Cell
PRESS

STAT3 Activation of miR-21 and miR-181b-1 via PTEN and CYLD Are Part of the Epigenetic Switch Linking Inflammation to Cancer

Dimitrios Iliopoulos,[1] Savina A. Jaeger,[3] Heather A. Hirsch,[1] Martha L. Bulyk,[2,3,4] and Kevin Struhl[1,*]
[1]Department of Biological Chemistry and Molecular Pharmacology
[2]Harvard/MIT Division of Health Sciences and Technology (HST)
[3]Division of Genetics, Department of Medicine, Brigham and Women's Hospital
[4]Department of Pathology, Brigham and Women's Hospital
Harvard Medical School, Boston, MA, 02115, USA
*Correspondence: kevin@hms.harvard.edu
DOI 10.1016/j.molcel.2010.07.023

SUMMARY

A transient inflammatory signal can initiate an epigenetic switch from nontransformed to cancer cells via a positive feedback loop involving NF-κB, Lin28, let-7, and IL-6. We identify differentially regulated microRNAs important for this switch and putative transcription factor binding sites in their promoters. STAT3, a transcription factor activated by IL-6, directly activates miR-21 and miR-181b-1. Remarkably, transient expression of either microRNA induces the epigenetic switch. MiR-21 and miR-181b-1, respectively, inhibit PTEN and CYLD tumor suppressors, leading to increased NF-κB activity required to maintain the transformed state. These STAT3-mediated regulatory circuits are required for the transformed state in diverse cell lines and tumor growth in xenografts, and their transcriptional signatures are observed in colon adenocarcinomas. Thus, STAT3 is not only a downstream target of IL-6 but, with miR-21, miR-181b-1, PTEN, and CYLD, is part of the positive feedback loop that underlies the epigenetic switch that links inflammation to cancer.

INTRODUCTION

In human cells, there are ~1000 microRNAs that collectively regulate the expression of more than 30% of protein-coding genes at the posttranscriptional and translational level (Bartel, 2009). Each microRNA represses multiple gene targets, and repression can occur by translational inhibition, mRNA cleavage, and mRNA decay initiated by microRNA-guided deadenylation. With respect to the regulation of gene expression patterns, microRNAs are analogous to DNA-binding transcription factors that directly regulate the expression of target genes. Thus, elucidation of transcriptional regulatory circuits requires the integration of transcription factors, microRNAs, and their direct targets into connected molecular pathways that are responsible for specific biological phenomena.

Bioinformatic programs can identify putative target genes for individual microRNAs, and many such microRNA-target interactions have been validated experimentally. However, there is limited information about how microRNAs are regulated at the transcriptional and posttranscriptional levels. The related RNA-binding proteins Lin28 and Lin28b that inhibit the let-7 family of tumor suppressor microRNAs are the only known factors that mediate posttranscriptional regulation of microRNAs (Viswanathan et al., 2008; Hagan et al., 2009; Iliopoulos et al., 2009). Although examples of transcription factors directly regulating microRNA expression have been described (O'Donnell et al., 2005; Löffler et al., 2007; Chang et al., 2008; Lin et al., 2009), microRNA promoters and transcription start sites were largely unknown until recently, thereby making it difficult to study transcriptional regulation of microRNAs. However, promoter regions for 175 microRNAs were identified by combining nucleosome mapping and chromatin signatures, and the DNA sequence of the linker regions was used to predict transcription factors regulating microRNA (Ozsolak et al., 2008). In addition, candidate transcriptional start sites of microRNAs were identified by analyzing H3-K4 trimethylation in multiple tissues at high resolution (Marson et al., 2008).

We recently described an inducible model of cellular transformation in order to identify transcriptional regulatory circuits important in oncogenesis (Iliopoulos et al., 2009). This model involves a nontransformed mammary epithelial cell line (MCF-10A) containing ER-Src, a derivative of the Src kinase oncoprotein (v-Src) that is fused to the ligand-binding domain of the estrogen receptor. Treatment of such cells with tamoxifen rapidly induces Src, and morphological transformation is observed within 36 hr. Unlike the parental cell line, the transformed cells form foci and colonies in soft agar, show increased motility and invasion, form mammospheres, and confer tumor formation in mouse xenografts. This model permits the opportunity to kinetically follow the pathway of cellular transformation in a manner similar to that used to study viral infection and other temporally ordered processes.

