Supplemental Information

Kinetic analysis reveals the fate of a microRNA following target regulation in mammalian cells

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Figure S1. Generation of conditional miR-223 expressing cells (c223). Related to Figure 1.

Figure S2. Deep-sequencing analysis of miR-223 target cleavage products in c223 cells, and endogenously expressed miR-223 isoforms in THP-1 monocytes. Related to Figure 2.

Figure S3. Evaluating the kinetics of target de-repression during miR-223 decay. Related to Figure 3.

Figure S4. Relative isoform abundance of select miRNAs in c223 cells. Related to Figure 4.

Supplemental Materials and Methods.

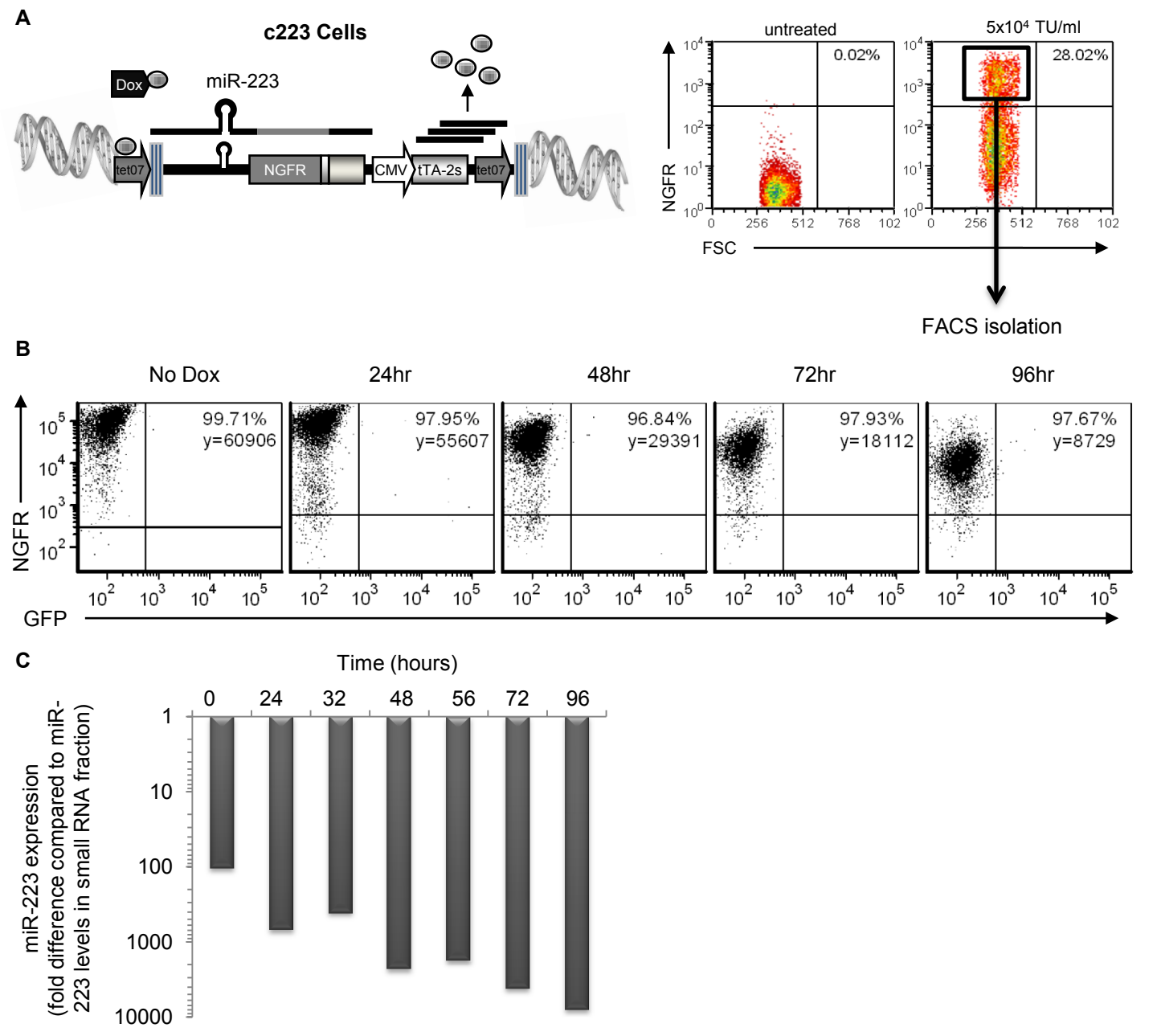
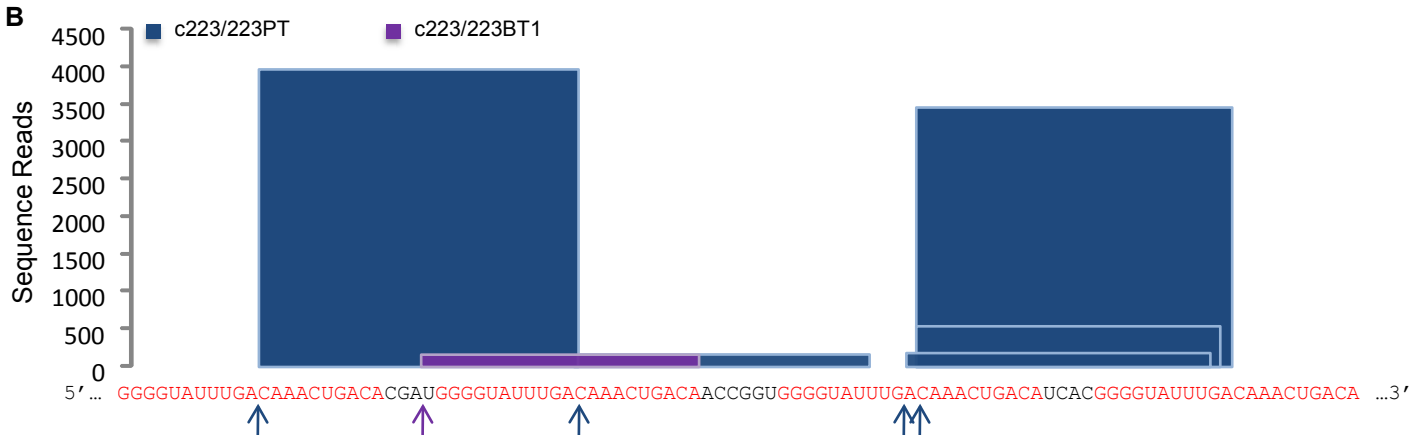


