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Supplemental Data

Widespread Shortening of 3′UTRs by Alternative Cleavage and Polyadenylation Activates Oncogenes in Cancer Cells

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Figure S1

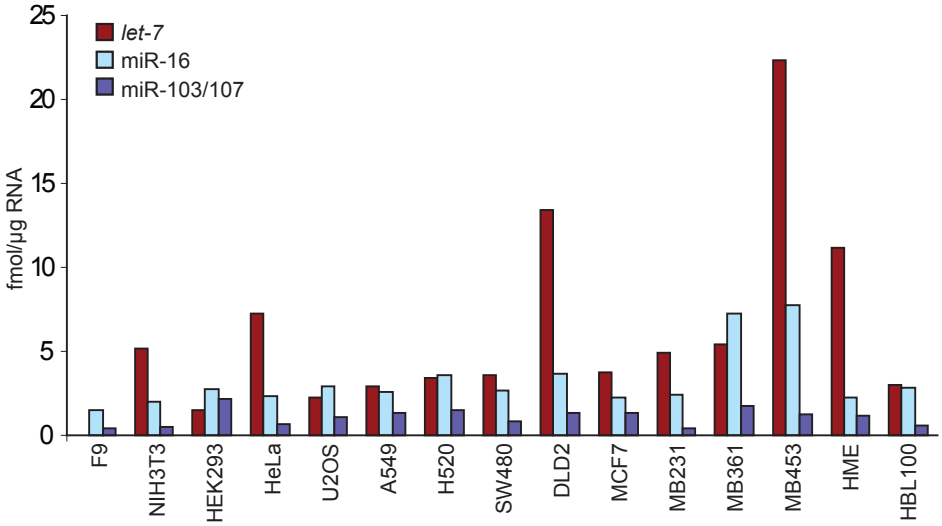

Figure S1. miRNA expression levels of *let-7*a–d, miR-16 and miR-103/107 in cell lines were determined by quantitative Northern blots.

Figure S2

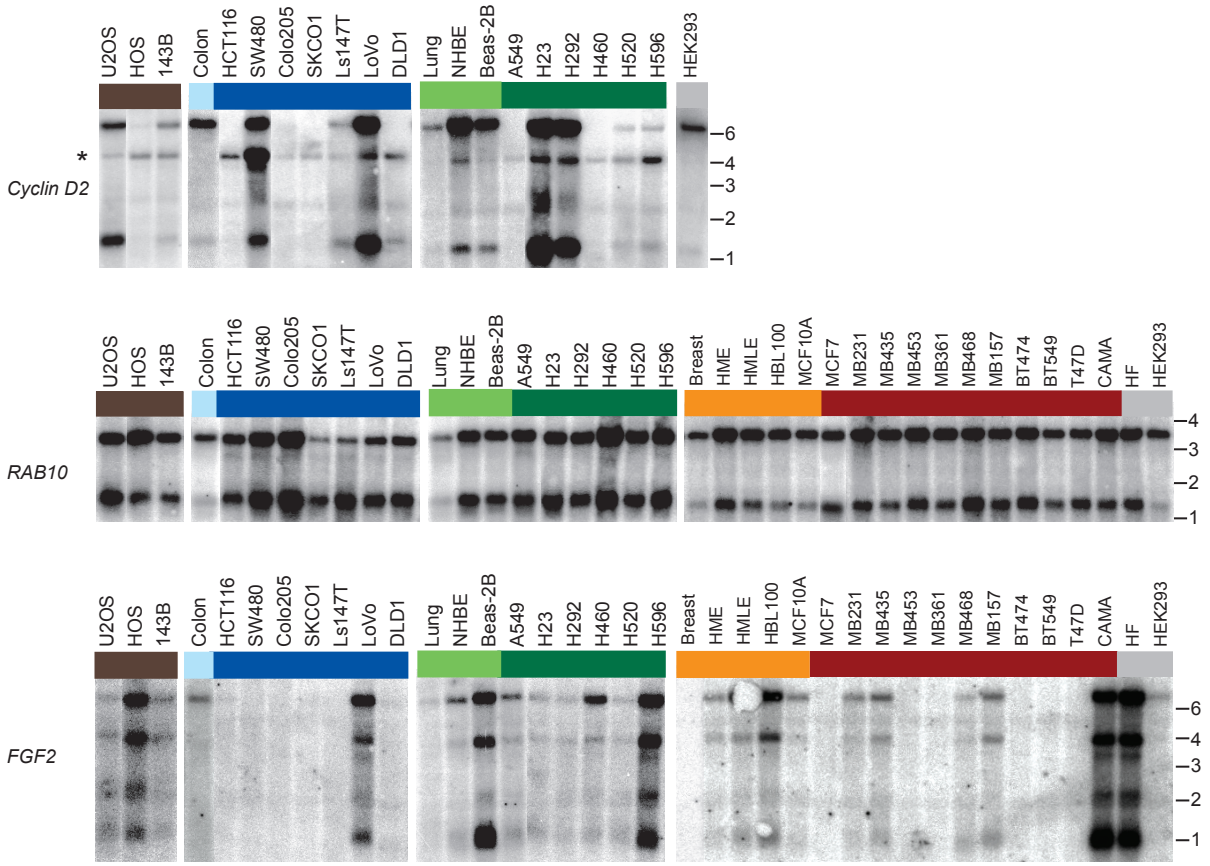

Figure S2. APA leads to shorter 3'UTRs in transformed cells.
(A) Northern blots of human cell lines and tissues. Sarcoma cell lines (brown), normal lung tissue, cultured human bronchial epithelial cells (NHBE) and immortalized lung epithelial cell line (Beas-2B, light green), lung cancer cell lines (dark green), normal colon tissue (light blue), colon cancer cell lines (dark blue), normal breast tissue and immortalized breast epithelial cell lines (orange), breast cancer cell lines (red), other cell lines (grey), immortalized fibroblasts (HF) and HEK293 (embryonic kidney cell line). In addition to the full-length annotated mRNA of *Cyclin D2* (6.5 kb), *RAB10* (3.5 kb) and *FGF2* (6.8 kb) shorter mRNAs are observed predominantly in cancer cells.
*, is cross hybridization to *Cyclin D1*.

Figure S3

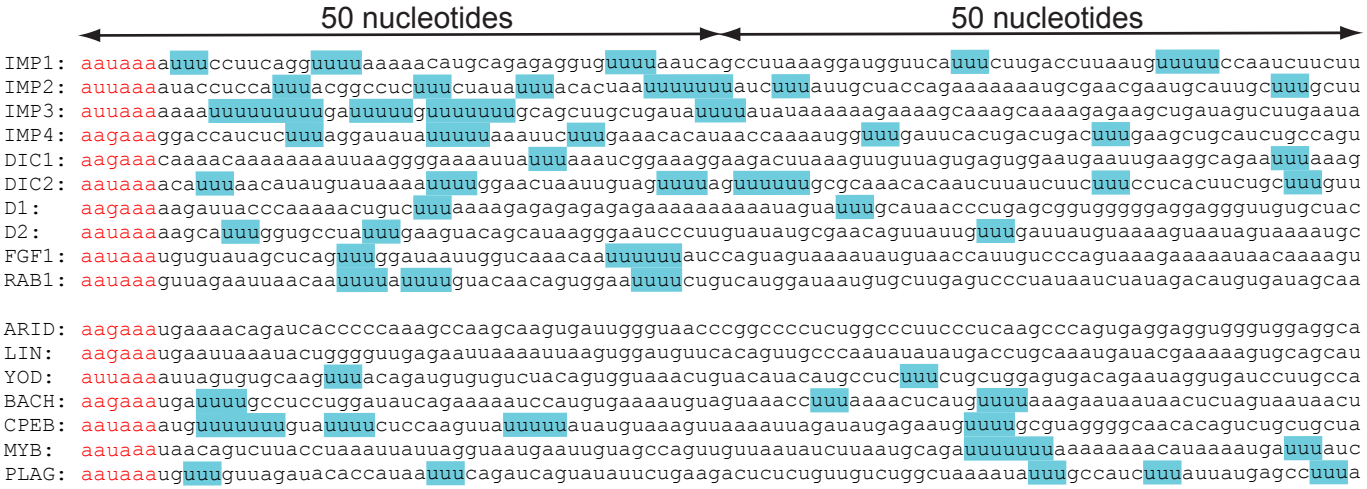

Figure S3. Sequence downstream of functional poly(A) signal.
Overrepresentation of U-rich sequences within 50 nucleotides of the APA signal (p = 0.02). Upper panel: genes with alternative mRNAs. Lower panel: genes without alternative mRNAs in cell lines and tissues investigated.