In this inducible transformation model, transient activation of Src triggers an inflammatory response that results in an
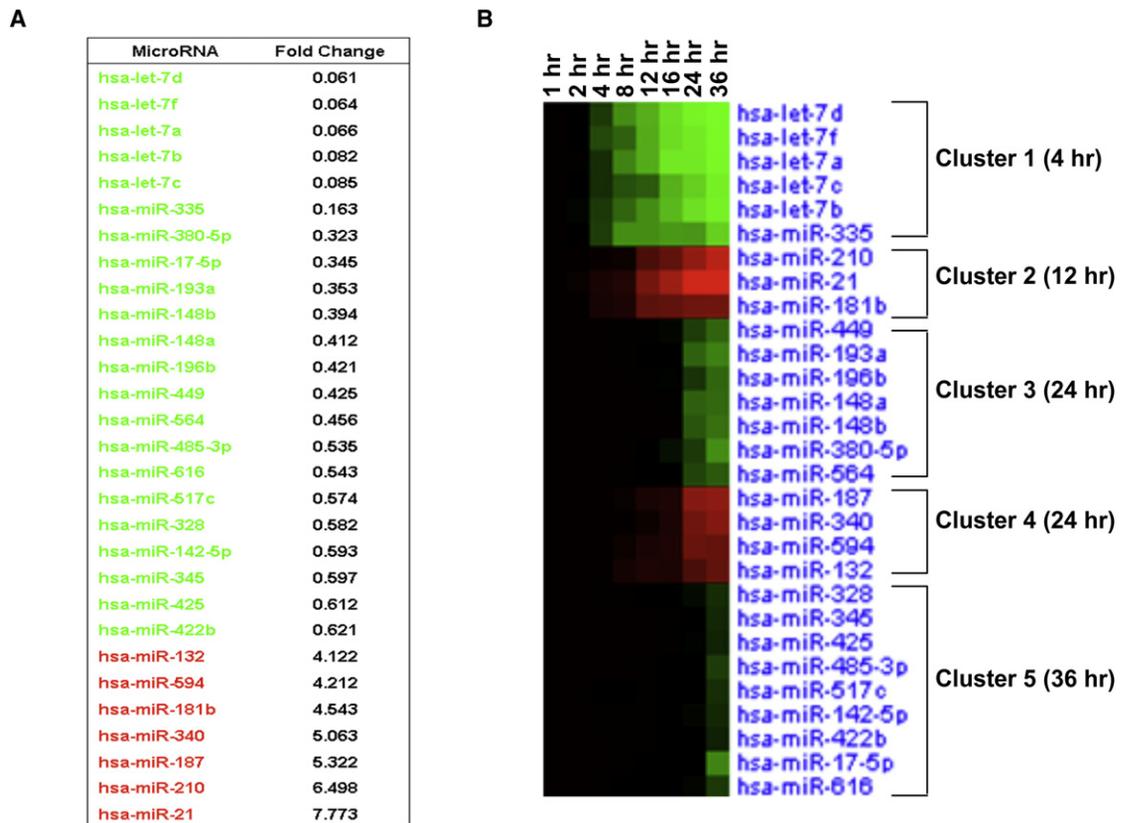
Figure 1. MicroRNA Dynamics during ER-Src Cellular Transformation
(A) Differentially expressed microRNAs between transformed (TAM 36 hr) and untransformed (0 hr) MCF10A ER-Src cells.
(B) Heatmap representation of differentially expressed microRNAs in different time points (1, 2, 4, 8, 12, 16, 24, and 36 hr) during ER-Src transformation. Upregulated microRNAs are shown in red, while downregulated microRNAs are shown in green.

epigenetic switch between nontransformed and transformed cells. The epigenetic switch is mediated by a positive feedback loop involving NF-κB, Lin28b, let-7 microRNA, and IL-6 (Iliopoulos et al., 2009). This regulatory circuit operates in other cancer cell lines, and its transcriptional signature is found in patient cancer tissues, indicating its importance in some human cancers. In addition to its role in the positive feedback loop, IL-6 activates STAT3, a transcription factor that is critical for transformation. This observation is consistent with the conventional view that STAT3 is a downstream effector of IL-6 (Frank, 2007; Yu et al., 2007, 2009), but not part of the central regulatory circuit that mediates the epigenetic switch.

Here, we use this inducible ER-Src model to study the regulation of microRNA expression throughout the process of cellular transformation. We identify differentially regulated microRNAs and show that many of them are important for transformation. Using computational algorithms, we identify individual and combinations of DNA sequence motifs that are overrepresented in the promoter regions of these differentially regulated microRNAs. We predict and experimentally validate transcription factor binding sites and hence transcription factor-microRNA interactions that are likely to be involved in the oncogenic process.

In particular, we demonstrate that STAT3 directly activates transcription of miR-21 and miR-181b-1 microRNAs during the transformation process. Remarkably, transient expression of either miR-21 or miR-181b-1 is sufficient to induce a stable transformed state, and this occurs by direct targeting of the phosphatase and tensin homolog (PTEN) and cylindromatosis (CYLD) tumor suppressor genes, respectively. The resulting inhibition of PTEN and CYLD expression leads to NF-κB activation, which is required to maintain the transformed state. Thus, STAT3 is not simply a downstream effector of IL-6 but, together with miR-21, miR-181b-1, PTEN, and CYLD, is part of the epigenetic switch that links inflammation to cancer.

RESULTS

Kinetic Analysis of Differentially Regulated MicroRNAs during the Process of Cellular Transformation
To identify differentially expressed microRNAs during transformation, we first measured the expression of 365 microRNAs in nontransformed and transformed (36 hr treatment with tamoxifen) MCF-10A-ER-Src cells by TaqMan microRNA analysis (Figure 1A). Twenty-nine differentially expressed microRNAs were identified, 22 of which are downregulated and 7 of which

are upregulated during transformation. Highly downregulated microRNAs include multiple members of the let-7 family (let-7d, let-7f, let-7a, let-7b, let-7c) and miR-335, while miR-21, miR-210, and miR-187 are highly upregulated.

