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SUPPLEMENTAL INFORMATION

MicroRNA Destabilization Enables Dynamic Regulation of the miR-16 Family In Response to Cell-Cycle Changes

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Supplemental Experimental Procedures

Plasmids. For a complete list of plasmids used in this study, see Supplemental Table 4.

miRNA Expression Constructs. To generate the miR-503~122 bicistronic construct, *pre-mmu-mir-503*, along with approximately 150 adjacent nucleotides, was cloned into pIS1 (Grimson et al., 2007) by PCR amplification using oligonucleotide primers Forward mmu-503 SacI and Reverse mmu-503 SpeI. The *pre-mir-503* hairpin was then cloned into pRetroX-Tight-Pur by PCR with primers BamHI 503 Fw and EcoRI SV40 Term and digesting the resulting product with BamHI and EcoRI. *pre-hsa-mir-122* was amplified using miR-122 SpeI Fw and miR-122 NotI Rev; the resulting product was cloned into pRetroX-Tight-Pur miR-503 with SpeI and NotI. To make pRetroX-Tight-Pur miR-497~mir-122, *pre-mmu-mir-497* (with surrounding nucleotides) was amplified with Forward mmu-miR-497 SacI and Reverse mmu-miR-497 SpeI; the product, digested with SacI and SpeI, was cloned into pRetroX-Tight-Pur miR-503~122, which had been similarly digested. In order to make various mutations, the following mutational scheme was performed with QuikII mutagenesis kit (Stratagene):

m1, m2, m08: Two-step mutagenesis was performed on pRetroX-Tight-Pur miR503. First the mature strand was mutated with 503 Fw/Rev1.1 (for m1), 503 Fw/Rev2.1 (for m2) or 503m09.1 Fw/Rev (for m08). The star strand was mutated with 503 Fw/Rev1.2 (for m2), 503 Fw/Rev2.2 (for m2) or 503m09.2 Fw/Rev (for m09).

m01, m02, m03, m04: One step mutagenesis of both the mature and star strands was performed on pRetroX-Tight-Pur miR503~122 with 503m01/2/3/4 Fw/Rev oligonucleotides.

m05, m06, m07: Two-step mutagenesis was performed on pRetroX-Tight-Pur miR503~122. First the mature arm was mutated with 503m05/6/7.1 Fw/Rev oligonucleotides; the star strand was then mutated with 503m05/6/7.2 Fw/Rev oligonucleotides.

In order to generate transient expression plasmids (pIS1miR-503~122 and pIS1miR-503m1~122), the miR-503~122 and miR-503m1~122 hairpins were amplified from the corresponding Tet plasmids with Forward mmu-503 SacI and miR-122 NotI Rev. The resulting product was digested with SacI and NotI and cloned into pIS1, which had been similarly digested.