Figure S4

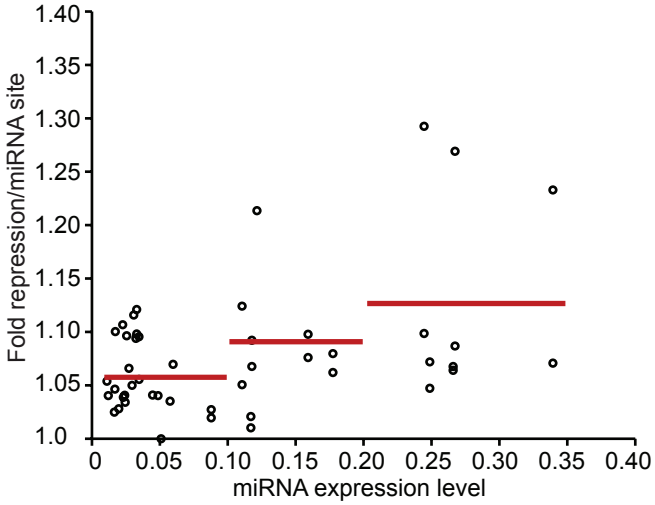

Figure S4. Correlation of endogenous miRNA expression in cell lines with the corresponding repression seen in luciferase reporter assays for three different 3'UTRs.

Relative miRNA expression was determined by small RNA sequencing. Luciferase reporter values were obtained by comparing reporter gene expression from constructs with intact miRNA sites and mutated miRNA sites. The repression per miRNA site was determined by taking the n^{th}-root of the total repression according to the number of miRNA sites (n) measured per 3'UTR. The average increase in luciferase expression per miRNA site (e) was determined by binning the relative miRNA expression in the tested cell lines (1–10%, 10–20% and >20%, red lines; Pearson's correlation coefficient, R = 0.43, p = 0.001).

The maximal increase in expression upon loss of all miRNA regulatory sites of miRNAs expressed higher than 1% in a given cell line was estimated by counting all conserved and non-conserved sites for a given 3'UTR as predicted by TargetScan for the miRNAs expressed higher than 1% in the cell line. According to the expression level of the miRNA, the corresponding luciferase reporter repression value (e) was applied. To determine the maximum repression, all possible repression values were multiplied.

Figure S5

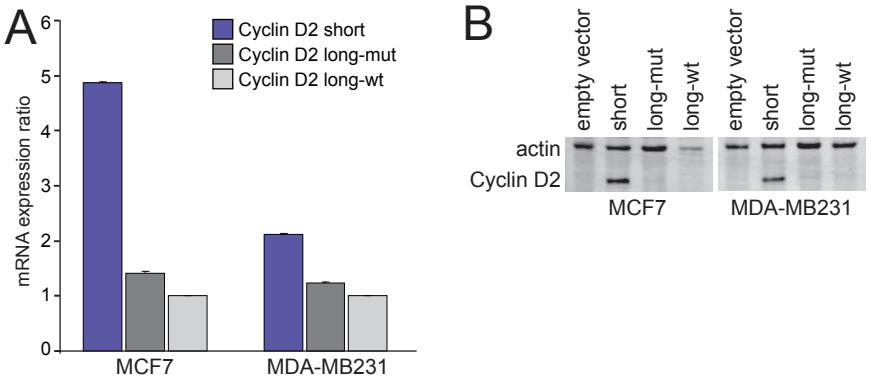

Figure S5. mRNA and protein expression of Cyclin D2 in breast cancer cell lines.
Figure S5A. mRNA expression of ectopically expressed mRNA isoforms of *Cyclin D2* measured by qRT-PCR. Shown is the average fold expression (± s. d.) of six experiments normalized to *GAPDH* and *Puromycin*, and the amount of Cyclin D2 expressed from the long isoform with wt miRNA sites (long-wt). Long-mut: the miRNA sites for miR-15/16 and *let-7* in the 3'UTR of Cyclin D2 were mutated.
Figure S5B. Protein expression of Cyclin D2 (samples as in Figure S5A).

Figure S6

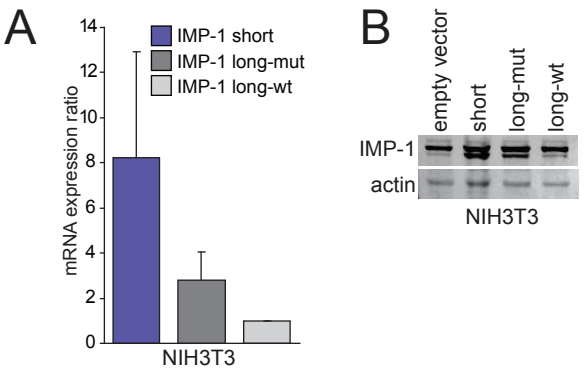

Figure S6. mRNA and protein expression of IMP-1 in NIH3T3 fibroblasts.
Figure S6A. mRNA expression of ectopically expressed mRNA isoforms of *IMP-1* measured by qRT-PCR. Shown is the average fold expression (± s. d.) of six experiments normalized to *GAPDH* and the amount of IMP-1 expressed from the long isoform with wild-type *let-7* sites (long-wt). Long-mut: mutated *let-7* sites.
Figure S6B. Protein expression of IMP-1 (samples as in Figure S6A). Endogenous IMP-1 is expressed as a doublet. Ectopic expression predominantly increases the lower migrating band.

Figure S7

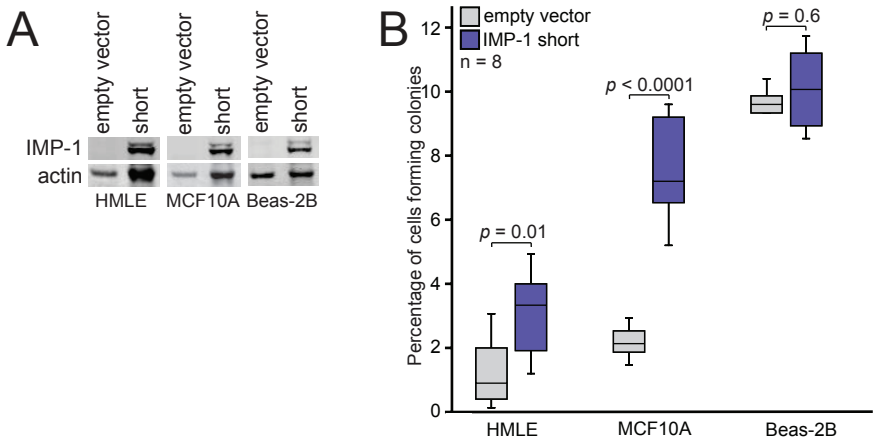

Figure S7. Protein expression of IMP-1 and oncogenic transformation of human epithelial cells by expression of the short mRNA of *IMP-1*.