To follow kinetically microRNA expression levels during cellular transformation, microRNA profiling was performed in cells isolated at various times (1, 2, 4, 8, 12, 16, 24 and 36 hr) after tamoxifen treatment. This analysis reveals five distinct microRNA clusters (Figures 1B and S1). The first cluster includes five microRNAs (let-7d, let-7f, let-7a, let-7c, let-7b, miR-335) downregulated very early (4 hr) during transformation. The second cluster consists of three early (12 hr) upregulated microRNAs (miR-210, miR-21, miR-181b-1; interestingly, miR-181a and miR-181b-2 are not differentially regulated). The third and fourth clusters consist of microRNAs downregulated and upregulated, respectively, 24 hr after tamoxifen treatment. The last cluster consists of nine downregulated microRNAs 36 hr after treatment. Overall, this kinetic analysis reveals two clusters of early microRNA responders and three clusters of late microRNA responders during transformation. Interestingly, microRNAs that show the earliest responses generally show the highest degree of differential regulation at later times.

Differentially Regulated MicroRNAs Are Important for Transformation, Tumorigenicity, and Chemotactic Cell Invasion

To assess the functional role of these differentially regulated microRNAs in transformation, we tested whether inhibition of upregulated microRNAs or downregulation of upregulated microRNAs blocks the transformation process. Nontransformed cells were transfected with antisense microRNAs for the seven upregulated microRNAs and with microRNA precursors for the 22 downregulated microRNAs. Tamoxifen was added 24 hr after transfection, and transformation efficiency was assayed by morphological changes 36 hr later (60 hr from the beginning of the experiment) (Figure 2A). Six out of seven antisense microRNAs (as-miR-21, as-miR-181b-1, as-miR-210, as-miR-132, as-miR-340, as-miR-187) and 10 out of 22 microRNA precursors (let-7a, let-7f, let-7b, let-7d, let-7c, miR-335, miR-148a, miR-148b, miR-449, miR-193a) show >50% inhibition of transformation efficiency (Figure 2B). Interestingly, microRNAs differentially expressed very early during transformation have the most significant effects on transformation, whereas late-responding microRNAs have little effect.

To extend these results, we used the same experimental procedure to test the 16 microRNAs that affected morphological transformation for effects on tumorigenicity and metastatic potential. All 16 microRNAs affected tumorigenicity, as assayed by the ability to form colonies in soft agar (Figure 2C). To analyze metastatic potential, we measured the infiltration of these cells through Matrigel in a modified Boyden chamber assay using 5% horse serum (HS) as a chemoattractant (Figure 2D). Under these conditions, 13 out of 16 tested microRNAs inhibit >50% of the invasive ability of transformed cells. Taken together, our results identify at least 13 microRNAs that are differentially regulated during the process of cellular transformation and are important for morphological alterations, tumorigenicity, and metastatic potential.

Enrichment of Transcription Factor Binding Sites in MicroRNA Promoters

To identify putative transcription factor binding sites associated with the 13 microRNAs described above, we used the Lever algorithm (Warner et al., 2008) to scan the flanking sequences (2 kb up- and 7 kb downstream) from the microRNA transcription start sites for overrepresented DNA sequence motifs. As shown in Table S1, this analysis was performed on 466 human transcription factor binding site motifs from the TRANSFAC database and on motifs derived by universal protein binding microarrays for 272 mouse transcription factors (Berger et al., 2008; Badis et al., 2009). DNA sequence motifs for STAT3 and Myc are among the top ranked motifs enriched in these microRNA promoter regions (Figure 3A), and both STAT3 and Myc are upregulated during cellular transformation (Figure 3B).

Putative STAT3 and Myc binding sites were further ranked according to their PhylCRM score (Warner et al., 2008) that combines binding affinity and evolutionary conservation across all sequenced mammalian species. We identified putative STAT3 binding sites in the promoters of miR-148a, miR-21, miR-132, miR-181b-1, miR-148b, miR-193a, miR-340, miR-335, miR-210, and miR-187 (ordered based on the maximum PhylCRM window score) (Tables S2 and S3). We also identified putative Myc binding sites in miR-181b-1, miR-148a, miR-335, and let-7a3 promoter regions (Tables S4 and S5), although PhylCRM scores for the Myc sites are generally lower than those for the STAT3 sites. As assayed by chromatin immunoprecipitation (ChIP), 9 out of 18 putative STAT3 sites and 3 out of 5 putative Myc sites with PhylCRM scores >1 are bound by their cognate proteins in vivo (Tables S3 and S5).

We extended our computational analysis by searching for overrepresented pairwise combinations of motifs within the microRNA promoter regions. We identified 267 protein binding microarray (PBM) motif pairs and 620 TRANSFAC motif pairs with z scores higher than 2.3 and AUC >0.7 (Table S1). A subset of these pairs contains a motif for one transcription factor differentially regulated during transformation (Figure S2), and there are 30 pairs (including STAT3 and Myc) in which both factors are differentially expressed (Figure 3B). Interestingly, one of the top-scoring motif pairs is for Max and RXRα, and this motif pair is predicted in the promoter regions of miR-148a, miR-132, miR-181b-1, miR-210, miR-193a, miR-148b, miR-335, and miR-340 (Tables S6 and S7). ChIP experiments validate the binding of both Max and RXRα to the miR-148a, miR-181b-1, and miR-148b promoter regions (Figure S2).

