also downregulated by miR-24, including MCM4 and MCM10 in the prereplication complex; RRM2, a ribonucleotide reductase that catalyzes deoxyribonucleotide synthesis from ribonucleotides; PCNA, which forms a moving platform to recruit replication enzymes to the replication fork; and FEN1, a flap endonuclease involved in rejoining Okasaki fragments. Other downregulated genes act principally to facilitate mitosis, including AURKB and CCNB1. Therefore, miR-24 may put the breaks on cell division at multiple steps in cell-cycle progression.

(B) miR-24 downregulates luciferase activity of a reporter gene containing wild-type (wt) *E2F2* MRE1. Mutations in the miR-24 pairing residues (mt) rescue luciferase expression (sequences and luciferase assays for candidate *E2F2* WT 3'UTR MREs are shown in Figure S3).

(C) miR-24 targets the 3'UTR of E2F-regulated genes (*AURKB*, *BRCA1*, *CCNA2*, *CDC2*, and *FEN1*) and *CDK4*, a MYC-regulated gene. *CHEK1* and *PCNA* 3'UTRs are not regulated by miR-24. HepG2 cells were cotransfected with a luciferase reporter containing the 3'UTR of the indicated gene and control miRNA (black) or synthetic miR-24 (white) for 48 hr. Expression of the unmodified luciferase vector (V) is unchanged by miR-24.

(D) Predicted binding sites in the 3'UTR of genes whose 3'UTR was repressed by miR-24 in (C) and binding site mutations tested (indicated in red).

(E) Expression of reporter genes containing wild-type (wt) *AURKB* MRE1, *BRCA1* MRE5, *CDC2* MRE1, *CDK4* MRE1, and *FEN1* MRE1 is significantly reduced upon cotransfection of HepG2 cells with miR-24 mimics (white) and not the control mimic (black). Mutations in the miR-24 pairing residues (mt) rescue luciferase expression (sequences and luciferase assays for all tested wt MREs for these genes are shown in Figures S6 and S7). The *CCNA2* MRE1 is not regulated.

(F) However, miR-24 regulates a 181 nt region containing the *CCNA2* MRE1 in the luciferase vector. Mutations in the binding residues of *CCNA2* MRE1 within the extended sequence restore luciferase activity.