Figure S7A. Protein expression of IMP-1 expressed from the short mRNA in human epithelial cells from breast (HMLE and MCF10A) and lung tissue (Beas-2B).

Figure S7B. Soft-agar assay of colony formation. The short mRNA isoform of *IMP-1* promotes oncogenic transformation. For breast epithelial cell lines stably transduced with retroviral vectors, the percentage that yielded colonies was plotted (median, horizontal line; 25th through 75th percentile, box; range, error bars; n = 8 from four independent experiments). The vectors expressed either the empty vector or the short mRNA isoform of *IMP-1*. In Beas-2B cells a high number of background colonies was observed that did not increase upon ectopic expression of IMP-1.

Figure S8

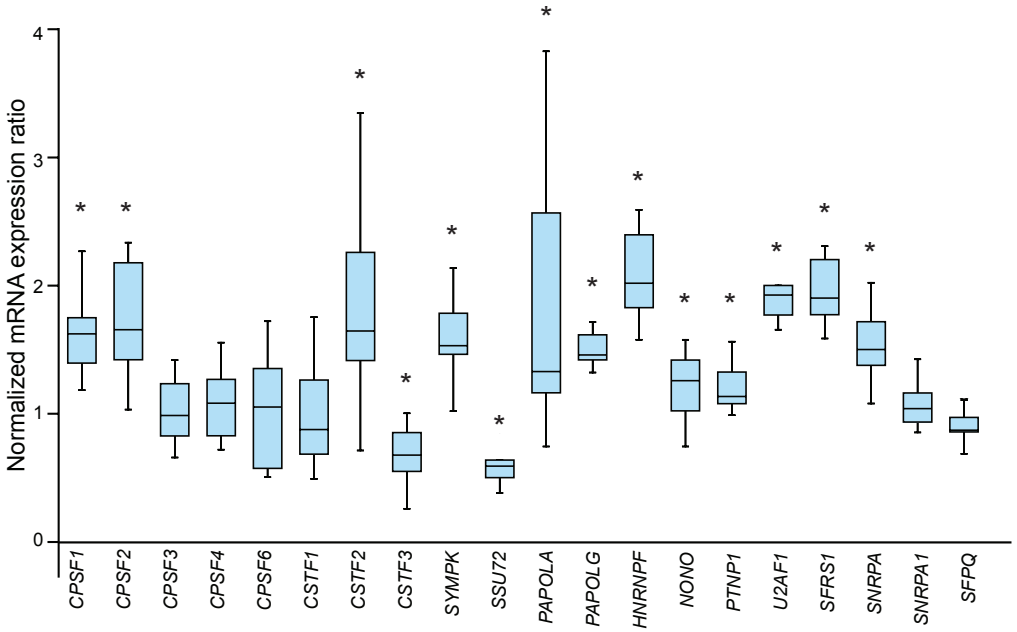

Figure S8. Normalized mRNA expression of proteins involved in polyadenylation. Shown is the mRNA expression of 20 potential *trans*-acting factors (Lou et al., 1999; Vagner et al., 2000; Millevoi et al., 2006; Hall-Pogar et al., 2007; Shi et al., 2009) from nine breast cancer cell lines normalized to the mRNA expression from MCF10A (n=3), a breast epithelial cell line (median, horizontal line; 25th through 75th percentile, box; range, error bars; *, $p<0.013$).

Table S1. miRNA expression in cell lines

Shown is the relative abundance of the 20 highest expressed miRNA families per cell line.

NIH3T3		MEF	
>mmu-let-7	0.249	>mmu-let-7	0.267
>mmu-mir-21	0.098	>mmu-mir-29	0.160
>mmu-mir-26	0.069	>mmu-mir-21	0.123
>mmu-mir-125	0.062	>mmu-mir-24	0.049
>mmu-mir-29	0.055	>mmu-mir-22	0.032
>mmu-mir-17/20/106	0.051	>mmu-mir-125	0.031
>mmu-mir-24	0.040	>mmu-mir-26	0.030
>mmu-mir-130	0.031	>mmu-mir-140	0.025
>mmu-mir-199	0.031	>mmu-mir-15/16	0.023
>mmu-mir-15/16	0.031	>mmu-mir-130	0.022
>mmu-mir-22	0.029	>mmu-mir-199	0.020
>mmu-mir-27	0.021	>mmu-mir-27	0.018
>mmu-mir-322	0.017	>mmu-mir-17/20/106	0.016
>mmu-mir-103/107	0.017	>mmu-mir-31	0.015
>mmu-mir-30	0.015	>mmu-mir-191	0.011
>mmu-mir-19	0.014	>mmu-mir-214	0.011
>mmu-mir-143	0.008	>mmu-mir-103/107	0.011
>mmu-mir-34c	0.008	>mmu-mir-23	0.009
>mmu-mir-23	0.006	>mmu-mir-143	0.008
>mmu-mir-503	0.006	>mmu-mir-221/222	0.007

	HEK293		HeLa
>hsa-mir-17/20/106	0.189	>hsa-let-7	0.339
>hsa-mir-15/16	0.144	>hsa-mir-21	0.184
>hsa-mir-103/107	0.060	>hsa-mir-27	0.052
>hsa-mir-221/222	0.049	>hsa-mir-17/20/106	0.048
>hsa-mir-19	0.047	>hsa-mir-26	0.040
>hsa-mir-26	0.039	>hsa-mir-24	0.034
>hsa-mir-25/32/92/363/367	0.039	>hsa-mir-30	0.027
>hsa-mir-191	0.035	>hsa-mir-92	0.024
>hsa-let-7	0.033	>hsa-mir-19	0.022
>hsa-mir-7	0.031	>hsa-mir-15/16	0.017
>hsa-mir-18	0.024	>hsa-mir-22	0.017
>hsa-mir-148	0.022	>hsa-mir-29	0.017
>hsa-mir-10	0.019	>hsa-mir-125	0.012
>hsa-mir-93	0.016	>hsa-mir-93	0.008
>hsa-mir-130	0.014	>hsa-mir-191	0.008
>hsa-mir-186	0.014	>hsa-mir-103/107	0.007
>hsa-mir-30	0.013	>hsa-mir-143	0.007
>hsa-mir-29	0.013	>hsa-mir-100	0.007
>hsa-mir-378	0.012	>hsa-mir-23	0.006
>hsa-mir-196	0.012	>hsa-mir-186	0.006

	U2OS		143B
>hsa-mir-21	0.445	>hsa-mir-21	0.280
>hsa-let-7	0.088	>hsa-let-7	0.117
>hsa-mir-26	0.041	>hsa-mir-29	0.101
>hsa-mir-130	0.037	>hsa-mir-103/107	0.051
>hsa-mir-15/16	0.035	>hsa-mir-15/16	0.045
>hsa-mir-103/107	0.030	>hsa-mir-27	0.041
>hsa-mir-29	0.028	>hsa-mir-24	0.039
>hsa-mir-27	0.027	>hsa-mir-26	0.035
>hsa-mir-7	0.026	>hsa-mir-17/20/106	0.034
>hsa-mir-24	0.024	>hsa-mir-125	0.027
>hsa-mir-17/20/106	0.024	>hsa-mir-221/222	0.026
>hsa-mir-22	0.023	>hsa-mir-22	0.018
>hsa-mir-221/222	0.015	>hsa-mir-25/32/92/363/367	0.013
>hsa-mir-148	0.011	>hsa-mir-19	0.012
>hsa-mir-93	0.011	>hsa-mir-148	0.012
>hsa-mir-135	0.009	>hsa-mir-130	0.010
>hsa-mir-25/32/92/363/367	0.007	>hsa-mir-146	0.010
>hsa-mir-186	0.007	>hsa-mir-100	0.008
>hsa-mir-19	0.007	>hsa-mir-320	0.007
>hsa-mir-151	0.005	>hsa-mir-101	0.006