STAT3 and Myc Are Important for Maintenance of the Transformed State

During the transformation process, STAT3 expression is strongly induced during transformation while Myc expression is induced to a lesser extent (Figure 3C). In previous work, we showed that STAT3 is important for cellular transformation in the ER-Src model (Iliopoulos et al., 2009). To address the role of STAT3 and Myc, we inhibited STAT3 and Myc expression by 24 hr pretreatment with siRNA (Figure S2), added tamoxifen for 36 hr to induce transformation, and then plated the resulting cells in soft agar to evaluate colony formation
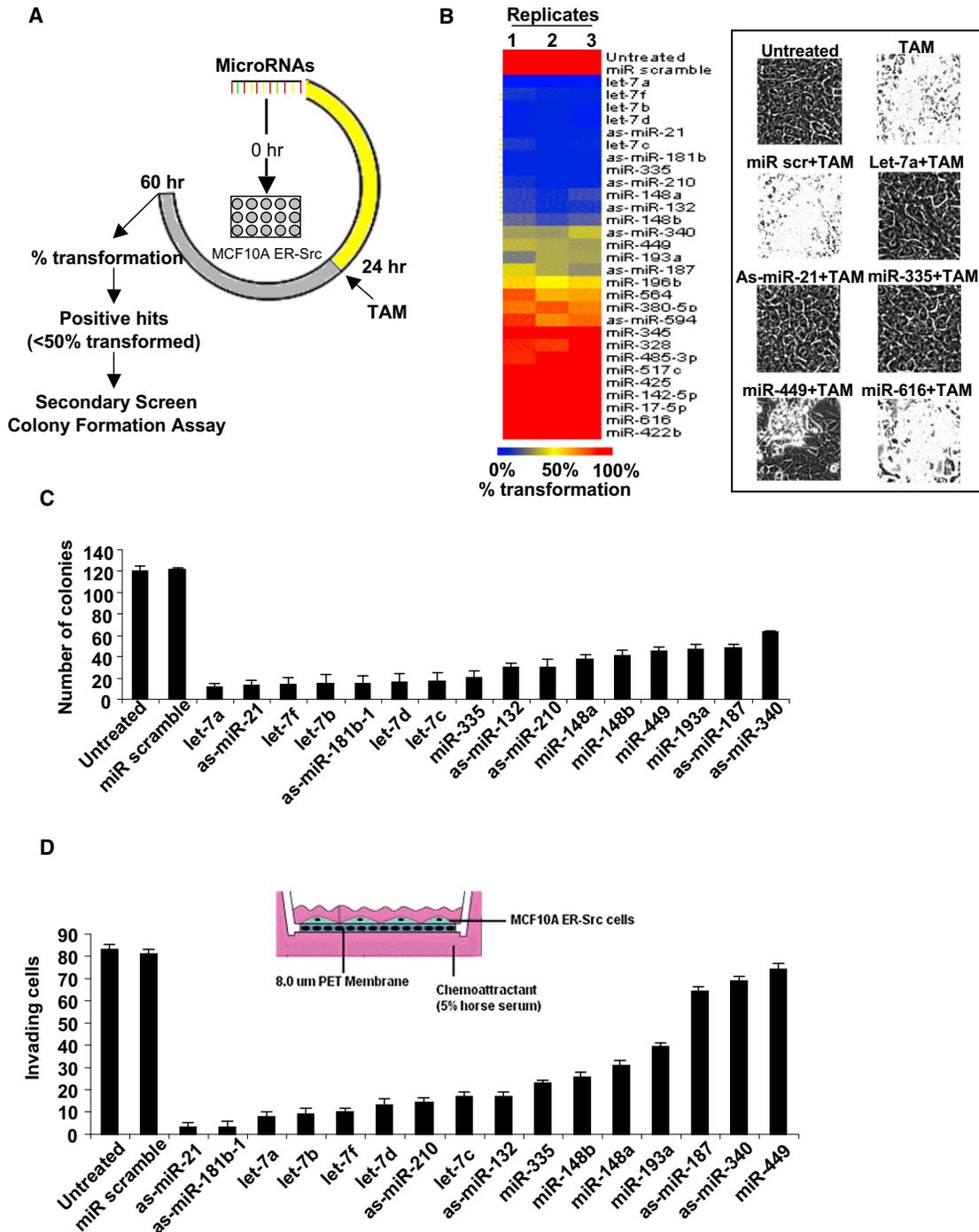
Figure 2. MicroRNAs Important for ER-Src Transformation

(A) Strategy for identifying microRNAs regulating ER-Src transformation.

(B) Heatmap representation of transformation efficiency (percent transformed cells as assayed by cell morphology) after transfection of microRNAs or antisense microRNAs in ER-Src cells.

(C and D) Soft agar colony assay (C) and invasion assay (D) in ER-Src cells transfected with sense or antisense microRNAs. Experiments performed in triplicate and data are shown as mean ±SD.

