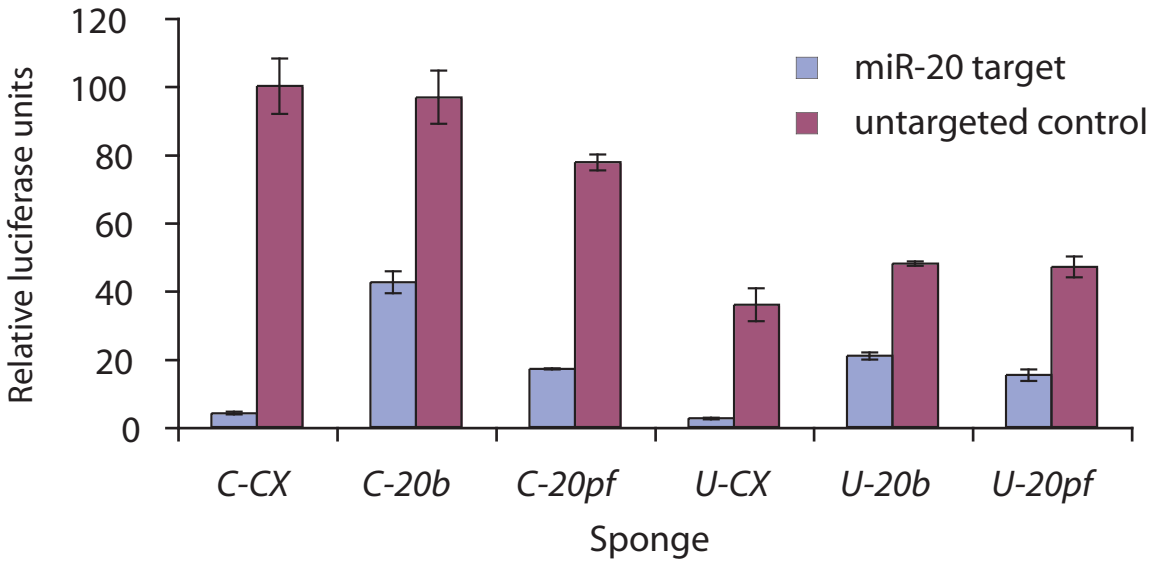
MicroRNA sponges: competitive inhibitors of small RNAs in mammalian cells

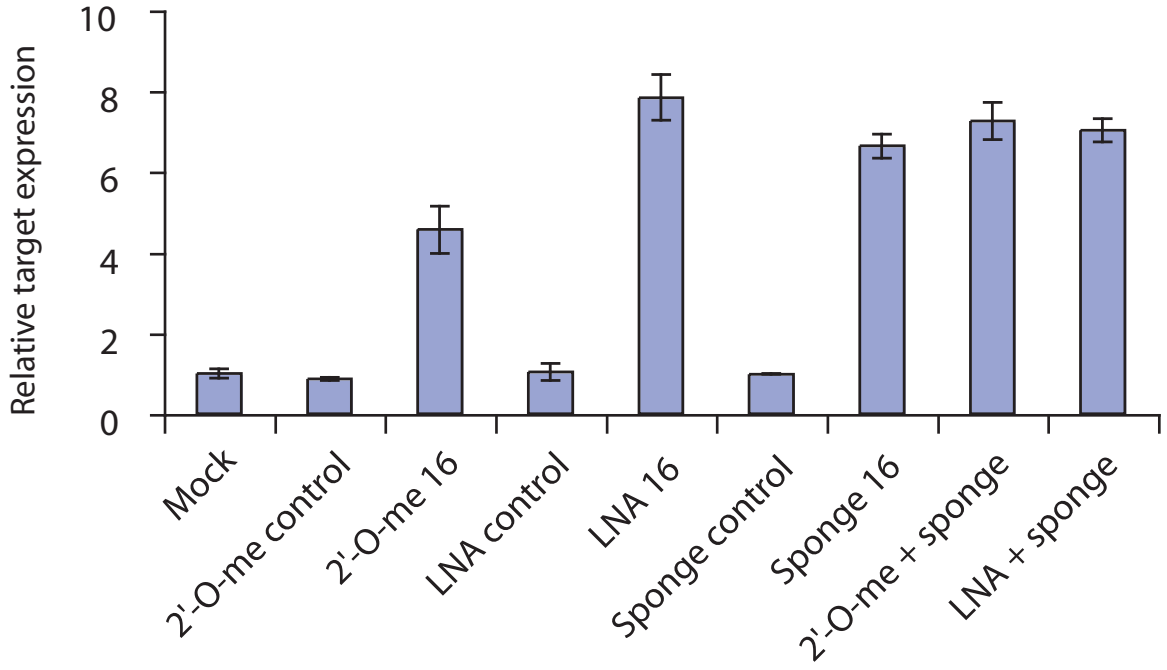
Margaret S Ebert, Joel R Neilson & Phillip A Sharp