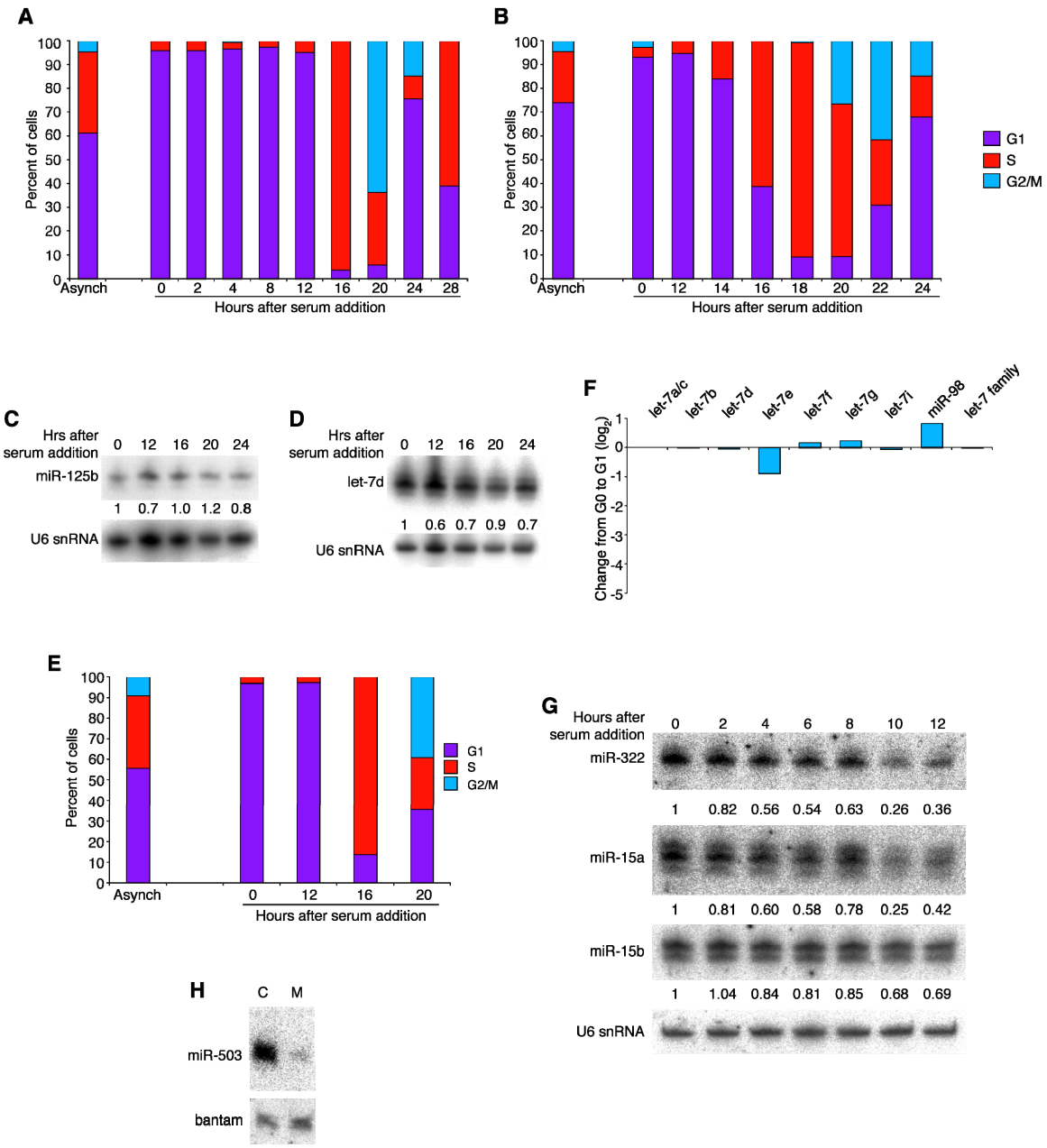
3' UTR reporter plasmids. In order to generate unstable *Renilla* luciferase, a PEST tag was amplified from pZsGreen1-DR vector (Clontech) using Forward luc-PEST and Reverse SacI PEST; *Renilla* luciferase was amplified from pISI using Reverse luc-PEST and Forward NheI luc. The two resulting products were stitched together and amplified using Forward NheI luc and Reverse SacI PEST. This product, digested with NheI and SacI, was cloned into similarly digested pISI and psiCheck2 (Promega); the resulting plasmids were called pIS1-DR and psiCheck2-DR, respectively. In order to generate, pIS1-DRx, pIS1-DR was digested with XbaI and NotI; annealed oligonucleotides containing an XhoI site (XhoI insert Fw and XhoI insert Rev) were then cloned into the linearized plasmid. In order to generate 3' UTR reporter plasmids, 3' UTR of *Cpeb2* was amplified from genomic DNA first with Cpeb2 Fw/Rev and then with Cpeb2 XbaI Fw and Cpeb2 NotI Rev. The 3' UTR of *Cdc25a* was amplified from genomic DNA with Cdc25a Fw/Rev. These UTRs were cloned into pIS1-DR and pIS1-DRx, respectively, with XbaI and NotI or, in the case of *Cdc25a*, with XhoI and NotI. The 3' UTR of *Ccne1* was amplified from genomic DNA with CcneI Fw and CcneI Rev (or, in the case of the mutant UTR, mCcneI Rev) and cloned into psiCheck2-DR with XhoI and NotI. miR-16 targets were then mutated with either QuikII (*Ccne1* and *Cdc25a*) or QuikMulti (*Cpeb2*) mutagenesis kits (Stratagene; see Supp Table for a complete list of oligonucleotides used).

pSilencer constructs. Target sequences were identified based on those available (and, where possible, validated) through Sigma; these were then used to generate the appropriate shRNA oligonucleotides for pSilencer2.1-U6, according the manufacturer's instructions (Ambion). These oligonucleotides were PAGE-purified and annealed, and the resulting duplexes (bearing BamHI and HindIII

overhangs) were then ligated into pSilencer2.1-U6 Scrambled (Ambion), which had been digested with BamHI and HindIII.

Knock-down experiments. Once stable lines had been selected with puromycin (2 μg/ml) as described in the Materials and Methods section, these were immediately used for analysis. Cells were first plated at low density (about 12% confluency) and 24 hours later were washed three times with 1x PBS. DMEM supplemented with P/S and 0.5% DCS was then added. After 48 hours, DMEM contained P/S and 20% DCS was added to release cells in G1. Serum-starved cells and cells that had been further incubated with serum for 12 hours were harvest. miR-503 levels were probed by northern blotting (see Materials and Methods section). To determine the degree of knock-down, 1 μg of total RNA was treated with DNase (Promega) according to manufacturer's instructions. One-tenth of this was then used for oligo-(dT)-primed reverse transcription with SuperScriptIII (Invitrogen) according to manufacturer's instructions. qPCR was performed with SYBR-Green on an ABI 7900 machine, according to manufacturer's instructions. *Gapdh* was used as an endogenous control, and fold change was calculated relative to the pSilencer Scrambled control line.