	A549		H520
>hsa-mir-21	0.423	>hsa-let-7	0.177
>hsa-let-7	0.087	>hsa-mir-29	0.106
>hsa-mir-24	0.056	>hsa-mir-21	0.102
>hsa-mir-27	0.045	>hsa-mir-103/107	0.058
>hsa-mir-29	0.038	>hsa-mir-200b,c	0.049
>hsa-mir-103/107	0.033	>hsa-mir-24	0.047
>hsa-mir-15/16	0.029	>hsa-mir-26	0.042
>hsa-mir-29	0.026	>hsa-mir-17/20/106	0.040
>hsa-mir-26	0.025	>hsa-mir-221/222	0.032
>hsa-mir-22	0.020	>hsa-mir-27	0.028
>hsa-mir-194	0.018	>hsa-mir-15/16	0.027
>hsa-mir-30	0.018	>hsa-mir-148	0.024
>hsa-mir-221/222	0.015	>hsa-mir-25/32/92/363/367	0.013
>hsa-mir-23	0.011	>hsa-mir-221/222	0.012
>hsa-mir-25/32/92/363/367	0.009	>hsa-mir-22	0.012
>hsa-mir-192	0.009	>hsa-mir-125	0.010
>hsa-mir-191	0.009	>hsa-mir-19	0.009
>hsa-mir-125	0.008	>hsa-mir-130	0.009
>hsa-mir-194	0.007	>hsa-mir-93	0.008
>hsa-mir-130	0.007	>hsa-mir-378	0.008

	SW480		DLD2
>hsa-mir-21	0.201	>hsa-let-7	0.266
>hsa-let-7	0.159	>hsa-mir-29	0.163
>hsa-mir-29	0.089	>hsa-mir-21	0.137
>hsa-mir-499	0.054	>hsa-mir-10	0.059
>hsa-mir-27	0.052	>hsa-mir-200c	0.054
>hsa-mir-200b,c	0.051	>hsa-mir-26	0.053
>hsa-mir-26	0.048	>hsa-mir-27	0.030
>hsa-mir-15/16	0.043	>hsa-mir-17/20/106	0.027
>hsa-mir-17/20/106	0.034	>hsa-mir-103/107	0.023
>hsa-mir-103/107	0.024	>hsa-mir-221/222	0.022
>hsa-mir-10	0.021	>hsa-mir-24	0.021
>hsa-mir-24	0.015	>hsa-mir-15/16	0.012
>hsa-mir-7	0.014	>hsa-mir-148	0.012
>hsa-mir-25/32/92/363/367	0.013	>hsa-mir-19	0.011
>hsa-mir-148	0.013	>hsa-mir-25/32/92/363/367	0.007
>hsa-mir-135	0.011	>hsa-mir-22	0.007
>hsa-mir-93	0.010	>hsa-mir-191	0.006
>hsa-mir-19b	0.009	>hsa-mir-210	0.005
>hsa-mir-221/222	0.008	>hsa-mir-93	0.005
>hsa-mir-96	0.008	>hsa-mir-320	0.005

	MCF7		MDA-MB231
>hsa-mir-21	0.570	>hsa-let-7	0.249
>hsa-let-7	0.121	>hsa-mir-29	0.153
>hsa-mir-26	0.033	>hsa-mir-21	0.126
>hsa-mir-103/107	0.030	>hsa-mir-15/16	0.049
>hsa-mir-200b,c	0.024	>hsa-mir-146	0.037
>hsa-mir-27	0.024	>hsa-mir-27	0.030
>hsa-mir-15/16	0.021	>hsa-mir-17/20/106	0.028
>hsa-mir-17/20/106	0.017	>hsa-mir-30	0.019
>hsa-mir-29	0.016	>hsa-mir-26	0.019
>hsa-mir-141	0.014	>hsa-mir-125	0.019
>hsa-mir-24	0.013	>hsa-mir-103/107	0.016
>hsa-mir-191	0.013	>hsa-mir-221/222	0.015
>hsa-mir-23	0.012	>hsa-mir-25/32/92/363/367	0.013
>hsa-mir-148	0.007	>hsa-mir-23	0.012
>hsa-mir-342	0.006	>hsa-mir-24	0.011
>hsa-mir-96	0.005	>hsa-mir-19	0.011
>hsa-mir-93	0.005	>hsa-mir-22	0.011
>hsa-mir-125	0.005	>hsa-mir-363	0.007
>hsa-mir-135	0.005	>hsa-mir-378	0.007
>hsa-mir-30	0.004	>hsa-mir-96	0.006

Table S2. Estimation of maximum repression due to miRNAs

	miRNA expression	repression per site	Number of miRNA sites per UTR			estimated repression per miRNA family		
	NIH3T3		**IMP-1**	**DICER1**	**Cyclin D2**	**IMP-1**	**DICER1**	**Cyclin D2**
>mmu-let-7	0.249	1.130	5	2	3	1.842	1.277	1.443
>mmu-mir-21	0.098	1.059	2	1	1	1.121	1.059	1.059
>mmu-mir-26	0.069	1.059	0	0	1	1.000	1.000	1.059
>mmu-mir-125	0.062	1.059	0	1	0	1.000	1.059	1.000
>mmu-mir-29	0.055	1.059	0	1	0	1.000	1.059	1.000
>mmu-mir-17/20/106	0.051	1.059	3	1	1	1.188	1.059	1.059
>mmu-mir-24	0.040	1.059	1	0	0	1.059	1.000	1.000
>mmu-mir-130	0.031	1.059	1	0	1	1.059	1.000	1.059
>mmu-mir-199	0.031	1.059	0	1	0	1.000	1.059	1.000
>mmu-mir-15/16	0.031	1.059	0	2	3	1.000	1.121	1.188
>mmu-mir-22	0.029	1.059	2	0	0	1.121	1.000	1.000
>mmu-mir-27	0.021	1.059	0	0	0	1.000	1.000	1.000
>mmu-mir-322	0.017	1.059	0	0	0	1.000	1.000	1.000
>mmu-mir-103/107	0.017	1.059	0	6	0	1.000	1.411	1.000
>mmu-mir-30	0.015	1.059	0	0	1	1.000	1.000	1.059
>mmu-mir-19	0.014	1.059	0	1	2	1.000	1.059	1.121

	miRNA expression	repression per site	Number of miRNA sites per UTR			estimated repression per miRNA family		
	MEF		**IMP-1**	**DICER1**	**Cyclin D2**	**IMP-1**	**DICER1**	**Cyclin D2**
mmu-let-7	0.267	1.130	5	2	3	1.842	1.277	1.443
mmu-mir-29	0.160	1.081	0	1	0	1.000	1.081	1.000
mmu-mir-21	0.123	1.081	2	1	1	1.169	1.081	1.081
mmu-mir-24	0.049	1.059	1	0	0	1.059	1.000	1.000
mmu-mir-22	0.032	1.059	2	0	0	1.121	1.000	1.000
mmu-mir-125	0.031	1.059	0	1	0	1.000	1.059	1.000
mmu-mir-26	0.030	1.059	0	0	1	1.000	1.000	1.059
mmu-mir-140	0.025	1.059	2	1	0	1.121	1.059	1.000
mmu-mir-15/16	0.023	1.059	0	2	3	1.000	1.121	1.188
mmu-mir-130	0.022	1.059	1	0	1	1.059	1.000	1.059
mmu-mir-199	0.020	1.059	0	1	0	1.000	1.059	1.000
mmu-mir-27	0.018	1.059	0	0	0	1.000	1.000	1.000
mmu-mir-17/20/106	0.016	1.059	3	1	1	1.188	1.059	1.059
mmu-mir-31	0.015	1.059	0	0	0	1.000	1.000	1.000
mmu-mir-191	0.011	1.059	0	0	0	1.000	1.000	1.000
mmu-mir-214	0.011	1.059	0	1	2	1.000	1.059	1.121
mmu-mir-103/107	0.011	1.059	0	6	0	1.000	1.411	1.000