Supplementary figures and text:

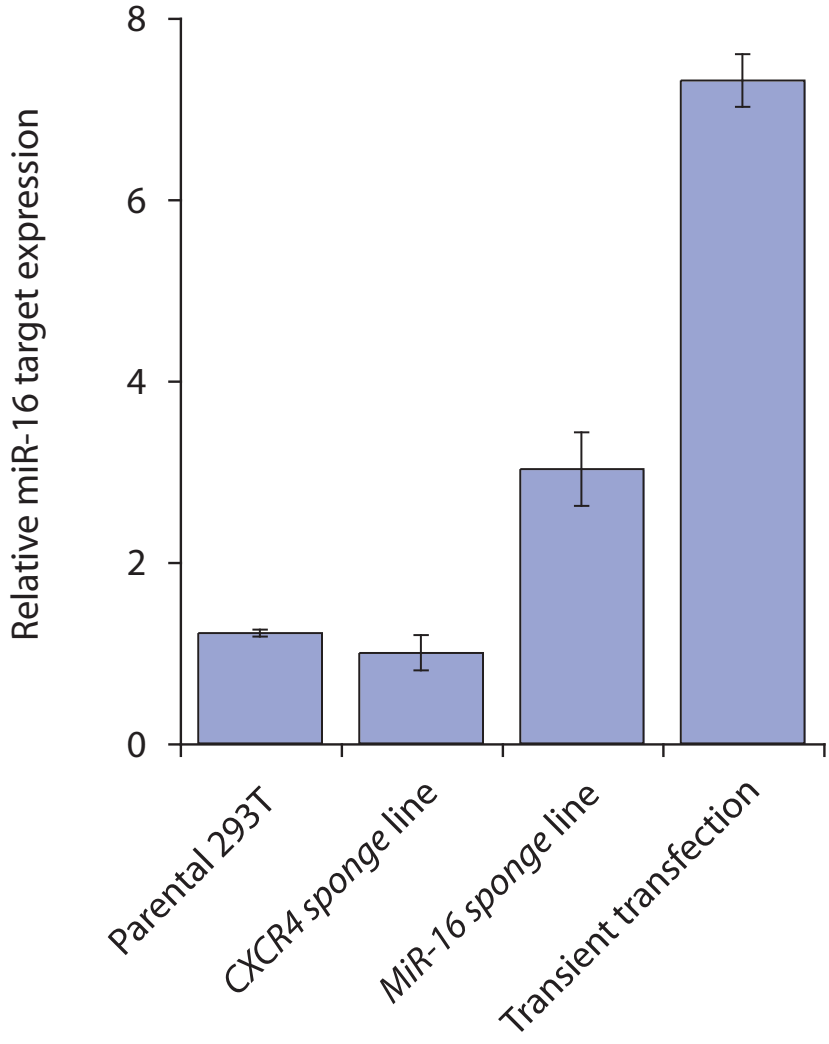
Supplementary Figure 1



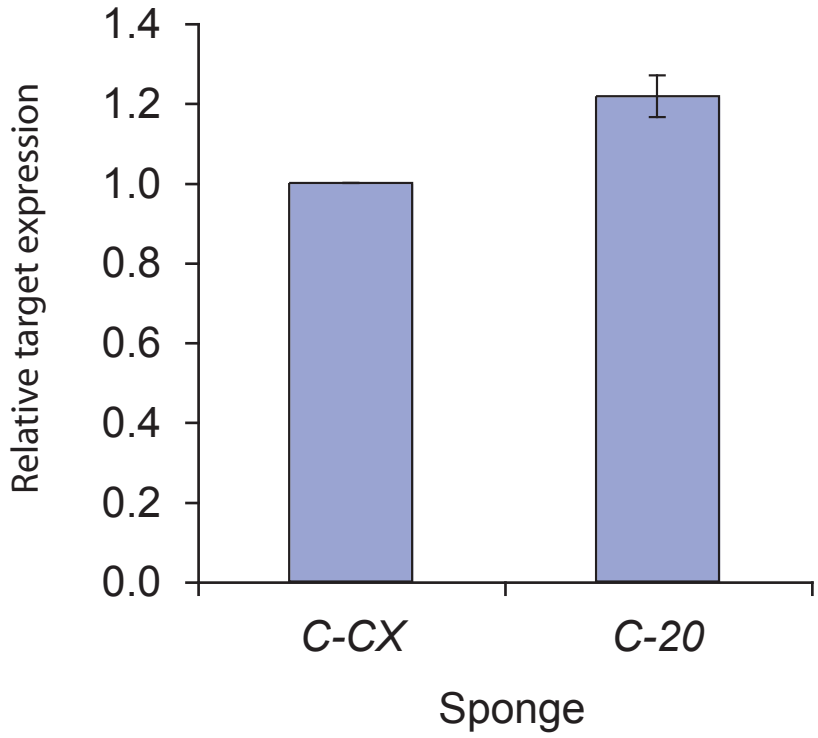
Supplementary Figure 1: Effect of sponges on a miR-20 target and an untargeted control. We cotransfected 293T cells with a *Renilla* luciferase vector containing seven miR-20 sites or an otherwise identical vector containing seven CXCR4 control sites and the sponge plasmids indicated. Bars represent *Renilla* luciferase unts relative to Firefly luciferase units. Error bars represent standard deviation among triplicate samples. Results are representative of a mimimum of three independent experiments.



Supplementary Figure 2: Comparison of sponges to antisense oligos. We transfected 293T cells with 20 nM antisense oligo (2'-O-methyl or LNA) or with the CMV bulged sponge against miR-16. Negative controls: mock (no oligos or sponges), 2'-O-methyl against miR-30, LNA against miR-122, CXCR4 sponge. The target reporter contains nine miR-16 sites and is derepressed slightly more strongly by the LNA and sponge than by the 2'-O-methyl oligo. Error bars denote standard deviation in triplicate samples. Results are representative of a minimum of three independent experiments.



Supplementary Figure 3: Inhibition of microRNA by a stably expressed sponge. We stably transfected 293T cells with the *miR-16 sponge* or *CXCR4 sponge* (control) plasmid, sorted for high GFP expression, and tested by dual luciferase assay with a *Renilla* luciferase reporter for miR-16 or an untargeted *Renilla* luciferase control. Bars represent expression of the miR-16 target relative to the untargeted control in each cell line. The miR-16 target is rescued about 40 percent as well by the stably expressed sponge as by the transiently transfected sponge. Error bars represent standard deviation in triplicate samples.



Supplementary Figure 4: Validation of new microRNA targets. We fused the E2F5 UTR (which contains a predicted miR-20 binding site) to a *Renilla* luciferase reporter. We transfected 293T cells with the UTR reporter, Firefly luciferase, and GFP sponges. Bars represent *Renilla* luciferase units relative to Firefly luciferase units. Error bars represent standard deviation among triplicate samples. Results are representative of a minimum of three independent experiments. Interestingly, *miR-20* is now shown to directly regulate at least two members of the E2F family of transcription factors.

Supplementary Table 1. Sequences of sponges and reporters, sites written 5' to 3'.