Rissland, et al. Figure S1



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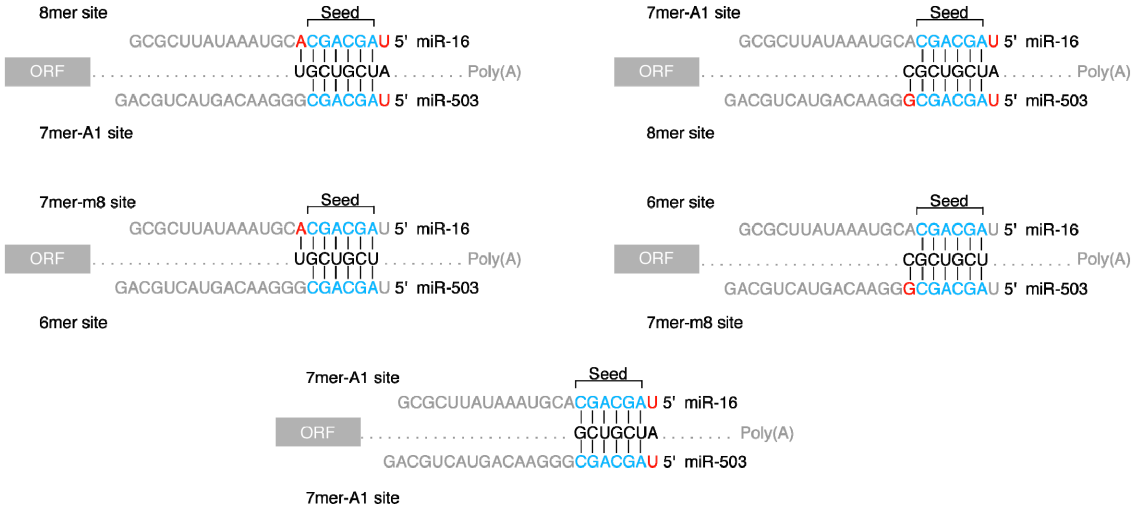
8mer site
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ORF
UGCUGCUA
Poly(A)
GACGUCAUGACAAGGGCGACGAU 5' miR-503
7mer-A1 site
7mer-A1 site
Seed
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ORF
CGCUGCUA
Poly(A)
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8mer site
7mer-m8 site
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ORF
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Poly(A)
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6mer site
6mer site
Seed
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ORF
CGCUGCU
Poly(A)
GACGUCAUGACAAGGGCGACGAU 5' miR-503
7mer-m8 site
7mer-A1 site
Seed
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ORF
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Poly(A)
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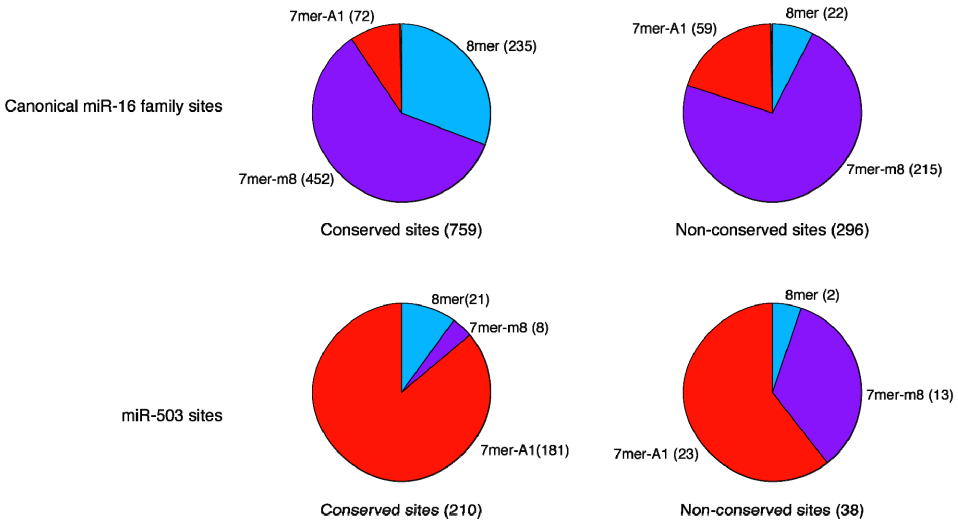
7mer-A1 site

J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7mer-A1 (72)
8mer (235)
7mer-m8 (452)
Conserved sites (759)
7mer-A1 (59)
8mer (22)
7mer-m8 (215)
Non-conserved sites (296)
miR-503 sites
8mer(21)
7mer-m8 (8)
7mer-A1(181)
Conserved sites (210)
8mer (2)
7mer-m8 (13)
7mer-A1 (23)
Non-conserved sites (38)