Figure S1. Generation of conditional miR-223 expressing cells (c223). Related to Figure 1. (**A**) Schematic of the conditional miR-223 cells (c223). The lentiviral vector (LV) constitutively expresses the tet-transactivator (tTA-2s), which binds to a tet07 element and drives expression of a transcript encoding human pri-miR-223 and the NGFR cDNA. Addition of Doxycycline (Dox) blocks the tTA-2s from binding the tet07 promoter, and terminates transcription of pri-miR-223 and NGFR. 293T cells were transduced with a low concentration of the vector, and FACS sorting was used to obtain a population of cells that contain 1 – 2 vector copies/cell. (**B**) The kinetics of NGFR protein turnover was monitored by treating c223 cells with Dox, and measuring NGFR expression by FACS over 96 hours. Dotplots are representative of n=>5. (**C**) Quantification of miR-223 levels in >200nt RNA fraction of c223/223T cells. Large (>200nt) and small (<200nt) RNA was column fractionated from c223/223T cells at the indicated time point after treatment with Dox or at the same time point from cells that were not treated with Dox. miR-223, miR-16, and Let-7a were quantified in all the samples, and the miR-223 data was normalized to the other miRNAs. The fold difference between the miR-223 levels in the small RNA and large fractions was calculated by ΔCT method. Specifically, for each time point the miR-223 threshold cycle (CT) from the large RNA fraction was subtracted from the value derived from the small RNA fraction, and raised to the power of 2. Of note, the miR-223 levels were more than 100-fold lower in the large RNA fraction than the small RNA fraction, and thus it does not appear that a significant amount of miR-223 is being trapped in the large RNA fraction by the target.

A

Search String: % AACUGACA %	seqlen	c223			c223/223PT			c223/223BT1		
		no Dox	24hr	48hr	no Dox	24hr	48hr	no Dox	24hr	48hr
CAAACUGACACGAUGGGGUAUUUGA	25	0	0	0	3980	2425	1052	1	0	0
CAAACUGACAUCACGGGGUAUUUGA	25	0	0	0	3436	1521	603	0	0	0
CAAACUGACAUCACGGGGUAUUUG	24	0	0	0	533	210	104	0	0	0
ACAAACUGACAUCACGGGGUAUUU	24	0	0	0	219	264	189	0	0	0
CAAACUGACACGAUGGGGUAUUUG	24	0	0	0	351	227	74	1	0	0
UGGGGUAUUUGUGAAACUGACAA	23	0	0	0	0	0	0	204	249	200
CAAACUGACAACCGGUGGGGUAU	23	0	0	0	210	259	164	0	0	0
CAAACUGACAUCACGGGGUAUUU	23	0	0	0	226	196	167	0	0	0
TOTAL		0	0	0	8,955	5,102	2,353	206	249	200

B



C

hsa-miR-223	seqlen	Chrom	start	THP-1 Reads	THP-1 %
UGUCAGUUUGUCAAAUACCCCA	22	chrX	65,238,778	108,627	30.42%
UGUCAGUUUGUCAAAUACCCCAU	23	-	-	5,745	1.61%
UGUCAGUUUGUCAAAUACCCCAG	23	-	-	875	0.25%
UGUCAGUUUGUCAAAUACCCCAUA	24	-	-	697	0.20%
UGUCAGUUUGUCAAAUACCCCAGA	24	-	-	198	0.06%
UGUCAGUUUGUCAAAUACCCCAA	23	chrX	65,238,778	230,086	64.43%
UGUCAGUUUGUCAAAUACCCCAAU	24	-	-	7,330	2.05%
UGUCAGUUUGUCAAAUACCCCAAA	24	-	-	2,435	0.68%
UGUCAGUUUGUCAAAUACCCCAAAA	25	-	-	577	0.16%
UGUCAGUUUGUCAAAUACCCCAAG	24	chrX	65,238,778	516	0.14%
TOTAL				357,086	100%