CXCR4 bulged Renilla luciferase reporter, 7 sites or 1 site; CMV sponge, 7 sites; U6 sponge, 4 sites.
AAGUUUUCAGAAAGCUAACA.

miR-16 bulged Renilla luciferase reporter, CMV sponge, and U6 sponge, 9 sites
AAUAUUCUAUGCUGCUA.

miR-16 perfect CMV sponge and U6 sponge, 2 sites
CGCCAAUAUUUACGUGCUGCUA.

miR-18 bulged CMV sponge, 8 sites
UAUCUGCACUUAGGCACCUUA.

miR-20 bulged Renilla luciferase reporter and CMV sponge, 7 sites; U6 sponge, 4 sites
UACCUGCACUCGCGCACUUUA.

miR-20 perfect CMV sponge and U6 sponge, 2 sites
CUACCUGCACUAUAAGCACUUUA.

miR-21 bulged Renilla luciferase reporter, 6 sites; CMV sponge, 7 sites
UCAACAUCAGGACAUAAGCUA.

miR-30c perfect Renilla luciferase reporter, 2 sites
GCUGAGAGUGUAGGAUGUUUACA.

miR-30e bulged CMV sponge, 6 sites
UCCAGUCCCUAUGUUUACA.

Method	2'-O-methyl antisense	LNA antisense	MicroRNA sponge
Composition	Modified RNA oligo	Modified RNA and DNA oligo	mRNA containing a 3' UTR
Number of binding sites	One	One	Multiple
Means of addition to cells	Transient transfection	Transient transfection	Transient transfection or stable expression from chromosomal insertions
Reporter function	None	None	GFP or other genetically encoded reporter proteins
Specificity	Single microRNA	Single microRNA	MicroRNA seed family

Supplementary Table 2. Comparison of microRNA sponges to antisense oligos.

Supplementary Methods

Primers
E2F1 UTR fragment: forward primer AATATTCTAGACTCTAACTGCACTTTCGGCC and reverse primer AATAAGGGCCCGAAGCAAATCAAAGTGCAGATTG.

CD69 UTR fragment: forward primer AGCTAGCTCGAGACTGTGCCATAGCACCACAG and reverse primer ATGCATGCGGCCGCACAGCTTAAACTTTATAGTGGGTTTT.

E2F5 UTR: forward primer GACTCGAGATTCCATGGAAACTTGGGAC and reverse primer CCGCGGCCGCAATGTTTTATACAATTTTATTTT.

Western blot
We transfected 293T cells two days in a row with Lipofectamine2000 and sponge plasmids. Fluorescence microscopy confirmed that 95-100 percent of the cells were GFP-positive 48 hours after the first transfection. We lysed cells in RIPA buffer and resolved the lysates on a Tris-HCl 4-20% gel, transferred to a nitrocellulose membrane, and probed with anti-E2F1 (Santa Cruz sc-193), stripped, and re-probed for beta-actin (Sigma A5441). We imaged the blots with Western Lightning chemiluminescence reagent (PerkinElmer) and film. We performed the experiment three times and have shown a representative result.

Northern blot
We transfected 293T cells with Lipofectamine2000 and sponge plasmids. We harvested total RNA by Trizol extraction 48 hours post-transfection. We ran 20 μg RNA on a 12% polyacrylamide gel, along with end-labeled 10-bp DNA ladder (Invitrogen), transferred to Hybond N+ membrane, and probed against miR-16, then stripped and reprobed for glutamine tRNA, then for the 3' end of the d2eGFP coding region, then for the 3' end of the U6 sponge RNA. We imaged the blots with a Storm scanner (Molecular Dynamics) and quantified the bands with ImageQuant software (Amersham Biosciences). We performed the experiment at least three times each for miR-16 and for miR-20 and have shown a representative result.

Construction of stable cell lines
We cotransfected 293T cells with linearized GFP sponge plasmids for miR-16 or the CXCR4 control at a 20:1 ratio to linear puromycin marker (Clontech). We cultured the cells in 2.5 μg/ml puromycin for about six weeks and sorted on a MoFlo FACS instrument (Cytomation) for the highest 10 percent of GFP expression. We cultured these fractions for another week before performing transfection assays.

Quantification of sponge RNAs
We transfected 293T cells with CMV sponges (CXCR4, 16, 20) and harvested total RNA 24-48 hours later. We treated RNA with DNaseI (Ambion) and reverse transcribed it with random primers using MMLV Reverse Transcriptase (Ambion). We used the cDNA samples or no-RTase controls as templates for real-time PCR with SYBRGreen detection

(Applied Biosystems) and primers in the coding region of GFP. We used a dilution series of GFP plasmid standards to estimate the number of GFP cDNAs present in each reaction. We ran each PCR experiment in triplicate and averaged the results of three experiments.