K

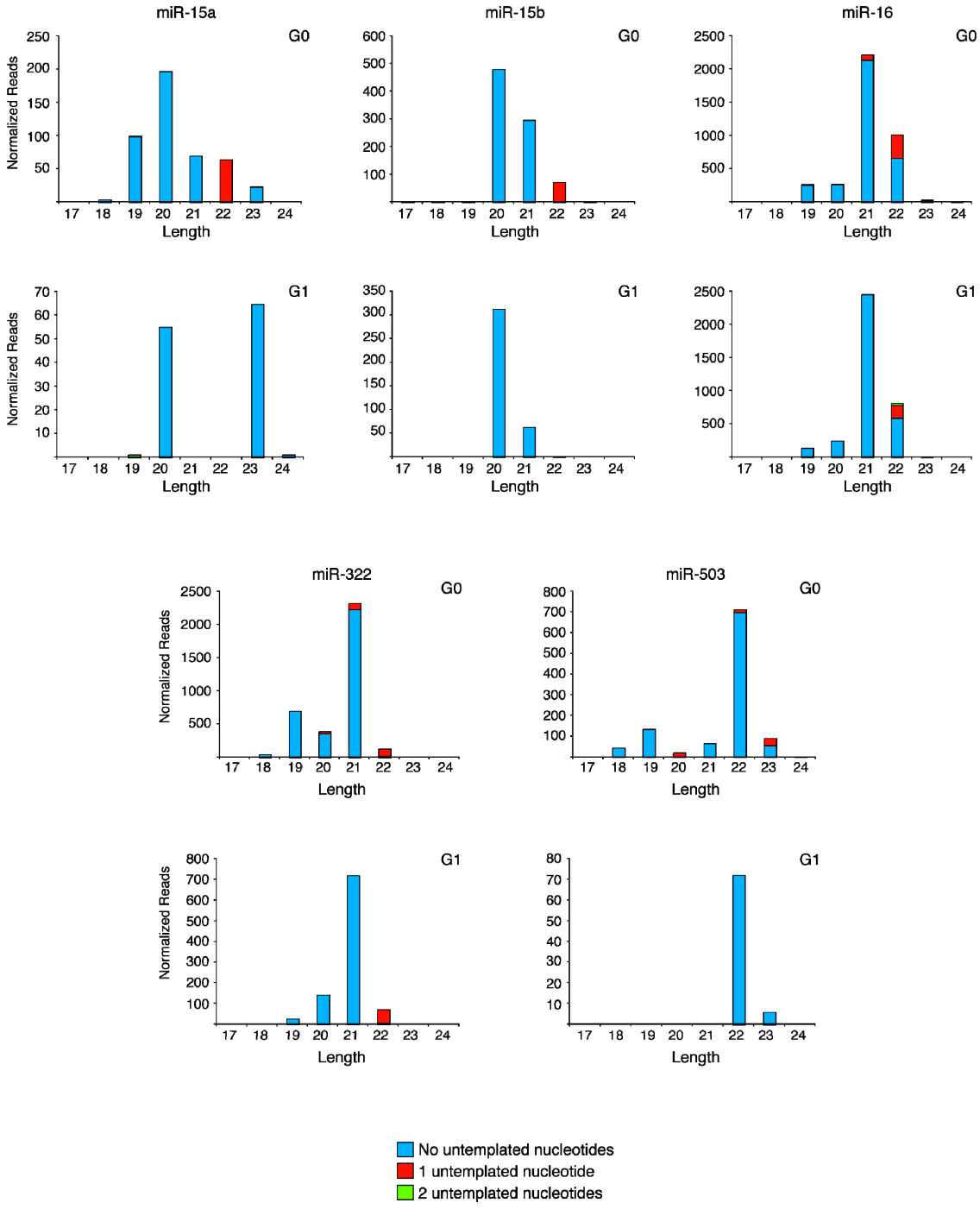
miR-15a
G0
Normalized Reads
Length
miR-15b
G0
miR-16
G0
G1
G1
G1
miR-322
G0
miR-503
G0
G1
G1
No untemplated nucleotides
1 untemplated nucleotide
2 untemplated nucleotides

Figure S1. The Response of miRNAs to Serum Starvation and Re-addition, Related to Figure 1

(A) FACS analysis of synchronized cells. Cells were arrested by serum starvation for 2 days (time 0) and then released into the cell cycle by addition of serum. Samples were harvested at the indicated time points and fixed in ice-cold ethanol; parallel samples were also harvested for RNA analysis. The DNA content was then determined by FACS following propidium iodide staining. For comparison, the cell cycle distribution of asynchronously growing cells was also determined. RNA samples from 0, 12, 16, 20 and 24-hour time points were used for small RNA libraries.

(B) As in (A), only cells were harvested at 0 hours and every two hours, starting twelve hours after the addition of serum. The RNA samples were then used for confirmatory northern blots in Figure 1E.

(C and D) miR-125a and let-7d do not change dramatically upon cell-cycle re-entry. NIH3T3 cells were arrested by serum starvation. After the addition of serum, cells were harvested at indicated time points. Levels of miR-125a (C) and let-7d (D) were determined by northern blotting and normalized to U6 snRNA (lower panel).

(E) The let-7 family is unaffected by cell-cycle re-entry. Plotted are the $\log_2$ fold-changes upon the transition from G0 to G1 in individual members of the let-7 family and the overall family, as determined by Illumina sequencing.

(F and G) Some members of the miR-16 family respond rapidly to cell cycle re-entry. (F) FACS analysis of synchronized cells, analyzed as described in (A). Cells were arrested by serum starvation for 2 days (time 0) and then released into the cell cycle by addition of serum. Samples were harvested at the indicated time points and fixed in ice-cold ethanol. The DNA content was then determined with FACS following propidium iodide staining. For comparison, the cell cycle distribution of asynchronously growing cells was also determined. (G) Dynamics of miR-16 family members during cell-cycle re-entry. In parallel to (F), samples were also isolated for RNA analysis at the various time points. Levels of the

indicated miRNAs were determined by northern blotting and normalized to U6 snRNA (bottom panel).

(H) miR-503 does not appear to be secreted. RNA was isolated from cells (C) serum starved for 24 hours and from the corresponding medium (M). S2 cell total RNA and glycoblue were used as carriers to ensure efficient precipitation of small RNAs. One-quarter of the isolated RNA from each sample was then separated by PAGE, and levels of miR-503 were determined by northern blotting (upper panel). As a control for RNA isolation, levels of bantam were also determined by northern blotting (lower panel).