D

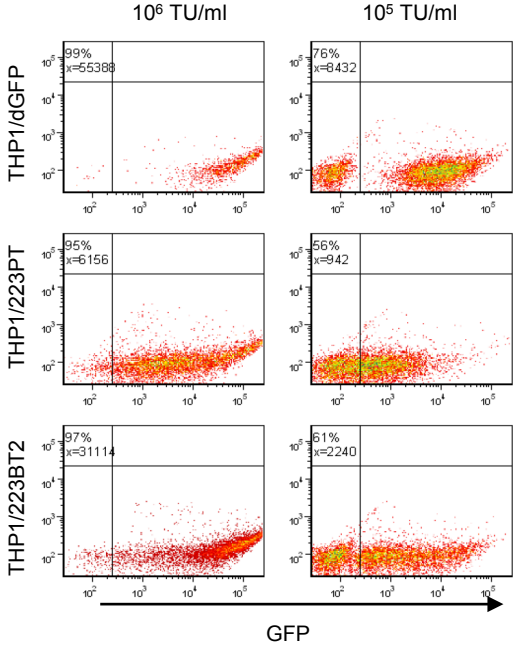


Figure S2. Deep-sequencing analysis of miR-223 target cleavage products in c223 cells, and endogenously expressed miR-223 isoforms in THP-1 monocytes. Related to Figure 2. (**A**) Small RNA deep-sequencing data from c223 cells or c223 cells stably expressing 223PT or 223BT1 was mined for sequences that contained the 8 nucleotide sequence AACUGACA, which pairs with the seed sequence of miR-223. Sequence and abundance of reads containing the sequence AACUGACA from the indicated sample are shown. (**B**) Graphical representation of the sequence distribution of the reads corresponding to the indicated portion of the vector-encoded transcript. Arrows indicate the 5' end of the read. (**C**) Deep-sequencing analysis of endogenously expressed miR-223 in human THP-1 cells. (**D**) Measurement of endogenous miR-223 activity in human monocytes. THP-1 cells were transduced with similar concentrations of the indicated vectors, or a control vector that expressed dGFP from the CAG promoter, but did not encode miR-223 target sites (dGFP). After 1 week, the cells were analyzed by FACS. The % GFP positive cells, and the mean florescence intensity (x) of GFP is indicated.

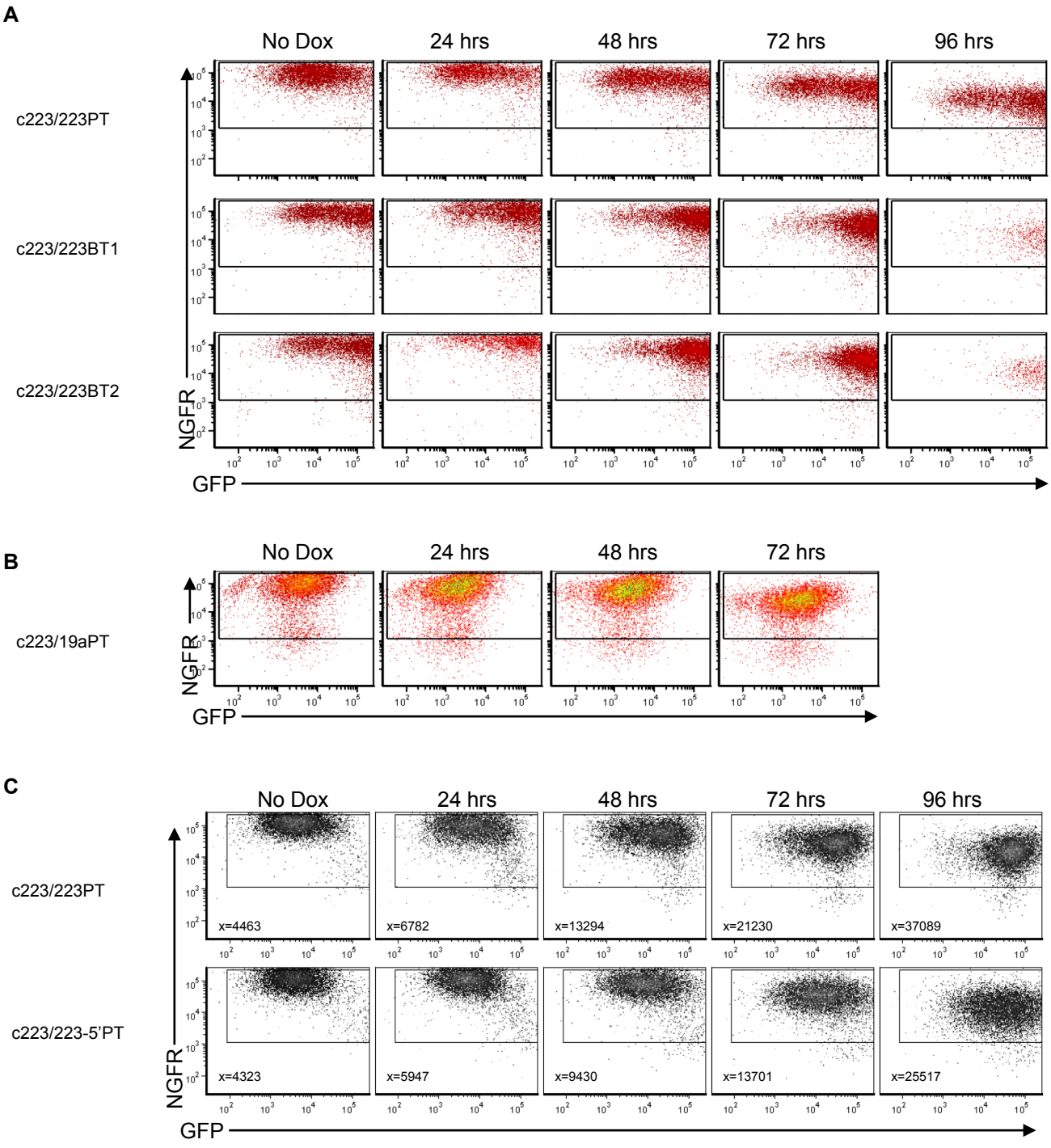
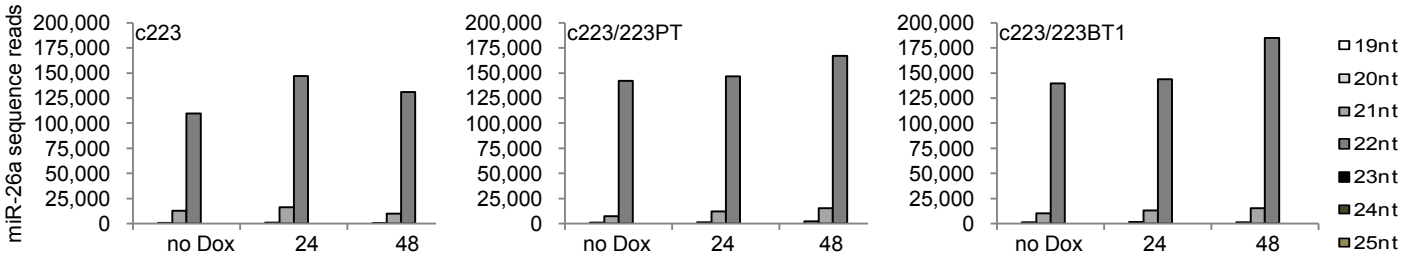