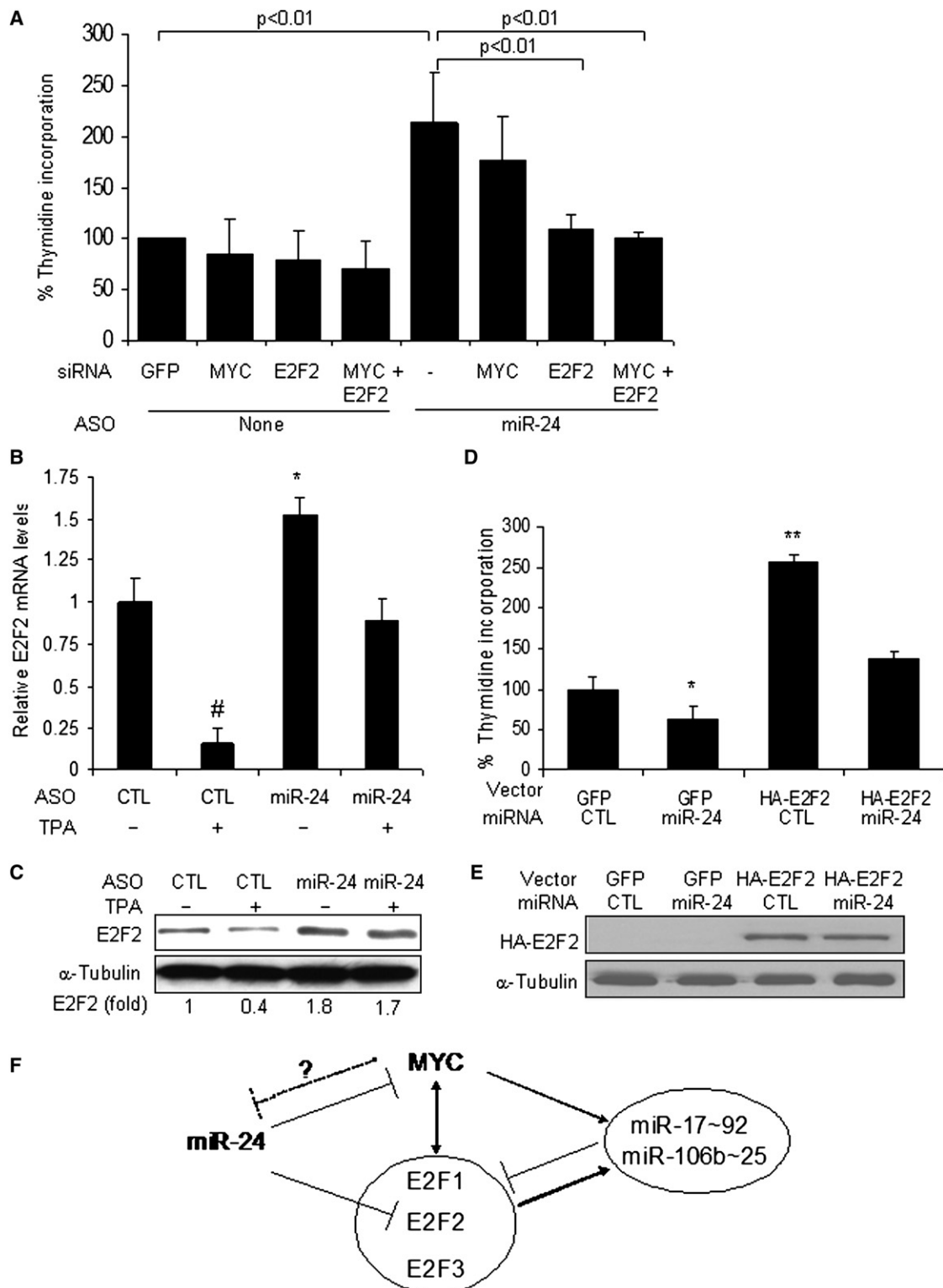Error bars in (A), (C), and (E) represent mean ± SD from three independent experiments. **p < 0.01, *p < 0.05.

Figure 7. *E2F2* **Is a Key miR-24 Target Gene**

(A) Increased cell proliferation from antagonizing miR-24 in K562 cells is blocked by siRNA-mediated knockdown of *E2F2*, but not *MYC*. Knockdown is shown by immunoblot (Figure S5). K562 cells were cotransfected with or without miR-24 ASO plus control siRNA or siRNAs targeting *E2F2* and/or *MYC*. The rate of cellular proliferation was determined 72 hr later by thymidine incorporation. Error bars represent mean ± SD from three independent experiments.

Silencing the genes mentioned above would be expected to inhibit cell division. However, suppressing other miR-24-downregulated genes would promote cell-cycle progression, especially in the context of DNA damage. These genes include CHEK1, which participates in the G2/M checkpoint and is activated by ATR in response to unresolved DNA damage, and BRCA1, which is in a surveillance complex that activates double-strand break repair. Prominent in the downregulated gene interaction network are the cyclin D inhibitor *CDKN1B* (*p27KIP1*) and *VHL*, a tumor suppressor protein. In addition, *p16INK4A*, a CDK inhibitor, is a validated direct miR-24 target (Lal et al., 2008). Thus, the role of miR-24 in regulating the cell cycle may be complex. If cells are unable to exit G1 and replicate their DNA, it may be economical to suppress the inhibitory genes that guard the genome from propagating damaged DNA. However, in some contexts, depending on the transcripts expressed in a particular cell, miR-24 might actually promote cell proliferation by suppressing these cell-cycle inhibitory genes. In fact, inhibiting miR-24 decreases proliferation of A549 lung cancer cells but has the opposite effect on HeLa cells (Cheng et al., 2005).