(I and J) Sites for miR-503 and miR-16 family members overlap. (I) Schematic summarizing recognition of various site types by miR-503 and canonical miR-16 family members. Nucleotides in blue represent those in the miRNA seed (nucleotides 2-7). Those in red are nucleotides (either position 8 or position 1) that also participate in target recognition for the indicated 3' UTR site. (J) The distribution of various site types for miR-503 and the canonical miR-16 family.

(K) Members of the miR-16 family do not show high levels of trimming and tailing. Plotted are the size distributions of genome-matching (blue) and non-genome matching reads for the indicated miRNAs in G0 and G1. Non-genome matching reads were progressively trimmed from the 3' end to determine the extent of untemplated nucleotide addition. Those reads with one untemplated (red) and two untemplated (green) nucleotides are shown.

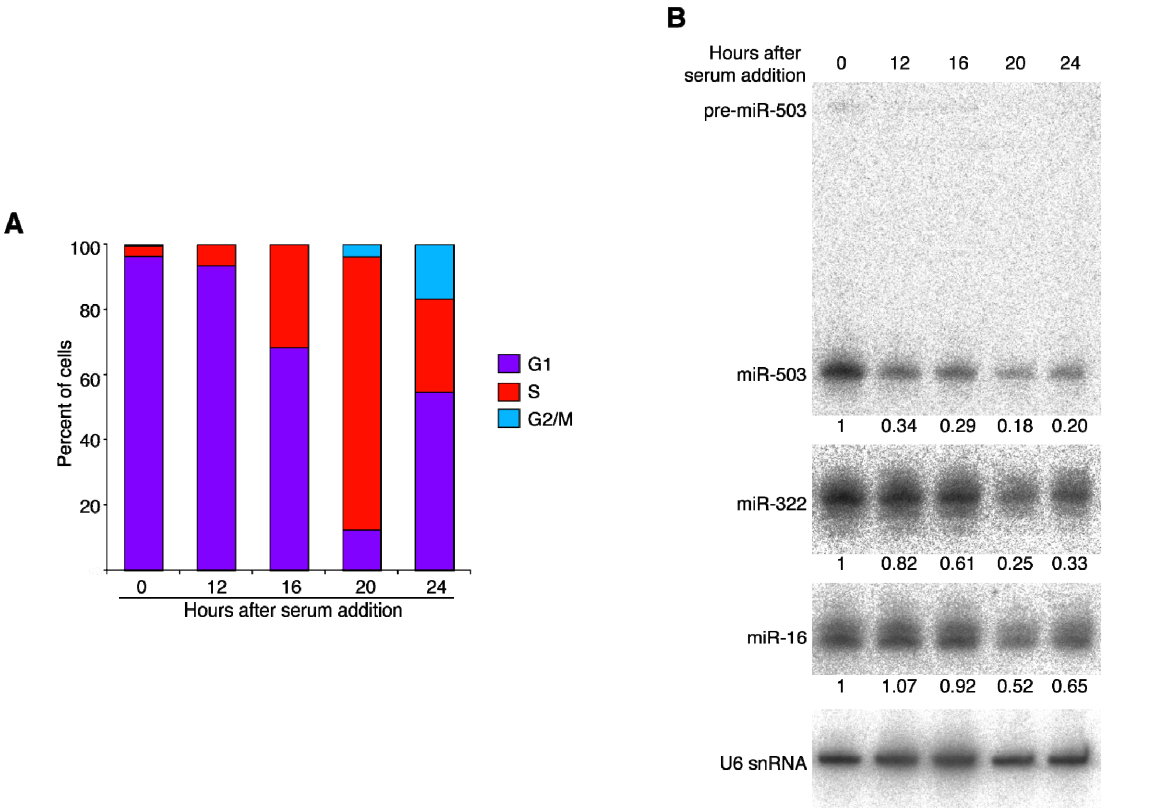

Figure S2. Tet-Off 3T3/MEFs Respond Normally to Serum Starvation and Re-addition, Related to Figure 2

(A) FACS analysis of synchronized cells. Tet-Off 3T3/MEF cells were arrested by serum starvation for 2 days (time 0) and then released into the cell cycle by addition of serum. Samples were harvested at the indicated time points and fixed in ice-cold ethanol. The DNA content was then determined with FACS following propidium iodide staining. For comparison, the cell cycle distribution of asynchronously growing cells was also determined.

(B) Dynamics of miR-16 family members during cell-cycle re-entry. In parallel to (A), samples were also isolated for RNA analysis at the various time points. Levels of the indicated miRNAs were determined by northern blotting and normalized to U6 snRNA (bottom panel).