Figure S3. Evaluating the kinetics of target de-repression during miR-223 decay. **Related to Figure 3.** (**A**) FACS analysis of miR-223 target expression in c223 cells. c223 cells transduced with 10^6 TU/ml of the indicated vector were treated with Dox, and GFP expression was monitored by FACS over 96 hours. (**B**) FACS analysis of miR-19a-regulated reporter expression in c223 cells. c223 cells were transduced with 10^6 TU/ml of a lentiviral vector utilizing the CAG promoter to express dGFP linked to four copies of a sequence that is perfectly complementary to miR-19a. GFP expression was monitored by FACS for 96 hours after Dox was added to the cells. (**C**) FACS analysis of miR-223 target expression in c223/223-5'PT cells after Dox addition. All dotplots are representative of 3 independent experiments.

A



B

					c223			C223/223PT			c223 /223BT1		
hsa-miR-10a	**seqlen**	**Chrom**	**start**	**stop**	**no Dox**	**24hr**	**48hr**	**no Dox**	**24hr**	**48hr**	**no Dox**	**24hr**	**48hr**
UACCCUGUAGAUCCGAAUUUG	21	chr17	46,657,287	46,657,267	0.21%	0.33%	0.13%	0.22%	0.52%	0.90%	0.38%	0.46%	0.63%
UACCCUGUAGAUCCGAAUUUGU	22	chr17	46,657,287	46,657,266	59.23%	63.28%	57.32%	70.19%	69.80%	68.79%	72.87%	70.67%	73.53%
UACCCUGUAGAUCCGAAUUUGUA	23	-	-	-	0.15%	0.16%	0.21%	0.14%	0.15%	0.16%	0.16%	0.12%	0.18%
UACCCUGUAGAUCCGAAUUUGUU	23	-	-	-	0.13%	0.15%	0.21%	0.10%	0.18%	0.18%	0.12%	0.18%	0.20%
UACCCUGUAGAUCCGAAUUUGUG	23	chr17	46,657,287	46,657,265	32.66%	26.72%	33.14%	21.12%	20.16%	20.73%	19.56%	19.43%	15.72%
UACCCUGUAGAUCCGAAUUUGUGA	24	-	-	-	1.35%	1.55%	2.64%	2.19%	1.92%	1.83%	1.94%	1.66%	1.78%
UACCCUGUAGAUCCGAAUUUGUGAA	25	-	-	-	0.09%	0.08%	0.15%	0.08%	0.08%	0.08%	0.07%	0.08%	0.10%
UACCCUGUAGAUCCGAAUUUGUGU	24	chr17	46,657,287	46,657,264	6.03%	7.40%	6.13%	5.94%	7.14%	7.28%	4.84%	7.31%	7.78%
UACCCUGUAGAUCCGAAUUUGUGUA	25	chr17	46,657,287	46,657,263	0.02%	0.02%	0.04%	0.02%	0.01%	0.02%	0.01%	0.02%	0.02%
TOTAL					211,601	198,663	171,005	225,068	206,016	263,764	224,468	193,386	211,036
hsa-miR-21	**seqlen**	**Chrom**	**start**	**stop**	**no Dox**	**24hr**	**48hr**	**no Dox**	**24hr**	**48hr**	**no Dox**	**24hr**	**48hr**
UAGCUUAUCAGACUGAUGUUG	21	chr17	57,918,633	57,918,653	2.70%	2.30%	1.45%	0.66%	1.13%	1.56%	0.96%	1.19%	1.61%
UAGCUUAUCAGACUGAUGUUGA	22	chr17	57,918,633	57,918,654	25.22%	23.17%	25.11%	18.72%	18.46%	19.32%	17.83%	16.62%	23.88%
UAGCUUAUCAGACUGAUGUUGAC	23	chr17	57,918,633	57,918,655	65.84%	68.11%	65.84%	75.94%	75.53%	73.87%	76.45%	76.67%	69.05%
UAGCUUAUCAGACUGAUGUUGACA	24	-	-	-	2.16%	2.20%	2.60%	2.12%	2.14%	2.43%	2.28%	2.86%	2.10%