15 days later. Inhibition of Myc results in a modest decrease in colony formation, whereas inhibition of STAT3 in parallel experiments confers a 10-fold decrease (Figure 3D). Similar results were obtained when siRNAs against STAT3 and Myc were added 10 days after cells were stably transformed, and the resulting cells tested for colony formation (Figure 3E). Thus,
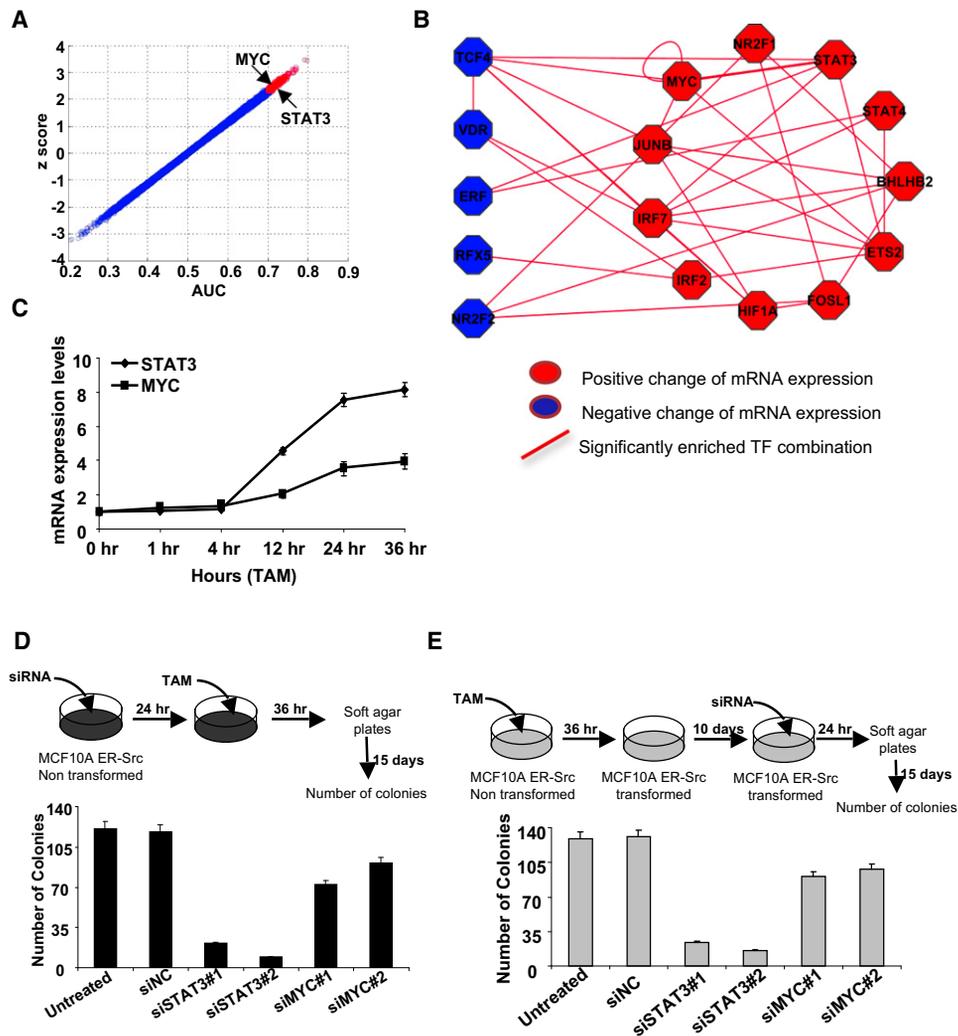
Figure 3. STAT3 and Myc Are Important Regulators of Transformation

(A) Lever algorithm analysis to identify transcription factor binding site motifs overrepresented in promoter areas of differentially expressed microRNAs. Motifs with AUC score higher than 0.7 and z-score higher than 2 are considered statistically significant (red color), with STAT3 and Myc indicated.

(B) Pairs of motifs (connected by lines) of TFs that are differentially regulated (upregulated in red and downregulated in blue) during transformation and overrepresented in microRNA promoters.

(C) STAT3 and Myc mRNA expression levels in ER-Src cells during transformation.

(D) Soft agar colony assay (mean ±SD) in TAM-treated (36 hr) cells transfected with siRNAs against STAT3, Myc, or control.

(E) Soft agar colony assay (mean ±SD) in transformed ER-Src cells (for 10 days) transfected with siRNAs against STAT3, Myc, or control.

STAT3 and (to a significantly lesser extent) Myc are important for cellular transformation and maintenance of the transformed phenotype.

STAT3- and Myc-Regulated MicroRNAs during Transformation

In transformed cells, STAT3 binds to three sites in the miR-21 promoter, three sites in the miR-181b-1 promoter, two STAT3 sites in the miR-132 promoter, and one STAT3 site in the miR-210 promoter during transformation (Figure 4A and Table S2). STAT3 binding to the target sites in the miR-21 promoter upon IL-6 induction has been reported previously