	miRNA expression	repression per site	Number of miRNA sites per UTR			estimated repression per miRNA family		
	HEK293		**IMP-1**	**DICER1**	**Cyclin D2**	**IMP-1**	**DICER1**	**Cyclin D2**
hsa-mir-17/20/106	0.189	1.081	3	1	1	1.263	1.081	1.081
hsa-mir-15/16	0.144	1.081	0	2	3	1.000	1.169	1.263
hsa-mir-103/107	0.060	1.059	0	6	0	1.000	1.411	1.000
hsa-mir-1221/222	0.049	1.059	0	1	1	1.000	1.059	1.059
hsa-mir-19	0.047	1.059	0	1	2	1.000	1.059	1.121
hsa-mir-26	0.039	1.059	0	0	1	1.000	1.000	1.059
hsa-mir-25/32/92/363/367	0.039	1.059	0	0	0	1.000	1.000	1.000
hsa-mir-191	0.035	1.059	2	0	1	1.121	1.000	1.059
hsa-let-7	0.033	1.059	5	2	3	1.332	1.121	1.188
hsa-mir-7	0.031	1.059	0	0	2	1.000	1.000	1.121
hsa-mir-18	0.024	1.059	0	2	2	1.000	1.121	1.121
hsa-mir-148	0.022	1.059	0	0	0	1.000	1.000	1.000
hsa-mir-10	0.019	1.059	2	0	0	1.121	1.000	1.000
hsa-mir-93	0.016	1.059	1	0	2	1.059	1.000	1.121
hsa-mir-130	0.014	1.059	1	0	1	1.059	1.000	1.059
hsa-mir-186	0.014	1.059	0	0	1	1.000	1.000	1.059
hsa-mir-30	0.013	1.059	0	0	1	1.000	1.000	1.059
hsa-mir-29	0.013	1.059	0	1	0	1.000	1.059	1.000
hsa-mir-378	0.012	1.059	1	1	3	1.059	1.059	1.188
hsa-mir-196	0.012	1.059	0	1	0	1.000	1.059	1.000

	miRNA expression	repression per site	Number of miRNA sites per UTR			estimated repression per miRNA family		
	HeLa		**IMP-1**	**DICER1**	**Cyclin D2**	**IMP-1**	**DICER1**	**Cyclin D2**
>hsa-let-7	0.339	1.130	5	2	3	1.842	1.277	1.443
>hsa-mir-21	0.184	1.081	2	1	1	1.169	1.081	1.081
>hsa-mir-27	0.052	1.059	0	0	0	1.000	1.000	1.000
>hsa-mir-17/20/106	0.048	1.059	3	1	1	1.188	1.059	1.059
>hsa-mir-26	0.040	1.059	0	0	1	1.000	1.000	1.059
>hsa-mir-24	0.034	1.059	1	0	0	1.059	1.000	1.000
>hsa-mir-30	0.027	1.059	0	0	1	1.000	1.000	1.059
>hsa-mir-25/32/92/363/367	0.024	1.059	0	0	0	1.000	1.000	1.000
>hsa-mir-19	0.022	1.059	0	1	2	1.000	1.059	1.121
>hsa-mir-15/16	0.017	1.059	0	2	3	1.000	1.121	1.188
>hsa-mir-22	0.017	1.059	2	0	0	1.121	1.000	1.000
>hsa-mir-29	0.017	1.059	0	1	0	1.000	1.059	1.000
>hsa-mir-125	0.012	1.059	0	1	0	1.000	1.059	1.000

	miRNA expression	repression per site	Number of miRNA sites per UTR			estimated repression per miRNA family		
	U2OS		**IMP-1**	**DICER1**	**Cyclin D2**	**IMP-1**	**DICER1**	**Cyclin D2**
>hsa-mir-21	0.445	1.130	2	1	1	1.277	1.130	1.130
>hsa-let-7	0.088	1.059	5	2	3	1.332	1.121	1.188
>hsa-mir-26	0.041	1.059	0	0	1	1.000	1.000	1.059
>hsa-mir-130	0.037	1.059	1	0	1	1.059	1.000	1.059
>hsa-mir-15/16	0.035	1.059	0	2	3	1.000	1.121	1.188
>hsa-mir-103/107	0.030	1.059	0	6	0	1.000	1.411	1.000
>hsa-mir-29	0.028	1.059	0	1	0	1.000	1.059	1.000
>hsa-mir-27	0.027	1.059	0	0	0	1.000	1.000	1.000
>hsa-mir-7	0.026	1.059	0	0	2	1.000	1.000	1.121
>hsa-mir-24	0.024	1.059	1	0	0	1.059	1.000	1.000
>hsa-mir-17/20/106	0.024	1.059	3	1	1	1.188	1.059	1.059
>hsa-mir-22	0.023	1.059	2	0	0	1.121	1.000	1.000
>hsa-mir-221/222	0.015	1.059	0	1	1	1.000	1.059	1.059
>hsa-mir-148	0.011	1.059	0	0	0	1.000	1.000	1.000
>hsa-mir-93	0.011	1.059	1	0	2	1.059	1.000	1.121

	miRNA expression	repression per site	Number of miRNA sites per UTR			estimated repression per miRNA family		
	143B		**IMP-1**	**DICER1**	**Cyclin D2**	**IMP-1**	**DICER1**	**Cyclin D2**
>hsa-mir-21	0.280	1.130	2	1	1	1.277	1.130	1.130
>hsa-let-7	0.117	1.081	5	2	3	1.476	1.169	1.263
>hsa-mir-29	0.101	1.081	0	1	0	1.000	1.081	1.000
>hsa-mir-103/107	0.051	1.059	0	6	0	1.000	1.411	1.000
>hsa-mir-15/16	0.045	1.059	0	2	3	1.000	1.121	1.188
>hsa-mir-27	0.041	1.059	0	0	0	1.000	1.000	1.000
>hsa-mir-24	0.039	1.059	1	0	0	1.059	1.000	1.000
>hsa-mir-26	0.035	1.059	0	0	1	1.000	1.000	1.059
>hsa-mir-17/20/106	0.034	1.059	3	1	1	1.188	1.059	1.059
>hsa-mir-125	0.027	1.059	0	1	0	1.000	1.059	1.000
>hsa-mir-221/222	0.026	1.059	0	1	1	1.000	1.059	1.059
>hsa-mir-22	0.018	1.059	2	0	0	1.121	1.000	1.000
>hsa-mir-25/32/92/363/367	0.013	1.059	0	0	0	1.000	1.000	1.000
>hsa-mir-19	0.012	1.059	0	1	2	1.000	1.059	1.121
>hsa-mir-148	0.012	1.059	0	0	0	1.000	1.000	1.000