UAGCUUAUCAGACUGAUGUUGACAA	25	-	-	-	0.01%	0.01%	0.02%	0.04%	0.05%	0.03%	0.06%	0.02%	0.04%
UAGCUUAUCAGACUGAUGUUGACC	24	-	-	-	0.07%	0.11%	0.17%	0.09%	0.15%	0.14%	0.08%	0.17%	0.16%
UAGCUUAUCAGACUGAUGUUGACG	24	-	-	-	0.15%	0.15%	0.17%	0.13%	0.20%	0.20%	0.20%	0.18%	0.26%
UAGCUUAUCAGACUGAUGUUGACU	24	chr17	57,918,633	57,918,656	3.57%	3.28%	4.57%	2.19%	2.22%	2.35%	1.98%	2.18%	2.70%
TOTAL					116,122	128,343	141,966	141,225	150,780	160,873	120,214	156,506	165,768
hsa-miR-22	**seqlen**	**Chrom**	**start**	**stop**	**no Dox**	**24hr**	**48hr**	**no Dox**	**24hr**	**48hr**	**no Dox**	**24hr**	**48hr**
AAGCUGCCAGUUGAAGAACU	20	chr17	1,617,228	1,617,209	0.04%	0.04%	0.02%	0.05%	0.05%	0.04%	0.03%	0.03%	0.07%
AAGCUGCCAGUUGAAGAACUG	21	chr17	1,617,228	1,617,208	0.05%	0.06%	0.06%	0.09%	0.12%	0.13%	0.14%	0.08%	0.12%
AAGCUGCCAGUUGAAGAACUGU	22	chr17	1,617,228	1,617,207	99.53%	99.35%	99.27%	98.64%	98.64%	98.72%	98.86%	99.17%	98.65%
AAGCUGCCAGUUGAAGAACUGUA	23	-	-	-	0.18%	0.26%	0.31%	0.47%	0.42%	0.37%	0.35%	0.25%	0.39%
AAGCUGCCAGUUGAAGAACUGUAA	24	-	-	-	0.03%	0.05%	0.05%	0.10%	0.10%	0.08%	0.08%	0.06%	0.08%
AAGCUGCCAGUUGAAGAACUGUAU	24	-	-	-	0.01%	0.01%	0.01%	0.04%	0.04%	0.05%	0.05%	0.02%	0.04%
AAGCUGCCAGUUGAAGAACUGUU	23	chr17	1,617,228	1,617,206	0.13%	0.21%	0.24%	0.56%	0.57%	0.56%	0.44%	0.35%	0.57%
AAGCUGCCAGUUGAAGAACUGUUU	24	-	-	-	0.01%	0.02%	0.02%	0.02%	0.05%	0.05%	0.03%	0.03%	0.06%
TOTAL					176,674	154,667	179,892	180,892	165,745	222,863	183,673	215,333	185,898
hsa-mir-103	**seqlen**	**Chrom**	**start**	**stop**	**no Dox**	**24hr**	**48hr**	**no Dox**	**24hr**	**48hr**	**no Dox**	**24hr**	**48hr**
AGCAGCAUUGUACAGGGCUAUG	22	chr20	3,898,187	3,898,208	5.77%	5.36%	5.78%	4.54%	5.14%	5.69%	4.28%	4.91%	5.32%
AGCAGCAUUGUACAGGGCUAUGA	23	chr20	3,898,187	3,898,209	89.14%	88.84%	87.40%	89.12%	88.13%	87.75%	89.84%	89.13%	87.26%
AGCAGCAUUGUACAGGGCUAUGAU	24	-	-	-	3.39%	4.05%	4.80%	3.33%	4.18%	4.22%	3.06%	3.68%	4.78%
AGCAGCAUUGUACAGGGCUAUGAUU	25	-	-	-	0.30%	0.32%	0.43%	0.12%	0.22%	0.18%	0.13%	0.16%	0.22%
AGCAGCAUUGUACAGGGCUAUGAG	24	-	-	-	0.21%	0.16%	0.23%	0.11%	0.10%	0.10%	0.11%	0.09%	0.10%
AGCAGCAUUGUACAGGGCUAUGAUA	25	-	-	-	0.07%	0.07%	0.09%	0.04%	0.05%	0.04%	0.04%	0.03%	0.05%
AGCAGCAUUGUACAGGGCUAUGAA	24	chr20	3,898,187	3,898,210	0.87%	0.93%	0.96%	2.52%	1.95%	1.84%	2.31%	1.77%	2.06%
AGCAGCAUUGUACAGGGCUAUGAAA	25	chr20	3,898,187	3,898,211	0.11%	0.09%	0.11%	0.12%	0.08%	0.07%	0.12%	0.09%	0.08%
TOTAL					1,284,235	1,503,587	1,484,627	1,257,872	1,311,394	1,246,033	1,407,381	1,613,328	1,094,326