miR-24 is most highly expressed in G1. This is consistent with our finding that miR-24 regulates the G1/S transition. The E2F family of transcription factors regulates progression through this checkpoint. It therefore makes sense that miR-24 acts, in large part, by directly targeting *E2F2* (and thereby indirectly suppressing *E2F1* and *E2F3* expression). The pattern of miR-24 expression is consistent with the known cell-cycle variation of E2F family members (Sears et al., 1997). When miR-24 is high in G1, E2F1 and E2F2 are low; the E2F family begins to be expressed in late G1 and peaks in S phase when miR-24 is turned down. The E2Fs continue to be expressed in G2 and M (where they also have important functions) when miR-24 levels remain low.

miR-24 is not the only miRNA that regulates the cell cycle and targets the *E2F* family (Figure 7F). For instance, the miR-17~92 cluster directly downregulates the *E2F* family (O'Donnell et al., 2005; Petrocca et al., 2008). However, unlike miR-24, whose expression varies inversely with E2F expression, miR-17~92 appears to be expressed uniformly except in quiescent cells. Moreover, E2F2 downregulation should antagonize the dominant effect of these miRNAs to promote cell proliferation. Thus, E2F downregulation is likely not a defining effect of miR-17~92 but, rather, a secondary effect that fine-tunes its major proliferative effect. Because miR-24 suppresses *MYC* and *E2F* expression and both MYC and the E2F family activate miR-17~92 and miR-106b~25 transcription (O'Donnell et al., 2005; Petrocca et al., 2008), miR-24 also likely inhibits proliferation by indirectly suppressing transcription of these cell-cycle-promoting miRNAs. A recent paper also suggests another layer of complexity to the miR-24, miR-17~92, MYC, and E2F network (Gao et al., 2009). MYC suppresses the transcription of miR-23b, which is encoded with miR-24. Although one recent study suggests that miR-24 and miR-23b are independently transcribed (Sun et al., 2009), MYC might also regulate miR-24 transcription. It is worth noting that *E2F1* mRNA and protein do not correlate during the cell cycle, consistent with posttranscriptional regulation by miRNAs (O'Donnell et al., 2005). Because *E2F2* mRNA has kinetics similar to *E2F1* (Sears et al., 1997), miRNA-dependent regulation of translation may be operating, possibly for all E2Fs that promote G1/S transition.

The integrated effect of miR-24 on a highly interacting set of key genes acts as a switch to stop cell division, rather than as a fine-tuning rheostat. It will be interesting to understand how expression of these two miR-24 gene clusters is regulated and to understand the function of the clustered miRNAs (miR-23 and miR-27). The only other miRNAs consistently upregulated during terminal differentiation are miR-22 and miR-125a (a mammalian ortholog of lin-4) (Lal et al., 2009). There are suggestions in the literature that these genes might also regulate important pathways of cell differentiation (Choong et al., 2007; Wu and Belasco, 2005).

miR-24 directly regulates both cell proliferation and DNA repair. Enhancing miR-24 function in cancer cells by introducing miR-24 mimics might be an attractive therapeutic, given that it could potentially block dysregulated cell proliferation and also sensitize cancer cells to DNA damage from chemo- and radiotherapy.

EXPERIMENTAL PROCEDURES

GO Analysis

The GO project provides structured controlled vocabularies, or ontologies, to describe genes relative to their biological processes. Each biological process consists of a series of events achieved by one or more molecular functions. The ontologies are stored in directed acyclic graphs in which each node represents a biological process and each subsequent node corresponds to a more specialized term. Overrepresented GO biological processes were determined using a MetaCore Analytical Suite (GeneGo Inc., St. Joseph, MI, http://www.genego.com), which utilizes the hypergeometric distribution to calculate the p value for genes showing enrichment in a biological process. The value is equivalent to the probability of a subset of genes from a specific

(B and C) Downregulation of *E2F2* mRNA (B) and protein (C) during TPA-mediated differentiation of K562 cells to megakaryocytes is mediated by miR-24 and can be completely inhibited by antagonizing miR-24. K562 cells were transfected with miR-24 or a control (CTL) ASO for 72 hr and then treated with TPA for 6 hr. mRNA was assessed by qRT-PCR normalized to *GAPDH* and normalized to control cells transfected with CTL ASO. E2F2 protein was quantified by densitometry and normalized to α-tubulin.