	miRNA expression	repression per site	Number of miRNA sites per UTR			estimated repression per miRNA family		
	A549		**IMP-1**	**DICER1**	**Cyclin D2**	**IMP-1**	**DICER1**	**Cyclin D2**
>hsa-mir-21	0.423	1.130	2	1	1	1.277	1.130	1.130
>hsa-let-7	0.087	1.081	5	2	3	1.476	1.169	1.263
>hsa-mir-24	0.056	1.059	1	0	0	1.059	1.000	1.000
>hsa-mir-27	0.045	1.059	0	0	0	1.000	1.000	1.000
>hsa-mir-29	0.038	1.059	0	1	0	1.000	1.059	1.000
>hsa-mir-103/107	0.033	1.059	1	6	0	1.000	1.411	1.000
>hsa-mir-15/16	0.029	1.059	1	2	3	1.000	1.121	1.188
>hsa-mir-26	0.025	1.059	0	0	1	1.000	1.000	1.059
>hsa-mir-22	0.020	1.059	2	0	0	1.121	1.000	1.000
>hsa-mir-194	0.018	1.059	0	0	0	1.000	1.000	1.000
>hsa-mir-30	0.018	1.059	0	0	1	1.000	1.000	1.059
>hsa-mir-221/222	0.015	1.059	0	1	1	1.000	1.059	1.059
>hsa-mir-23	0.011	1.059	1	0	0	1.059	1.000	1.000

	miRNA expression	repression per site	Number of miRNA sites per UTR			estimated repression per miRNA family		
	H520		**IMP-1**	**DICER1**	**Cyclin D2**	**IMP-1**	**DICER1**	**Cyclin D2**
>hsa-let-7	0.177	1.081	5	2	3	1.476	1.169	1.263
>hsa-mir-29	0.106	1.081	0	1	0	1.000	1.081	1.000
>hsa-mir-21	0.102	1.081	2	1	1	1.169	1.081	1.081
>hsa-mir-103/107	0.058	1.059	0	6	0	1.000	1.411	1.000
>hsa-mir-200b,c	0.049	1.059	1	0	0	1.059	1.000	1.000
>hsa-mir-24	0.047	1.059	1	0	0	1.059	1.000	1.000
>hsa-mir-26	0.042	1.059	0	0	1	1.000	1.000	1.059
>hsa-mir-1720/106	0.040	1.059	3	1	1	1.188	1.059	1.059
>hsa-mir-221/222	0.032	1.059	0	1	1	1.000	1.059	1.059
>hsa-mir-27	0.028	1.059	0	0	0	1.000	1.000	1.000
>hsa-mir-15/16	0.027	1.059	0	2	3	1.000	1.121	1.188
>hsa-mir-148	0.024	1.059	0	0	0	1.000	1.000	1.000
>hsa-mir-25/32/92/363/367	0.013	1.059	0	0	0	1.000	1.000	1.000
>hsa-mir-221/222	0.012	1.059	0	1	1	1.000	1.059	1.059
>hsa-mir-22	0.012	1.059	2	0	0	1.121	1.000	1.000
>hsa-mir-125	0.010	1.059	0	1	0	1.000	1.059	1.000

	miRNA expression	repression per site	Number of miRNA sites per UTR			estimated repression per miRNA family		
	SW480		**IMP-1**	**DICER1**	**Cyclin D2**	**IMP-1**	**DICER1**	**Cyclin D2**
>hsa-mir-21	0.201	1.130	2	1	1	1.277	1.130	1.130
>hsa-let-7	0.159	1.081	5	2	3	1.476	1.169	1.263
>hsa-mir-29	0.089	1.059	0	1	0	1.000	1.059	1.000
>hsa-mir-499	0.054	1.059	0	0	0	1.000	1.000	1.000
>hsa-mir-27	0.052	1.059	0	0	0	1.000	1.000	1.000
>hsa-mir-200c	0.051	1.059	1	0	0	1.059	1.000	1.000
>hsa-mir-26	0.048	1.059	0	0	1	1.000	1.000	1.059
>hsa-mir-/1516	0.043	1.059	0	2	3	1.000	1.121	1.188
>hsa-mir-17/20/106	0.034	1.059	3	1	1	1.188	1.059	1.059
>hsa-mir-103/107	0.024	1.059	0	6	0	1.000	1.411	1.000
>hsa-mir-10	0.021	1.059	2	0	0	1.121	1.000	1.000
>hsa-mir-24	0.015	1.059	1	0	0	1.059	1.000	1.000
>hsa-mir-7	0.014	1.059	0	0	2	1.000	1.000	1.121
>hsa-mir-25/32/92/363/367	0.013	1.059	0	0	0	1.000	1.000	1.000
>hsa-mir-148	0.013	1.059	0	0	0	1.000	1.000	1.000
>hsa-mir-135	0.011	1.059	2	0	0	1.121	1.000	1.000

	miRNA expression	repression per site	Number of miRNA sites per UTR			estimated repression per miRNA family		
	DLD2		**IMP-1**	**DICER1**	**Cyclin D2**	**IMP-1**	**DICER1**	**Cyclin D2**
>hsa-let-7	0.266	1.130	5	2	3	1.842	1.277	1.443
>hsa-mir-29	0.163	1.081	0	1	0	1.000	1.081	1.000
>hsa-mir-21	0.137	1.081	2	1	1	1.169	1.081	1.081
>hsa-mir-10	0.059	1.059	2	0	0	1.121	1.000	1.000
>hsa-mir-200c	0.054	1.059	1	0	0	1.059	1.000	1.000
>hsa-mir-26	0.053	1.059	0	0	1	1.000	1.000	1.059
>hsa-mir-27	0.030	1.059	0	0	0	1.000	1.000	1.000
>hsa-mir-17/20/106	0.027	1.059	3	1	1	1.188	1.059	1.059
>hsa-mir-103/107	0.023	1.059	0	6	0	1.000	1.411	1.000
>hsa-mir-221222	0.022	1.059	0	1	1	1.000	1.059	1.059
>hsa-mir-24	0.021	1.059	1	0	0	1.059	1.000	1.000
>hsa-mir-15/16	0.012	1.059	0	2	3	1.000	1.121	1.188
>hsa-mir-148	0.012	1.059	0	0	0	1.000	1.000	1.000
>hsa-mir-19	0.011	1.059	0	1	2	1.000	1.059	1.121

	miRNA expression	repression per site	Number of miRNA sites per UTR			estimated repression per miRNA family		
	MCF7		**IMP-1**	**DICER1**	**Cyclin D2**	**IMP-1**	**DICER1**	**Cyclin D2**
>hsa-mir-21	0.570	1.130	2	1	1	1.277	1.130	1.130
>hsa-let-7	0.121	1.081	5	2	3	1.476	1.169	1.263
>hsa-mir-26	0.033	1.059	0	0	1	1.000	1.000	1.059
>hsa-mir-103/107	0.030	1.059	0	6	0	1.000	1.411	1.000
>hsa-mir-200b,c	0.024	1.059	1	0	0	1.059	1.000	1.000
>hsa-mir-27	0.024	1.059	0	0	0	1.000	1.000	1.000
>hsa-mir-15/16	0.021	1.059	0	2	3	1.000	1.121	1.181
>hsa-mir-29	0.016	1.059	0	1	0	1.000	1.059	1.000
>hsa-mir-24	0.013	1.059	1	0	0	1.059	1.000	1.000
>hsa-mir-191	0.013	1.059	2	0	1	1.121	1.000	1.059
>hsa-mir-23	0.012	1.059	1	0	0	1.057	1.000	1.000
>hsa-mir-17/20/106	0.010	1.059	3	1	1	1.188	1.059	1.059