C

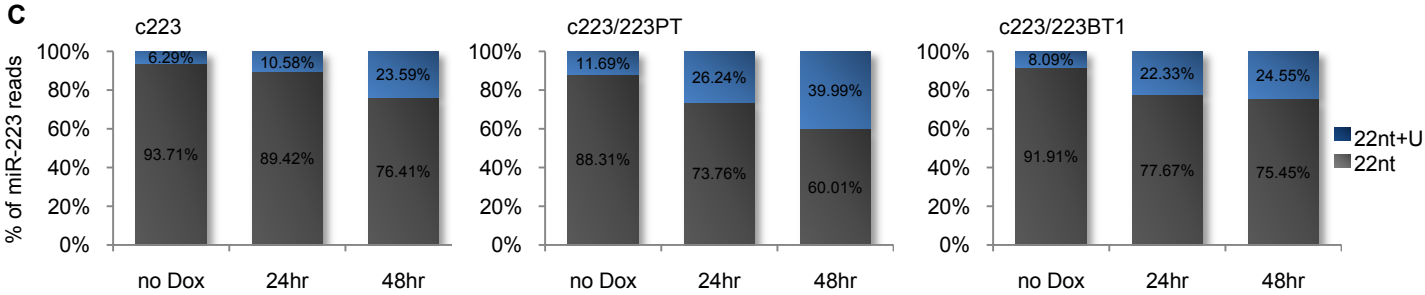


Figure S4. Relative isoform abundance of select miRNAs in c223 cells. Related to Figure 4. Deep-sequencing was performed on small RNA libraries generated from c223 cells and c223 cells ovexpressing a perfect target (c223/223PT) or bulge target (c223/223BT), in steady-state or 24 and 48 hours after treatment with Dox. (**A**) Comparison of miR-26a isoforms before and after Dox addition. Sequence reads mapping to the miR-26a with lengths varying from 19 – 25 nucleotides starting from the 5' end of the miRNA were quantitated and graphed. (**B**) Sequence and abundance of various miRNAs in c223 cells. In the far left column are the sequences that map to the mature form of the indicated miRNA. The relative contribution (in percentage) of each isoform was calculated by dividing the number of reads of the specific isoform by the total reads mapping to the microRNA. The sequences that do not have mapping coordinates are isoforms that closely match the underlined sequence but contain a non-templated oligonucleotide, which is highlighted in red. (**C**) Relative contribution of the 22nt isoform of miR-223 (5'-UGUCAGUUUGUCAAAUACCCCA-3') to the uridylated (+U) form during miR-223 decay in c223 cells The percent contribution of each isoform to the total is shown on the graph. Note that the (+A) form can not be graphed because it corresponds to the 23nt isoform of miR-223.

Materials and Methods

Cell Culture

Human Embryonic Kidney 293T cells were maintained in Iscove's modified Dulbecco's medium (IMDM, Fisher Scientific, Pittsburgh, PA) supplemented with 10% of heat-inactivated fetal bovine serum, 1% of penicillin-streptomycin and 1% glutamine. Human THP-1 monocytic cells were maintained in RPMI medium (Fisher Scientific) supplemented with 10% of heat-inactivated fetal bovine serum, 1% of penicillin-streptomycin and 1% glutamine. Doxycycline (Clontech, Mountview, CA) was used at a final concentration of 500ng/ml. Transduction of 293T and THP-1 cells were performed as previously described [1]. For experiments in which cell division was arrested: on Day -1 the media was aspirated from a 15 cm plate of c223 cells, the cells were washed with PBS, and replaced with fresh media containing mitomycin C (25µg/ml). After 25 minutes at 37°C, the media was aspirated, the cells were washed with PBS, and trypsinized for 2 minutes. Trypsin was inactivated with complete IMDM, and the cells were washed, centrifuged, and resuspended in fresh media. Finally, 1,000,000 cells/well were seeded in a 6 well plate. The following day (Day 0), Doxycycline was added to initiate the time course.

Vector Construction

Target sequences were designed based on miRNA sequences obtained from the miRNA Registry [2], and cloned as previously described [3]. Oligonucleotides used for constructing the bulge and perfect miR-223 target sequences were:

223PT.sense1: CTAGATAAGGGGTATTTGACAAACTGACACGATGGGGTATTTGACAAACTGACAACCGGT
223PT.sense2: GGGGTATTTGACAAACTGACATCACGGGGTATTTGACAAACTGACAC,
223PT.antisense1: TGTCAGTTTGTCAAATACCCCATCGTGTCAGTTTGTCAAATACCCCTTAT,
223PT.antisense2: CCGGGTGTCAGTTTGTCAAATACCCCGTGATGTCAGTTTGTCAAATACCCCACCGGT,
223BT1.sense1: CTAGATAAGGGGTATTTGTGAAACTGACACGATGGGGTATTTGTGAAACTGACAACCGGT,
223BT1.sense2: GGGGTATTTGTGAAACTGACATCACGGGGTATTTGTGAAACTGACAC,
223BT1.antisense1: TGTCAGTTTCACAAATACCCCATCGTGTCAGTTTCACAAATACCCCTTAT,
223BT1.antisense2: CCGGGTGTCAGTTTCACAAATACCCCGTGATGTCAGTTTCACAAATACCCCACCGGT,
223BT2.sense1: CTAGATAAGGGGTATTTTGAAACTGACACGATGGGGTATTTTGAAACTGACAACCGGT,
223BT2.sense2: GGGGTATTTTGAAACTGACATCACGGGGTATTTTGAAACTGACAC,
223BT2.antisense1: TGTCAGTTTCAAAATACCCCATCGTGTCAGTTTCAAAATACCCCTTAT,
223BT2.antisense2: CCGGGTGTCAGTTTCAAAATACCCCGTGATGTCAGTTTCAAAATACCCCACCGGT,
223-5'PT.sense1: CTAGCATGCATTTTTCTATTTGACAAACTGACAAACCAATTTTCTATTTGACAAACTGACAAACCAA,
223-5'PT.sense2: TTTTCTATTTGACAAACTGACAAACCAATTTTCTATTTGACAAACTGACACTGCAGA,
223-5'PT.antisense1: TGTCAGTTTGTCAAATAGAAAATTGGTTTGTCAGTTTGTCAAATAGAAAAATGCATG,
223-5'PT.antisense2: CCGGTCTGCAGTGTCAGTTTGTCAAATAGAAAATTGGTTTGTCAGTTTGTCAAATAGAAAATTGGTT
19aPT.sense1: CTAGCATGCATTCAGTTTTGCATAGATTTGCACAACTAACCATCAGTTTTGCATAGATTTGCACAACTAACCA
19aPT.sense2: TCAGTTTTGCATAGATTTGCACAACTAACCATCAGTTTTGCATAGATTTGCACACTGCAGA
19aPT.antisense1: TGTGCAAATCTATGCAAAACTGATGGTTAGTTGTGCAAATCTATGCAAAACTGAATGCATG
19aPT.antisense2: CCGGTCTGCAGTGTGCAAATCTATGCAAAACTGATGGTTAGTTGTGCAAATCTATGCAAAACTGATGGTTAGT

The sense1/antisense1 (s1/as1) and sense2/antisense2 (s2/as2) oligonucleotides were annealed together. The 5' end of the s1/as1 had NheI and XbaI compatible overhangs, and the 3' end of the s2/as2 had AgeI and XmaI compatible overhangs. The 3' end of s1/as1 and the 5' end of s2/as2 have compatible overhangs. The oligonucleotides were combined and ligated into the XbaI/XmaI or NheI/XmaI site downstream of the WPRE in the 3'UTR of the expression cassette of one of the following lentiviral vector constructs: pCCL.sin.cPPT.PGK.eGFP.WPRE.XbaI/XmaI, pCCL.sin.cPPT.CAG.dGFP.WPRE.NheI/Xma. The self-regulated, conditional miR-223 expressing lentiviral vector was previously described [4]. All constructs are based on a third generation self-inactivating lentiviral vector platform.

Vector Production

Lentiviral vectors were produced as previously described [5]. Briefly, 293T cells were seeded approximately 24 hours before transfection in a 15cm plate and incubated at 37°C with 5% CO_2. The following day, the cells were transfected with a third generation packaging system (pVSV, pMDLg/pRRE, pRSV-REV) and the appropriate transfer plasmid using calcium phosphate. The media was changed 14 hours later, and 30 hours later the cell supernatant containing the vector was passed through a 0.22um filter and ultracentrifuged (20,000 RPM for 2 hours) to concentrate the vector 300 – 500-fold. The vector particles were resuspended in sterile PBS, aliquoted and stored at -80°C. All vectors were titered on 293T cells, as previously described [6].

miRNA Quantification

miRNAs were extracted from cells using the RT^2 qPCR-Grade miRNA Isolation Kit (SABiosciences, Frederick, MD) according to the manufacturer's instructions. The concentration of each miRNA was determined by 260/280nm optical density reading on a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE). For analysis of miRNA expression, reverse transcription (RT) was carried out on 2ng of small RNA using the Reverse Transcription miRNA Taqman Kit (Applied Biosystem, Foster City, CA) specific for the miRNA (hsa-Let-7a, hsa-miR-16, and hsa-miR-223). For each cDNA sample, real-time quantitative PCR (qPCR) was performed in duplicate using miRNA-specific primers/probe on an ABI Prism 7900HT Real Time PCR System (Applied Biosystem). Using a previously described protocol[1,7], we generated a standard curve for miR-223 using a synthetic miR-223 RNA oligonucleotide corresponding to the 22 nucleotide form of the miRNA (5'-UGUCAGUUUGUCAAAUACCCCA-3'). We also compared detection of a synthetic miR-223 RNA oligonucleotide corresponding to the 23 nucleotide form of miR-223 (5'-UGUCAGUUUGUCAAAUACCCCAA-3'), and found that it was detected with similar efficiency, indicating that the miR-223 Taqman is able to quantitate both common isoforms of miR-223. For each time point, samples were collected from cells treated with Dox or cells left untreated. miR-223 values were normalized using miR-16 and Let-7a values in the corresponding sample (ΔCT), and calibrated against the untreated (no Dox) time point ($\Delta\Delta$CT). The fold change was calculated using the formula $2^{\Delta\Delta CT}$.