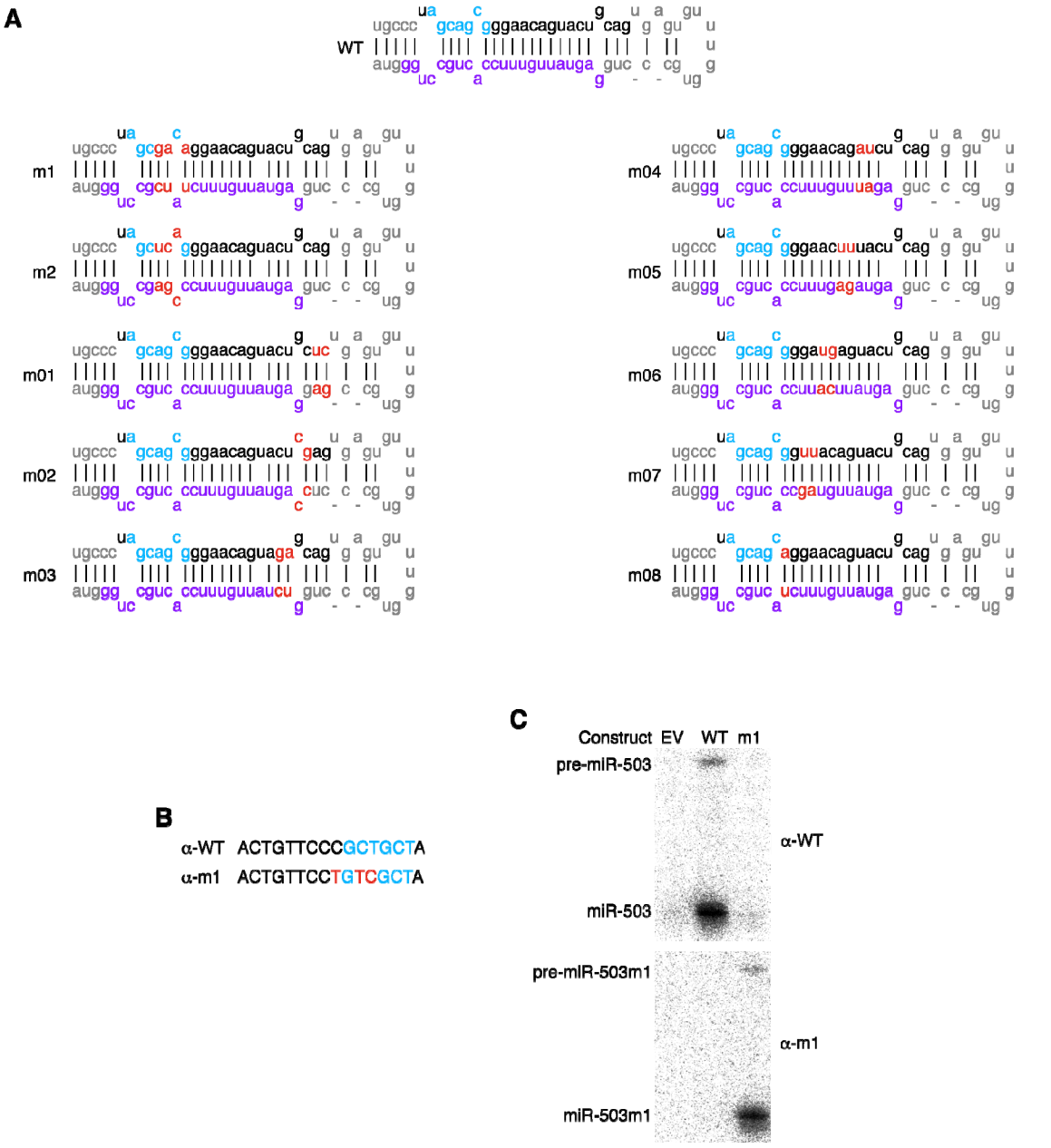

Figure S3. Some miR-503 Mutants Can Be Distinguished by Northern Blotting, Related to Figure 3

(A) Predicted hairpins of the miR-503 mutants used in this study. Nucleotides in blue are positions 1-8 of the mature miRNA, which is denoted in black. Those in

purple are the miRNA*; those in red are residues mutated in the miR-503 derivative hairpin.
(B) Northern blot probes used to distinguish wild-type (WT) and m1 miR-503.
(C) WT and m1 can be distinguished by northern blotting. 293 cells were transiently transfected either with pIS1, a construct expressing either luciferase (EV), or with pIS1-miR-503/pIS1-miR-503m1, constructs expressing luciferase followed by a 3' UTR containing either miR-503 (WT) or miR-503m1. Expression of the miRNAs was then determined with the indicated probes.