(Löffler et al., 2007). STAT3 binding to these sites is induced upon transformation, because it is not detected in nontransformed cells. Inhibition of STAT3 by siRNA or by a pharmacological inhibitor (JSI-124) strongly reduces miR-21 and miR-181b-1 expression levels, but has only a very modest effect on miR-132 and miR-210 expression levels (Figure 4B). Conversely, STAT3 activation by IL-6 treatment in MCF-10A cells (i.e., lacking the ER-Src construct) results in upregulation of these microRNAs (Figures 4C and S3). Interestingly, inhibition of miR-21 and miR-181b-1 had a major effect while miR-132 and miR-210 had a minor effect on colony formation ability of the ER-Src cells (Figure 4D). These results strongly suggest
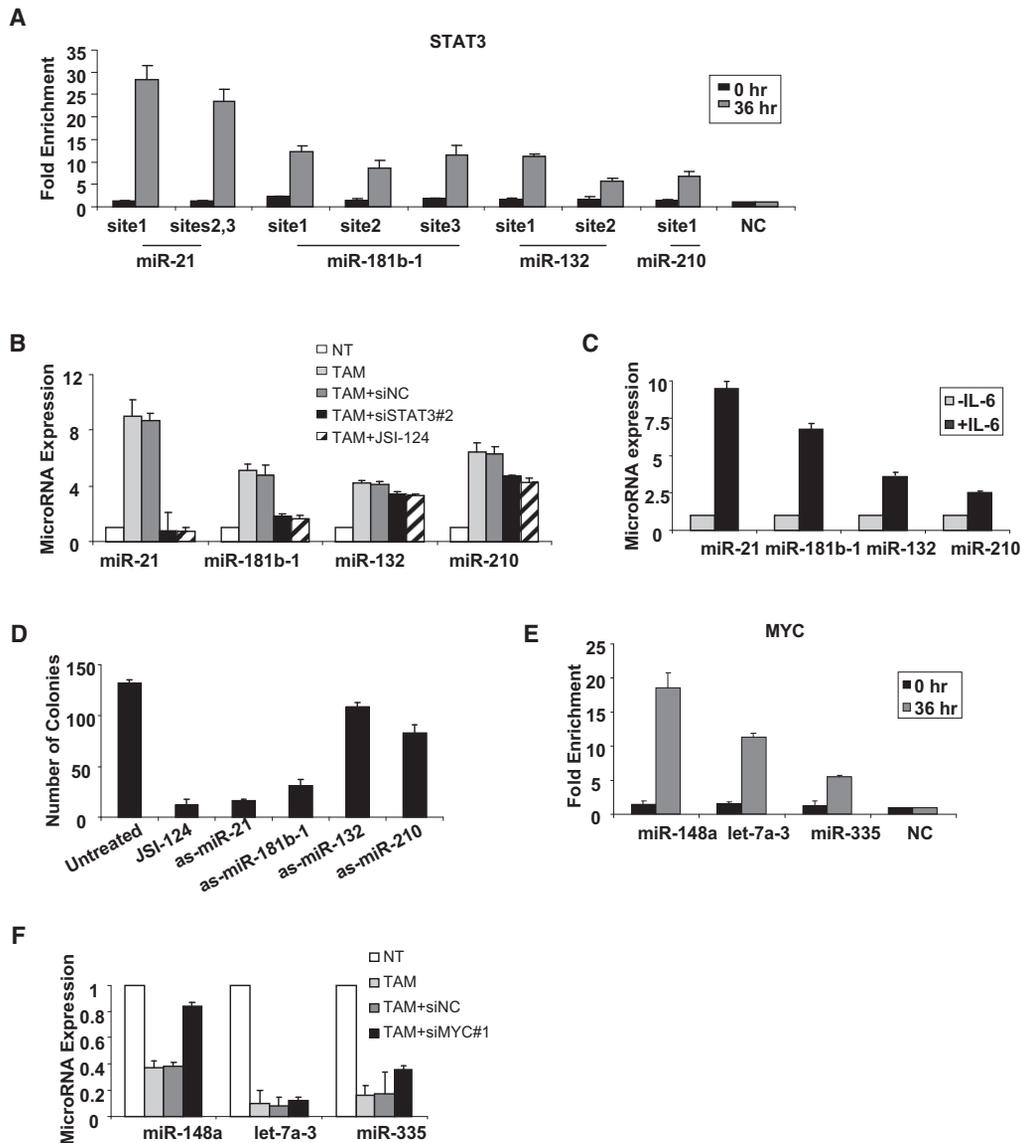
Figure 4. STAT3 and Myc-Regulated MicroRNAs during ER-Src Transformation
(A) STAT3 occupancy (fold enrichment) at the indicated miR loci in cells that were or were not treated with TAM, as determined by ChIP.
(B) MicroRNA expression levels in TAM-treated ER-Src cells transfected in the presence of STAT3 inhibitors (siSTAT3#2 or 8 μM JSI-124).
(C) MicroRNA expression levels in MCF-10A cells treated with 50 ng/ml IL-6.
(D) Soft agar colony assay in MCF10A-IL-6 transformed cells transfected with the indicated antisense microRNAs.
(E) Myc occupancy (fold enrichment) at the indicated miR loci that were or were not treated with TAM.
(F) MicroRNA expression levels in TAM-treated ER-Src cells transfected with siRNA negative control or siMyc. In all experiments, data are presented as mean ±SD of three independent experiments.

that STAT3 function in transformation involves transcriptional activation of miR-21 and miR-181b-1.

ChIP revealed one Myc binding site in the let-7a3 promoter, one site in the miR-335 promoter, and one site in the miR-148a promoter (Figure 4E). Inhibition of Myc expression by siRNA did not significantly affect let-7a-3 and miR-335 expression, but it did result in increased levels of miR-148a (Figure 4F). These data suggest that Myc directly inhibits miR-148a expression levels during transformation.