It is important to note that this protocol minimizes the possibility that target-bearing transcripts can interfere with miRNA detection because we extract small RNA (<200 nucleotides), and the RT reaction utilizes a primer specific for each miRNA, and thus, only the miRNA is transcribed into cDNA. To determine if the miRNA was being trapped by the target during extraction, we performed qPCR analysis on the large RNA fraction of the samples, and did not find evidence of miR-223 enrichment (**Figure S1C**). The miRNA half-life was determined by averaging the decay constant from the exponential decay curve of each biological replicate. Significance was determined by student's t-test using Graphpad Prism (La Jolla, CA).

mRNA Quantification

Total RNA was extracted from cells using Trizol reagent (Invitrogen, Carlsbad, CA), and quantitated on a NanoDrop ND-1000 Spectrophotometer. Reverse transcription was carried out on 1000ng total RNA using the high capacity RNA to cDNA kit (Applied Biosystems). Real-time PCR analysis of GFP, NGFR, and PGK expression was performed by Taqman assay using the following primers and probe: GFP: F - 5'–CAGCTCGCCGACCACTA–3', R - 5'–GGGCCGTCGCCGAT–3' and P - 5'–6FAM–CCAGCAGAACACCCCC–MGB–3', For the human growth factor receptor (NGFR) and the human phosphoglycerate kinase 1 (PGK1) Endogenous Control primers/probes set were used (Applied Biosystem). PGK was used to normalize the GFP and NGFR values by the ΔCT method.

To determine the absolute concentration of GFP and NGFR transcripts, we generated a standard curve using in vitro transcribed GFP or NGFR mRNA. To do this, we linearized a pBlue plasmid containing the transgene.upstream of a T3 site, and performed in vitro transcription using the MEGAscript® T3 Kit (Ambion, Austin, TX). The resulting RNA was purified by RNeasy (Qiagen, Valencia, CA), visualized on a gel to confirm the presence of the RNA, and quantitated by NanoDrop. Serial dilutions of the RNA were made into 1000ng of 293T total RNA starting from 1pg RNA/ng total RNA to 0.00782pg/ng total RNA.

Samples were analyzed on an ABI Prism 7900HT Real Time PCR System. Two technical replicates, and at least three biological replicates were carried out for each experiment. A no RT control was performed on selected samples to ensure that there was no DNA contamination.

Small RNA Sequencing (miR-seq)

For small RNA deep-sequencing (miR-seq), small RNA libraries were prepared using a protocol from the Hannon Laboratory[8], which is based on previous protocols [9,10]. Briefly, total RNA was extracted from untreated or Doxycycline-treated c223 cells using Trizol reagent (Invitrogen). Ten micrograms of total RNA was run on a 15% polyacrylamide/urea gel alongside a ^{32}P-labeled Decade marker (Ambion), and the 15 – 30 nucleotide fraction of the sample RNA was excised from the gel, and purified. The resulting small RNA was ligated to a 5'-adenylated 3' adapter oligonucleotide (5'-AppCTGTAGGCACCATCAATdideoxyC-3', Integrated DNA Technologies, Coralville, IA) using the Rnl2 Air™ Ligase (BIOO Scientific, Austin, Texas) in the absence of ATP. The ligation product was separated from the unligated adapter by gel purification, and ligated to the 5' adapter RNA oligonucleotide using T4 RNA ligase 1 (NEB) in the presence of ATP. After gel isolation, the final

ligation product was reverse transcribed, and PCR amplified for 22 cycles, and the amplified library was purified by gel electrophoresis. The small RNA library quality was assessed on the Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA), and the quantity was determined using a PicroGreen assay. Each small RNA library was sequenced on an Illumina Genome Analyzer II Platform (Illumina, San Diego, CA). The sequences were mapped using the computational programs we previously generated [8,11,12], and analyzed on the small RNA dashboard (http://katahdin.mssm.edu/html/scripts/resources.pl).

Accession Numbers

The processed sequencing data has been deposited with NCBI GEO under the accession number .

Flow Cytometry

Sorting of 293T cells transduced with miR-pri-223-NGFR was performed on a FACS Vantage sorter (Becton-Dickinson). Adherent cells were detached from the plate with 0.05% trypsin –EDTA, washed and resuspend in sterile PBS. The cells were identified by NGFR marking in a sterile condition. Between 10^6 -10^7 purified cells were obtained in this way. Sorted cells were expanded for more than 2 weeks before initiating studies.

For analysis of NGFR and GFP expression in steady-state and Dox-treated cells, adherent cells were first detached with 0.05% trypsin-EDTA, washed and resuspended in PBS containing 2% FBS. For immune staining 10^5 cells were blocked in PBS/2%FBS for 15 minutes at 4°C. After blocking, the cells were incubated with R-phycoerythrin (RPE)-conjugated anti-LNGFR antibody (BD Pharmingen, San Diego, CA) for 25 minutes at 4°C, washed, and resuspended in PBS. The cells were analyzed on either the BD FACScan or BD LSRII (Becton-Dickinson, Franklin Lakes, NJ). Analysis was performed on FCSExpress Software (De Novo Software, Los Angeles, CA).

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