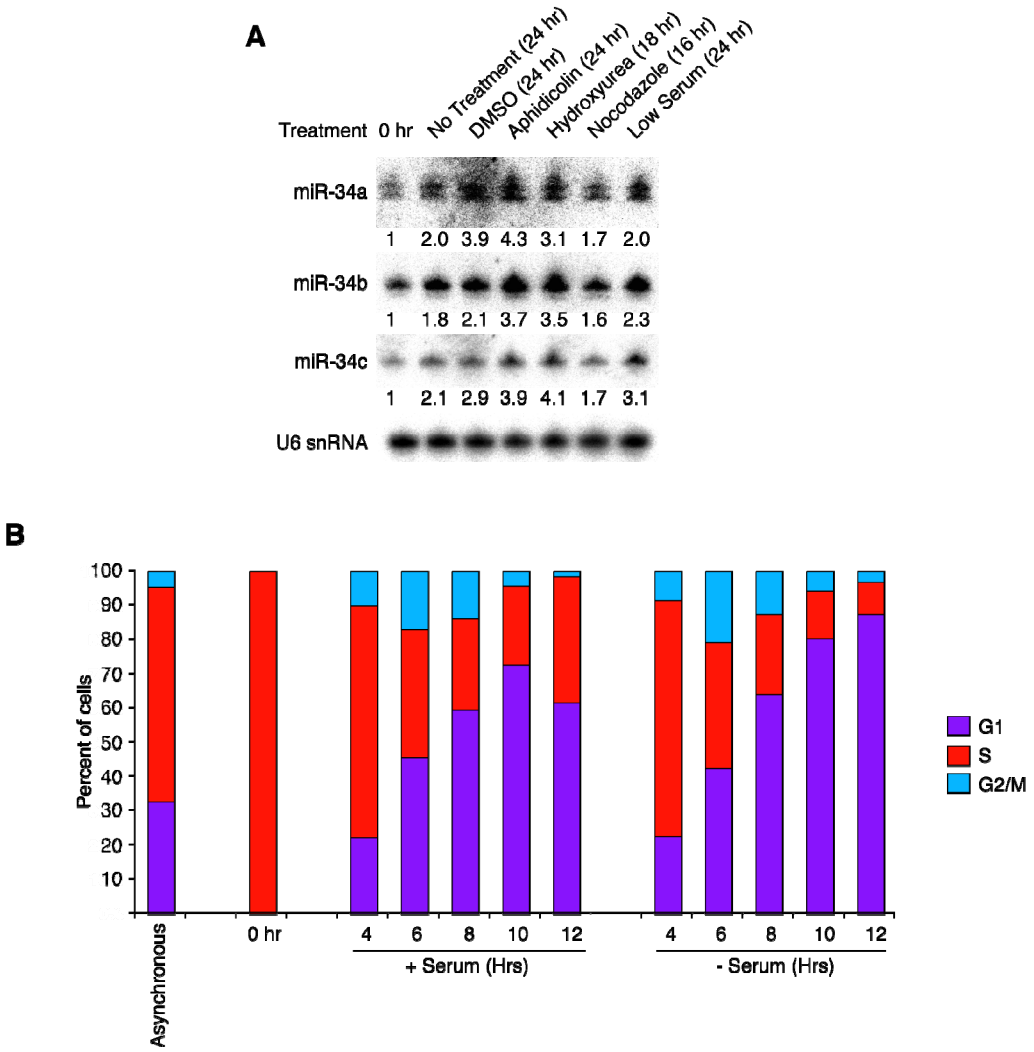

Figure S4. The Response of miRNAs to Different Cell-cycle Arrests, Related to Figure 4

(A) miR-34 family members are up-regulated in response to many different types of cell cycle arrest. The response of miR-34 family members in NIH3T3 cells to aphidicolin, hydroxyurea, nocodazole and serum starvation was determined by northern blotting with normalization to U6 snRNA (bottom panel).

(B) NIH3T3 cells were treated with 2 mM thymidine for 14 hours (0 hours) and then released into media containing normal serum (+serum) or low serum (-serum). Samples were harvested at the indicated time points and fixed in ice-cold ethanol. The DNA content was then determined with FACS following propidium iodide staining. For comparison, the cell cycle distribution of asynchronously growing cells was also determined.

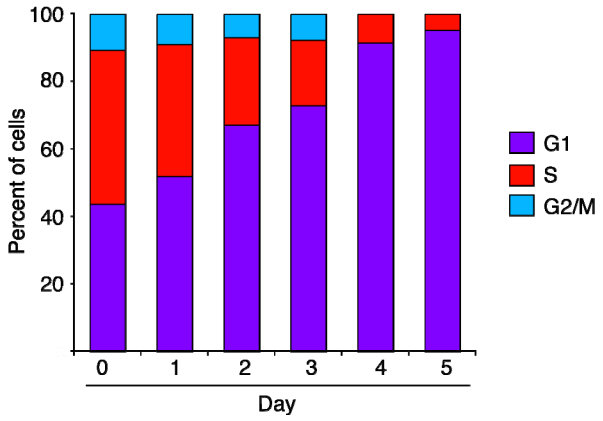

Figure S5. The Cell-cycle Distribution of NIH3T3 Cells in Response to Contact Inhibition, Related to Figure 5

NIH3T3 were plated for 24 hours, and then media was exchanged with fresh media every subsequent day. Samples were harvested at the indicated time points and fixed in ice-cold ethanol. The DNA content was then determined with FACS following propidium iodide staining.

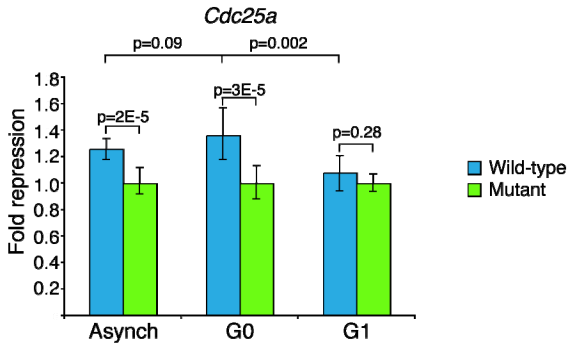

Figure S6. Repression of *Cdc25a* by the miR-16 Family Is Alleviated upon Cell-cycle Re-entry, Related to Figure 6

After transfection of a reporter expressing unstable *Renilla* luciferase followed by the 3' UTR of *Cdc25a*, NIH3T3 cells were grown in the presence of serum to measure repression during asynchronous growth (asynch), arrested in the absence of serum (G0) or arrested by serum starvation and then released into the cell cycle by the addition of serum (G1). Fold repression was calculated relative to that of mutant 3' UTR (that where the miR-16 sites had been mutated). Plotted are the normalized values, with error bars representing the third largest and third smallest values (n=12).