Transient Expression of Either miR-21 or miR-181b-1 Is Sufficient to Induce the Epigenetic Switch to a Stable Transformed State

STAT3 is phosphorylated in response to IL-6 during cellular transformation (Frank, 2007), and in our experimental system, this occurs only at later time points after IL-6 superactivation (Iliopoulos et al., 2009). Based on these observations, we proposed that STAT3 is a downstream effector of IL-6 required for transformation, but not part of the epigenetic switch that is
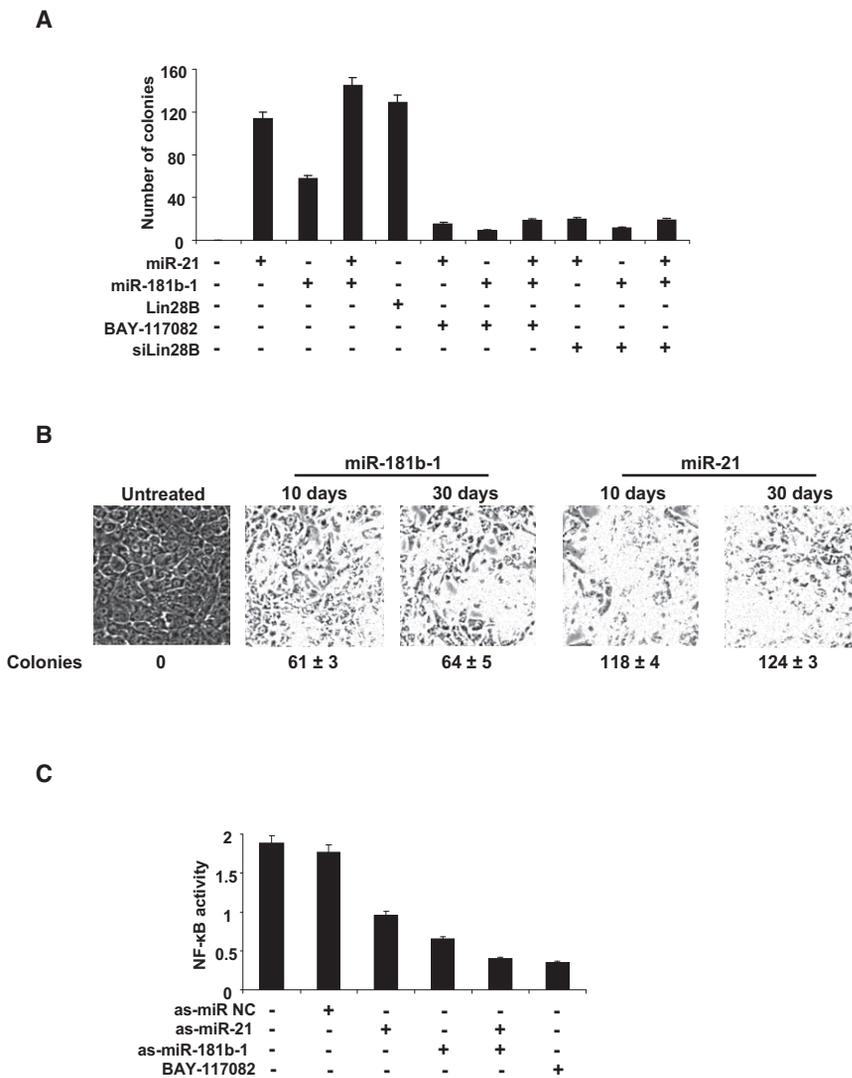
A

B

C

suggestion, treatment with BAY-117082 (NF-κB inhibitor) or siRNA against Lin28b reduces the tumorigenicity of cells transformed by miR-21 and/or miR-181b-1. Furthermore, ER-Src-transformed cells treated with antisense RNAs against miR-21 and/or miR-181b-1 show reduced levels of NF-κB activation (Figure 5C) that are roughly comparable to those obtained upon inhibition by BAY-117082. The ability of miR-21 and miR-181b-1 to induce a stable transformed state in a manner dependent on NF-κB and Lin28b indicates that these microRNAs and STAT3 are part of the positive feedback loop mediating the epigenetic switch and are not simply downstream effectors of IL-6.

miR-21 Targets the PTEN Tumor Suppressor Gene that Functions through the Akt Pathway

The ability of miR-21 or miR-181b-1 to induce a stable transformed state indicates that these microRNAs must, in some manner, activate NF-κB to sustain the inflammatory positive feedback loop. In accord with a previous study identifying the PTEN tumor suppressor gene as a direct target of miR-21 (Meng et al., 2007), expression levels of miR-21 and PTEN mRNA expression levels are inversely correlated during ER-Src transformation (Figure S4A). In addition, inhibition of miR-21 expression results in upregulation of PTEN mRNA expression (Figure S4B), suggesting that PTEN expression is regulated by miR-21 in ER-Src cells. PTEN is an upstream regulator of Akt kinase pathway that is involved in oncogenesis (Yuan and Cantley, 2008). In this regard, Akt activity (assayed by phosphorylation) increases during ER-Src transformation in a manner that depends upon miR-21 expression (Figure S4C), and inhibition of Akt expression reduces tumorigenicity of ER-Src-transformed cells (Figure S4D). Lastly, the PTEN/Akt pathway regulates NF-κB activity in cancer cells, PTEN deletion results in increased NF-κB activity (García et al., 2009; Guigon et al., 2009), and inhibition of Akt expression reduces NF-κB activity and IL-6

needed to convert a nontransformed cell into a stable transformed line (Iliopoulos et al., 2009). If STAT3 and its target microRNAs are simply downstream effectors of IL-6, they should be insufficient for cellular transformation, because they would not affect the function of NF-κB, Lin28, or let-7, all of which are required for the process.