(D and E) Transfection of K562 cells with a miR-24 mimic reduces cell proliferation, which can be rescued by expressing miR-24-insensitive *E2F2* lacking the 3'UTR. K562 cells were cotransfected with a vector expressing HA-tagged E2F2 or GFP and miR-24 or cel-miR-67 (CTL) mimics for 72 hr before measuring thymidine uptake.

(E) Immunoblot probed for HA tag. Error bars represent mean ± SD from three independent experiments. *p < 0.05, **p < 0.01, and #p < 0.005.

(F) Model of the miR-24, miR-17~92, MYC, and E2F network of cell-cycle regulators. Here, we show that miR-24 directly suppresses expression of *MYC* and *E2F2* (and indirectly suppresses *E2F1* and *E2F3*) and thereby regulates the G1/S transition. Expression of the opposing miRNAs encoded by the miR-17~92 and miR-106b~25 clusters that promote cell proliferation is transcriptionally activated by the same transcription factors that miR-24 suppresses (O'Donnell et al., 2005; Petrocca et al., 2008). Therefore, miR-24 would be predicted to reduce expression of the proliferation-promoting miRNA clusters indirectly. These miRNAs also knock down the *E2F* genes but probably to fine-tune their proliferative effect. MYC may also suppress miR-24 transcription (Gao et al., 2009).

experiment (i.e., miR-24 overexpression) to arise by chance given the total number of genes associated with the biological process.

Network Analysis

We developed a graphical representation of the molecular relationships between proteins from the 248 genes downregulated in miR-24-overexpressing cells. Network analysis was performed using the MetaCore Analytical Suite, and visualization was performed using Ingenuity Pathways Analysis (Ingenuity Inc., http://www.ingenuity.com). Proteins are represented as nodes, and the biological relationship between two nodes is represented as an edge. Functional and pathway analysis was based on support for direct interaction edges in the networks from at least one reference from the literature, a textbook, or from canonical information stored in the network generation software database, manually curated for experimentally verified human protein-protein interactions and protein-DNA interactions.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, nine figures, and five tables and can be found with this article online at http://www.cell.com/molecular-cell/supplemental/S1097-2765(09)00600-5.

REFERENCES

Baek, D., Villen, J., Shin, C., Camargo, F.D., Gygi, S.P., and Bartel, D.P. (2008). The impact of microRNAs on protein output. Nature 455, 64–71.

Bar-Joseph, Z., Siegfried, Z., Brandeis, M., Brors, B., Lu, Y., Eils, R., Dynlacht, B.D., and Simon, I. (2008). Genome-wide transcriptional analysis of the human cell cycle identifies genes differentially regulated in normal and cancer cells. Proc. Natl. Acad. Sci. USA 105, 955–960.

Bartel, D.P. (2009). MicroRNAs: target recognition and regulatory functions. Cell 136, 215–233.

Bracken, A.P., Ciro, M., Cocito, A., and Helin, K. (2004). E2F target genes: unraveling the biology. Trends Biochem. Sci. 29, 409–417.

Calin, G.A., Ferracin, M., Cimmino, A., Di Leva, G., Shimizu, M., Wojcik, S.E., Iorio, M.V., Visone, R., Sever, N.I., Fabbri, M., et al. (2005). A MicroRNA signature associated with prognosis and progression in chronic lymphocytic leukemia. N. Engl. J. Med. 353, 1793–1801.

Chang, T.C., Wentzel, E.A., Kent, O.A., Ramachandran, K., Mullendore, M., Lee, K.H., Feldmann, G., Yamakuchi, M., Ferlito, M., Lowenstein, C.J., et al. (2007). Transactivation of miR-34a by p53 broadly influences gene expression and promotes apoptosis. Mol. Cell 26, 745–752.