	miRNA expression	repression per site	Number of miRNA sites per UTR			estimated repression per miRNA family		
	MDA-MB231		**IMP-1**	**DICER1**	**Cyclin D2**	**IMP-1**	**DICER1**	**Cyclin D2**
>hsa-let-7	0.249	1.130	5	2	3	1.842	1.277	1.443
>hsa-mir-29	0.153	1.081	0	1	0	1.000	1.081	1.000
>hsa-mir-21	0.126	1.081	2	1	1	1.169	1.081	1.081
>hsa-mir-15/16	0.049	1.059	0	2	3	1.000	1.121	1.188
>hsa-mir-146	0.037	1.059	0	0	0	1.000	1.000	1.000
>hsa-mir-27	0.030	1.059	0	0	0	1.000	1.000	1.000
>hsa-mir-17/20/106	0.028	1.059	3	1	1	1.188	1.059	1.059
>hsa-mir-30	0.019	1.059	0	0	1	1.000	1.000	1.059
>hsa-mir-26	0.019	1.059	0	0	1	1.000	1.000	1.059
>hsa-mir-125	0.019	1.059	0	1	0	1.000	1.059	1.000
>hsa-mir-103/107	0.016	1.059	0	6	0	1.000	1.411	1.000
>hsa-mir-222	0.015	1.059	0	1	1	1.000	1.059	1.059
>hsa-mir-92	0.013	1.059	0	0	0	1.000	1.000	1.000
>hsa-mir-23	0.012	1.059	1	2	0	1.059	1.121	1.000
>hsa-mir-24	0.011	1.059	1	0	0	1.059	1.000	1.000
>hsa-mir-19	0.011	1.059	0	1	2	1.000	1.059	1.121
>hsa-mir-22	0.011	1.059	2	0	0	1.121	1.000	1.000

Supplemental Experimental procedures

Cell lines

The following cell lines were purchased from ATCC and cultured as indicated: HEK293, HEK293T, HeLa, NIH3T3, MEF, F9, human sarcoma cell lines (U2OS, Saos-2, HOS, 143B), human breast cancer cell lines (A549, NCI-H23, NCI-H292, NCI-H460, NCI-H520, NCI-H596), the human immortalized bronchial epithelial cell line Beas-2B, human immortalized fibroblasts (HF) and human colon cancer cell lines (HCT116, SW480, HT-29, Colo-205, Lovo and DLD2). NHBE (normal human bronchial epithelial cells) were purchased from Lonza and cultured as indicated. The colon cancer cell lines SKCO1 and Ls147T were a gift from Thijn Brummelkamp (Whitehead Institute, Cambridge, USA). Immortalized breast epithelial cell lines (HME, HMLE, HBL-100, MCF10A) and breast cancer cell lines MCF7, MDA-MB231, MDA-MB435, MDA-MB453, MDA-MB361, MDA-MB468, MDA-MB157, BT474, BT549, T47D, CAMA) were a gift from Robert Weinberg's lab (Whitehead Institute, Cambridge, USA). Phoenix cells were a gift from Michael Hemann (MIT, Cambridge, USA).

Prediction of miRNA sites

Targetscan (version 4.2) was used for prediction of miRNA targets (Lewis et al., 2005).

Northern blots to detect miRNAs

Total RNA was isolated from the above mentioned cell lines using Tri-reagent (Ambion) and 20 µg was loaded per lane. RNA blotting was performed as described (Mayr et al., 2007) (http://web.wi.mit.edu/bartel/pub/protocols/), with the following DNA oligo probes:

let-7a,b,c,d 5'-CAACCTACTACCTCA

let-7e 5'-ACTATACAACCTCCTACCTCA

let-7f 5'-ACTATACAATCTACTACCTCA

let-7g,i 5'-ACAAACTACTACCTCA

miR-15a/b 5'-AAACCATGATGTGCTGCTA
miR-16 5'-CGCCAATATTTACGTGCTGCTA
miR-103/107 5'-ATAGCCCTGTACAATGCTGCT
U6 snRNA 5'-TTGCGTGTCATCCTTGCGCAGG

Northern blots to detect mRNAs

Total RNA was isolated from the above mentioned cell lines using Tri-reagent (Ambion). Polyadenylated RNA was purified with Oligotex (Qiagen) and 1.5-2 µg was used per sample. The protocol for the Northern blot was adapted from Cold Spring Harbor Protocols (Sambrook & Russell, 2006). 2 µl of poly(A)$^+$ RNA was incubated with 10 µl glyoxal reaction mixture [6 ml DMSO, 2 ml of deionized glyoxal, 1.2 ml of 10X BPTE electrophoresis buffer (100 mM PIPES, 300 mM Bis-Tris, 10 mM EDTA), 0.6 ml of 80% glycerol in H_2O, 0.2 ml ethidium bromide (10 mg/ml in H_2O)] at 55 °C for 1 hour. Glyoxal-treated RNA was separated on an agarose gel and transferred onto Nytran SuPerCharge Turboblotter Membrane (Whatman) overnight. The glyoxal reaction was reversed by incubation of the membrane in 20 mM Tris-HCL (pH = 8), then crosslinked and baked. Blots were prehybridized in UltraHyb solution (Ambion), hybridized and washed according to the manufacturer's instructions. PCR probes were gel-purified and labeled with ^{32}P according to the manufacturer's instructions using the Megaprime labeling kit (Amersham). Blots were scanned on a Phosphoimager, and bands were quantified using MultiGauge V2.2.

mRNA stability

Cell lines were treated with Actinomycin D (10 µg/ml), total RNA was isolated at 0h, 2h, 4h, 6h and 8h and Northern blots were performed as described above. Half-life was calculated: $t_{1/2} = 0.693/[(\ln c1-\ln c2)/t]$, with t = time interval between c1 and c2, c1 = amount of mRNA at t = 0 and c2 = mRNA amount at t = 2h, 4h or 6h.

3' RACE

This protocol was adapted from Cold Spring Harbor Protocols (Sambrook & Russell, 2006). 1 µg total RNA was used to generate cDNA with Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's instructions using TAP as a primer. The first PCR was done with a gene-specific forward primer and AP as reverse primer. Nested PCR was done with a nested gene-specific forward primer and MAP as reverse primer. The PCR product from the nested PCR was separated on an agarose gel, cloned and sequenced.

TAP: 5'-GACTCGAGTCGACATCGATTTTTTTTTTTTTTTT;

AP: 5'-GACTCGAGTCGACATCG;

MAP: 5'-CGACATCGATTTTTTTTTT.

If only full-length mRNAs were found by 3' RACE, Northern blots were reprobed with different probes in the ORF to find alternatively spliced isoforms.

Constructs

The long and the short 3'UTRs of the human *IGF2BP1/IMP-1, DICER1* and *Cyclin D2* were PCR amplified with Pfu UltraII (Stratagene) and subcloned into the *Renilla* luciferase vector pIS1 (Mayr et al., 2007). The following primers were used:

IMP-1 fw: 5'-CAAACCGGTCCAGCCCCTCCCTGTCCCTTCG

IMP-1 long rev: 5'-CGTGCGGCCGCTTGTCTTTCAATATAAGTTTCCT

IMP-1 short rev: 5'-CGTGCGGCCGCAAATGAACCATCCTTTAAGGC

DICER1 fw: 5'-GCCTCTAGAAACCGCTTTTTAAAATTC

DICER1 long rev: 5'-GATGCGGCCGCGAACAGACGATAAC

DICER1 short rev: 5'- GATGCGGCCGCTATACATATGTTAAATGTTTTATTCTG

Cyclin D2 fw: 5'-CAATCTAGAGGATGCCAGTTGGGCCGAAAG

Cyclin D2 long rev: 5'-CGTGCGGCCGCAGGTCAAGGTGAGTTTATTGTCC

Cyclin D2 short rev: 5'-CGTGCGGCCGCTTCAAATAGGCACCAAATGC

To generate only the long mRNAs poly(A) signals were mutated to a C at positions 2 and 4. MiRNA complementary sites were mutated at nucleotides

complementary to positions 3 and 5 of the seed of the miRNA using Quikchange Multi-site kit (Stratagene).