Table S3. Knockdown of Candidate Genes and the Effect on miR-503

Gene Class	Gene	shRNA	Fold change in targeted gene	Fold change in miR-503
N/A	Scrambled	1	N/A	0.28
SDN exonucleases	Aen	1	0.32	0.32
		2	0.27	0.25
	Eri-1	1	0.01	0.22
		2	0.02	0.21
	Isg20	1	0.39	0.40
		2	0.45	0.15
	Rexo1	1	0.29	0.31
		2	0.18	0.21
5'→3' exonuclease	Xrn1	1	0.33	0.17
		2	0.05	0.14
Exosome subunits	Rrp41	1	0.23	0.10
		2	0.22	0.25
	Ski2	1	0.48	0.18
		2	0.59	0.19
		3	0.29	0.18
Cytoplasmic terminal transferases	mGld-2	1	0.22	0.34
	Zcchc6	1	0.58	0.11
		2	0.26	0.40
	Zcchc11	1	0.72	0.14
		2	0.59	0.19
Endosome maturation	Hps4	1	0.09	0.15
		2	0.04	0.16

Knock-down of candidate genes does not stabilize miR-503. Based on proteins described in previous studies, we focused on several classes of enzymes: those related to the SDN exonucleases (Ramachandren and Chen, 2008); known cytoplasmic mRNA exonucleases (both 5'→3' and 3'→5'); known cytoplasmic terminal transferases; and Hps4, which is involved in endosome maturation and has been implicated in RISC recycling (Lee et al., 2009). We further refined our list, based on those expressed in our system, giving us four SDN candidates (*Aen*, *Eri-1*, *Isg20* and *Rexo1*), *Xrn1* (which encodes the primary 5'→3' cytoplasmic exonuclease), two components of the cytoplasmic exosome (*Ski2* and *Rrp41*), three terminal transferases (*mGld-2*, *Zcchc7* and *Zcchc11*), and *Hps4*. Knock-down of the targeted gene was determined by qRT-PCR. Levels miR-503 upon cell cycle re-entry were determined by northern blotting and normalized to U6 snRNA, comparing levels in G0 to those 12 hours after the re-addition of serum. Values represent the mean fold-change from independently derived lines (when applicable).

Table S5. Small RNA Probes Used in This Study

Name	Sequence
anti-hsa-mir-122	CAAACACCATTGTCACACTCCA
anti-mmu-mir-125b	TCACAAGTTAGGGTCTCAGGGA
anti-mmu-mir-503	CTGCAGTACTGTTCCCGCTGCTA
anti-mmu-mir-322	TCCAAAACATGAATTGCTGCTG
anti-mmu-mir-15a	CACAAACCATTATGTGCTGCTA
anti-mmu-let-7d	AACTATGCAACCTACTACCTCT
anti-mmu-mir-15b	TGTAAACCATGATGTGCTGCTA
anti-mmu-mir-16	CGCCAATTTTACGTGCTGCTA
anti-mmu-mir-497	TACAAACCACAGTGTGCTGCTG
anti-503 short	ACTGTTCCCGCTGCTA
anti-503.1 short	ACTGTTCCTGTCGCTA
anti-503.2 short	ACTGTTCCCTGAGCTA
anti-503m05	AAAGTTCCCGCTGCTA
anti-503m06	ACTCATCCCGCTGCTA
anti-503m07	ACTGTAACCGCTGCTA
anti-503m08	ACTGTTCCTGCTGCTA
anti-mmu-mir-34a	ACAACCAGCTAAGACACTGCCA
anti-mmu-mir-34b	ACAATCAGCTAATTACACTGCCT
anti-mmu-mir-34c	GCAATCAGCTAACTACACTGCCT
anti-bantam	AGTCAAACCAAATCGAAAACCGG

Table S6. Plasmids Used in This Study

Name	Source
pRetroX-Tight-Pur	Clontech
pRetroX-Tight-Pur-miR-503~122	This study
pRetroX-Tight-Pur-miR-503m1~122	This study
pRetroX-Tight-Pur-miR-503m01~122	This study
pRetroX-Tight-Pur-miR-503m02~122	This study
pRetroX-Tight-Pur-miR-503m03~122	This study
pRetroX-Tight-Pur-miR-503m04~122	This study
pRetroX-Tight-Pur-miR-503m05~122	This study
pRetroX-Tight-Pur-miR-503m06~122	This study
pRetroX-Tight-Pur-miR-503m07~122	This study
pRetroX-Tight-Pur-miR-503m08~122	This study
pIS0	(Grimson et al., 2007)
pIS1	(Grimson et al., 2007)
pIS1-miR-503~122	This study
pIS1-miR-503m1~122	This study
pIS1xDR	This study
pIS1xDR-Cpeb2	This study
pIS1xDR-mCpeb2	This study
pIS1xDR-Cdc25a	This study
pIS1xDR-mCdc25a	This study
psiCheck2	Promega
psiCheck2DR-Ccne1	This study
psiCheck2DR-mCcne1	This study
pSilencer2.1 U6 Scrambled	Ambion
pSilencer Aen shRNA.1	This study
pSilencer Aen shRNA.2	This study
pSilencer Eri-1 shRNA.1	This study
pSilencer Eri-1 shRNA.2	This study
pSilencer Isg20 shRNA.1	This study
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pSilencer Gld-2 shRNA	This study
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