Cheng, A.M., Byrom, M.W., Shelton, J., and Ford, L.P. (2005). Antisense inhibition of human miRNAs and indications for an involvement of miRNA in cell growth and apoptosis. Nucleic Acids Res. 33, 1290–1297.

Choong, M.L., Yang, H.H., and McNiece, I. (2007). MicroRNA expression profiling during human cord blood-derived CD34 cell erythropoiesis. Exp. Hematol. 35, 551–564.

Didiano, D., and Hobert, O. (2006). Perfect seed pairing is not a generally reliable predictor for miRNA-target interactions. Nat. Struct. Mol. Biol. 13, 849–851.

Didiano, D., and Hobert, O. (2008). Molecular architecture of a miRNA-regulated 3′ UTR. RNA 14, 1297–1317.

Diederichs, S., and Haber, D.A. (2007). Dual role for argonautes in microRNA processing and posttranscriptional regulation of microRNA expression. Cell 131, 1097–1108.

Doench, J.G., and Sharp, P.A. (2004). Specificity of microRNA target selection in translational repression. Genes Dev. 18, 504–511.

Duursma, A.M., Kedde, M., Schrier, M., le Sage, C., and Agami, R. (2008). miR-148 targets human DNMT3b protein coding region. RNA 14, 872–877.

Easow, G., Teleman, A.A., and Cohen, S.M. (2007). Isolation of microRNA targets by miRNP immunopurification. RNA 13, 1198–1204.

Fernandez, P.C., Frank, S.R., Wang, L., Schroeder, M., Liu, S., Greene, J., Cocito, A., and Amati, B. (2003). Genomic targets of the human c-Myc protein. Genes Dev. 17, 1115–1129.

Fukuda, Y., Kawasaki, H., and Taira, K. (2005). Exploration of human miRNA target genes in neuronal differentiation. Nucleic Acids Symp. Ser. (Oxf.) 49, 341–342.

Gao, P., Tchernyshyov, I., Chang, T.C., Lee, Y.S., Kita, K., Ochi, T., Zeller, K.I., De Marzo, A.M., Van Eyk, J.E., Mendell, J.T., and Dang, C.V. (2009). c-Myc suppression of miR-23a/b enhances mitochondrial glutaminase expression and glutamine metabolism. Nature 458, 762–765.

Hermeking, H., Rago, C., Schuhmacher, M., Li, Q., Barrett, J.F., Obaya, A.J., O'Connell, B.C., Mateyak, M.K., Tam, W., Kohlhuber, F., et al. (2000). Identification of CDK4 as a target of c-MYC. Proc. Natl. Acad. Sci. USA 97, 2229–2234.

Johnson, S.M., Grosshans, H., Shingara, J., Byrom, M., Jarvis, R., Cheng, A., Labourier, E., Reinert, K.L., Brown, D., and Slack, F.J. (2005). RAS is regulated by the let-7 microRNA family. Cell 120, 635–647.

Johnson, C.D., Esquela-Kerscher, A., Stefani, G., Byrom, M., Kelnar, K., Ovcharenko, D., Wilson, M., Wang, X., Shelton, J., Shingara, J., et al. (2007). The let-7 microRNA represses cell proliferation pathways in human cells. Cancer Res. 67, 7713–7722.

Lal, A., Kim, H.H., Abdelmohsen, K., Kuwano, Y., Pullmann, R., Jr., Srikantan, S., Subrahmanyam, R., Martindale, J.L., Yang, X., Ahmed, F., et al. (2008). p16(INK4a) translation suppressed by miR-24. PLoS ONE 3, e1864.

Lal, A., Pan, Y., Navarro, F., Dykxhoorn, D., Moreau, L., Meire, E., Bentwich, Z., Lieberman, J., and Chowdhury, D. (2009). miR-24-mediated down-regulation of H2AX suppresses DNA repair in terminally differentiated blood cells. Nat. Struct. Mol. Biol. 16, 492–498.