For functional analyses, the ORF of human IMP-1 together with either the short or the long 3'UTR (with mutation of all four poly(A) signals) with either wild-type *let-7* sites or mutant *let-7* sites was cloned into pPIG (which was a gift of Michael Hemann, MIT, USA), which was derived from pMSCVpuro (Clontech) and modified by cloning the IRES of encephalomyocarditis virus (ECMV) and GFP downstream of puromycin into the vector. The ORF of the human *Cyclin D2* gene with 120 bp of the 5'UTR (fw primer: 5'-TATTTAGCCAAAGGAAGGAGGTC) together with either the short or the long 3' UTR (with mutation of the first AAUAAA) with either wild-type miR-15/16 and *let-7* sites or mutant miR-15/16 and *let-7* sites was cloned into pMSCVpuro (Clontech).

Luciferase assays

All cell lines were transfected using Lipofectamine 2000 (Invitrogen) in 24-well plates with 100 ng firefly luciferase control reporter plasmid pISO (Mayr et al., 2007) and 400 ng *Renilla* luciferase reporter plasmid per well for the full-length mRNA. For the shorter mRNA same molar amounts were transfected. Firefly and *Renilla* luciferase activities were measured 24 hours after transfection with the Dual-luciferase assay (Promega). *Renilla* activity was normalized to firefly activity to control for transfection efficiency. To account for differences in plasmid preparations, values were then normalized to those of the reporter in F9 cells for the *let-7* miRNA, in DLD2 cells for miR-15/16 and in 143B for miR-103/107.

Cell culture

To produce retroviral particles for ectopic expression of Cyclin D2 HEK293T cells were transfected with Lipofectamine 2000 (Invitrogen) together with plasmids for VSV-G and MCV to transduce human cell lines. Supernatant containing viral particles was harvested 48 hours later. The breast cell lines were transduced

(with comparable MOIs) and 24 hours later puromycin (1 µg/ml) was added. Marker-selected populations were obtained after 7-10 days.

To produce retroviral particles for ectopic expression of IMP-1 Phoenix cells were transfected with Lipofectamine 2000 (Invitrogen) and supernatant containing viral particles was harvested 48 hours later. NIH3T3 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FCS (Invitrogen). NIH3T3 were transduced (with comparable MOIs) and 24 hours later puromycin (2 µg/ml) was added. Marker-selected populations were obtained after 7-10 days. To transduce human cell lines HEK293T cells were used as packaging cells and transfected together with plasmids for VSV-G and MCV. The transcript for expression of the short isoform of IMP-1 is 6,124 bp (5' PCMV LTR, ψ+ packaging signal, IMP-1, PGK, Puromycin, IRES, GFP) and the long isoform of IMP-1 is 12,412 bp. For transduction of mouse cells (NIH3T3) we used Phoenix cells as the packaging cell line and obtained NIH3T3 cells expressing human IMP-1. For transduction of human epithelial cell lines we used HEK293T cells for packaging. For the short isoform we obtained comparable viral titers as for transduction of NIH3T3 cells, but for the longer isoforms the titers obtained were low. Visualization of the mRNA transcripts on a Northern blot showed IMP-1 transcripts including the ORF for the NIH3T3 cells as well as for the shorter IMP-1 isoforms in the human cells, but splice variants without the IMP-1 ORF for the long isoforms. These spliced transcripts (including the antibiotic selection marker) were about 5 kb. We suspect that the transcript including the full-length IMP-1 is rather large for packaging in HEK293T cells and we were unable to express it in human cells.

Western blot

Cells were lysed in Laemmli buffer (Biorad). Samples were run on 4-12% Tris-HCL gels, transferred to PVDF membrane (Biorad), blocked with Odyssey blocking buffer (LI-COR) and probed with anti-IMP-1 antibody (1:200; Santa Cruz), anti-Cyclin D2 antibody (1:200; Santa Cruz) and anti-actin antibody

(1:10,000; Sigma). Blots were scanned and bands were quantified using the Odyssey Imager (LI-COR).

RT–PCR

RNA was extracted with Tri-reagent (Ambion), treated with DNase using the DNA-free kit (Ambion). DNA-free RNA was reverse-transcribed with random hexamers and Superscript II (Invitrogen) according to manufacturer's instructions. Quantitative RT–PCR was performed on an ABI 7900HT Real-Time PCR system with ABI SYBR Green reagents. The following primer pairs were used to amplify the specified mRNAs:
IMP-1 fw: 5'-GAAGAAGGTAGAGCAAGATACCG,
IMP-1 rev: 5'-CCCGAACTTTCTTCATTATTTCC,
Cyclin D2 fw: 5'- GAGCTGCTGGCTAAGATCACC,
Cyclin D2 rev: 5'-ATATCCCGCACGTCTGTAGG,
Puromycin fw: 5'-CACCAGGGCAAGGGTCTG,
Puromycin rev: 5'-GCTCGTAGAAGGGGAGGTTG,
human GAPDH fw: 5'- GGTCTCCTCTGACTTCAACAGC,
human GAPDH rev: 5'- GCTGTAGCCAAATTCGTTGTCATACC,
mouse GAPDH fw: 5'- CTCACTCAAGATTGTCAGCAATG,
mouse GAPDH rev: 5'-CACATTGGGGGTAGGAACAC.
Threshold cycle (Ct) and baseline were detected by ABI 7900HT SDS2.3 software.

Cell cycle analysis by FACS

Cells were plated at comparable densities, harvested after 48h, fixed with ethanol and stained with propidium iodide (50μg/ml) and RNase A (40 U/ml) and DNA content was measured on a FACS Calibur HTS (Becton Dickinson). The percentage of diploid cells in G1, S and G2 were analyzed by ModFitLT V3.1.

Soft-agar Assay

Soft-agar assays were performed as described (Mayr et al., 2007). 2×10^5 transduced NIH3T3 cells were suspended in 0.5% Noble Agar (Sigma) in Ham's F12 medium (Cellgro), supplemented with 12% FCS and puromycin (1 µg/ml), and plated over a first layer of 0.5% Noble Agar in Ham's F12 medium. The cells were grown at 37° C and 5% $C0_2$, and colonies were counted at day 28. Colonies of eight cells or more were counted. Six independent transductions were done for each of the constructs. Each transduction was plated in triplicate. For each plate, 30 fields of 30 cells were counted. HMLE and MCF10A cells were suspended in 0.5% Noble Agar (Sigma) in MEGM (Lonza) and Beas-2B in BEGM (Lonza) and supplemented with puromycin (1 µg/ml). Colonies (five cells or more) from at least three independent experiments were counted.

mRNA expression of *trans*-acting factors

Published Affymetrix array data (GSE12790) were analyzed. Three replicates of MCF10A were used to normalize the expression of 20 *trans*-acting factors from nine breast cancer cell lines (MCF7, MDA-MB231, MDA-MB361, MDA-MB468, MDA-MB453, BT474, BT549, T47D and CAMA).

Statistics

The Kruskal-Wallis test was used to analyze the difference between several independent subgroups. Mann Whitney test was applied to analyze the difference between two independent supgroups. The Wilcoxon test was used to make pairwise comparisons using SPSS 14.0.

Supplemental References

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