Lebofsky, R., and Walter, J.C. (2007). New Myc-anisms for DNA replication and tumorigenesis? Cancer Cell 12, 102–103.

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.

Lewis, B.P., Burge, C.B., and Bartel, D.P. (2005). Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell 120, 15–20.

Lim, L.P., Lau, N.C., Garrett-Engele, P., Grimson, A., Schelter, J.M., Castle, J., Bartel, D.P., Linsley, P.S., and Johnson, J.M. (2005). Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. Nature 433, 769–773.

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.

Neilson, J.R., Zheng, G.X., Burge, C.B., and Sharp, P.A. (2007). Dynamic regulation of miRNA expression in ordered stages of cellular development. Genes Dev. 21, 578–589.

O'Donnell, K.A., Wentzel, E.A., Zeller, K.I., Dang, C.V., and Mendell, J.T. (2005). c-Myc-regulated microRNAs modulate E2F1 expression. Nature 435, 839–843.

Olsen, P.H., and Ambros, V. (1999). The lin-4 regulatory RNA controls developmental timing in Caenorhabditis elegans by blocking LIN-14 protein synthesis after the initiation of translation. Dev. Biol. *216*, 671–680.

Petrocca, F., Visone, R., Onelli, M.R., Shah, M.H., Nicoloso, M.S., de Martino, I., Iliopoulos, D., Pilozzi, E., Liu, C.G., Negrini, M., et al. (2008). E2F1-regulated microRNAs impair TGFbeta-dependent cell-cycle arrest and apoptosis in gastric cancer. Cancer Cell *13*, 272–286.

Polager, S., and Ginsberg, D. (2008). E2F - at the crossroads of life and death. Trends Cell Biol. *18*, 528–535.

Sears, R., Ohtani, K., and Nevins, J.R. (1997). Identification of positively and negatively acting elements regulating expression of the E2F2 gene in response to cell growth signals. Mol. Cell. Biol. *17*, 5227–5235.

Selbach, M., Schwanhausser, B., Thierfelder, N., Fang, Z., Khanin, R., and Rajewsky, N. (2008). Widespread changes in protein synthesis induced by microRNAs. Nature *455*, 58–63.

Sun, Q., Zhang, Y., Yang, G., Chen, X., Cao, G., Wang, J., Sun, Y., Zhang, P., Fan, M., Shao, N., and Yang, X. (2008). Transforming growth factor-beta-regulated miR-24 promotes skeletal muscle differentiation. Nucleic Acids Res. *36*, 2690–2699.

Sun, F., Wang, J., Pan, Q., Yu, Y., Zhang, Y., Wan, Y., Wang, J., Li, X., and Hong, A. (2009). Characterization of function and regulation of miR-24-1 and miR-31. Biochem. Biophys. Res. Commun. *380*, 660–665.

Tay, Y., Zhang, J., Thomson, A.M., Lim, B., and Rigoutsos, I. (2008). MicroRNAs to Nanog, Oct4 and Sox2 coding regions modulate embryonic stem cell differentiation. Nature *455*, 1124–1128.

Vella, M.C., Choi, E.Y., Lin, S.Y., Reinert, K., and Slack, F.J. (2004). The C. elegans microRNA let-7 binds to imperfect let-7 complementary sites from the lin-41 3′UTR. Genes Dev. *18*, 132–137.

Vernell, R., Helin, K., and Muller, H. (2003). Identification of target genes of the p16INK4A-pRB-E2F pathway. J. Biol. Chem. *278*, 46124–46137.

Wu, L., and Belasco, J.G. (2005). Micro-RNA regulation of the mammalian lin-28 gene during neuronal differentiation of embryonal carcinoma cells. Mol. Cell. Biol. *25*, 9198–9208.

Zhang, L., Ding, L., Cheung, T.H., Dong, M.Q., Chen, J., Sewell, A.K., Liu, X., Yates, J.R., III, and Han, M. (2007). Systematic identification of C. elegans miRISC proteins, miRNAs, and mRNA targets by their interactions with GW182 proteins AIN-1 and AIN-2. Mol. Cell *28*, 598–613.