In striking contrast to our previous model, individual expression of miR-21 or miR-181b-1 is sufficient for cellular transformation (Figure 5A). As assayed by colony formation, miR-21 is ∼2-fold more effective than miR-181b-1 and of comparable efficacy to that occurring upon overexpression of Lin28b. Remarkably, even though transiently transfected microRNAs are likely to be present only for a few days, the transformed cells can be passaged for at least 30 days without losing their ability to form colonies in soft agar, indicating that cells are stably transformed (Figure 5B).

These observations indicate that transient expression of either miR-21 or miR-181b-1 is sufficient to induce the epigenetic switch to the stable transformed state. In accord with this
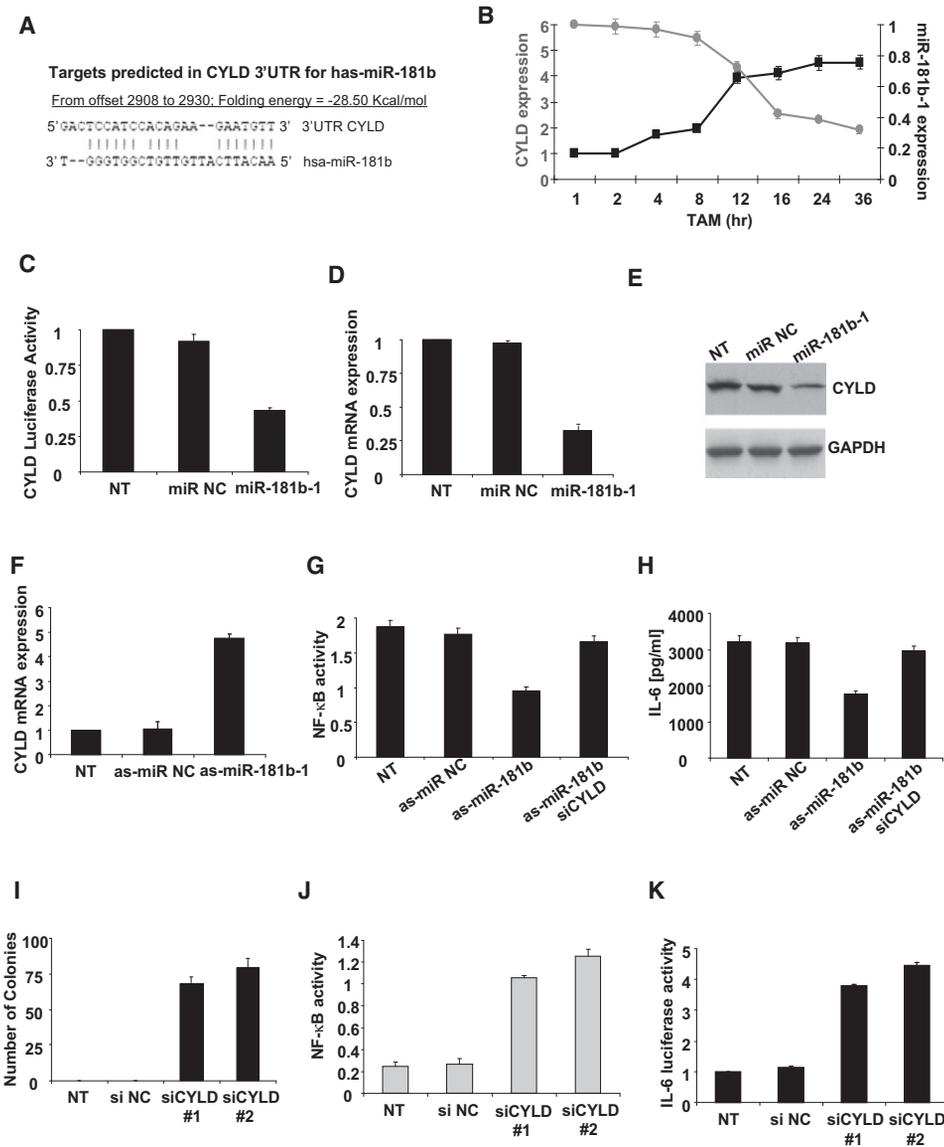
Figure 6. miR-181b-1 Regulates CYLD Expression during ER-Src Transformation

(A) Sequence complementarity between miR-181b-1 and the 3′ UTR of CYLD gene.

(B) miR-181b-1 and CYLD mRNA expression levels at the indicated times during ER-Src transformation.

(C) Luciferase activity of a reporter containing the 3′ UTR of CYLD 24 hr after transfection with miR-181b-1 or miR negative control.

(D) CYLD mRNA levels after treatment with miR-181b-1 or miR negative control.

(E) CYLD protein levels after treatment with miR-181b-1 or miR negative control.

(F) CYLD mRNA levels after treatment with as-miR-181b-1 or as-miR-NC.

(G) NF-κB activity (ELISA assay) in ER-Src cells untreated (NT) or treated with as-miR-181b-1, si-CYLD, or as-miR-NC.

(H) IL-6 production (ELISA assay) in ER-Src cells treated with as-miR-181b-1, si-CYLD, or as-miR-NC.

(I–K) Number of colonies (I), NF-κB activity assessed by ELISA assay (J), and IL-6 luciferase activity (K) in ER-Src cells treated with two different siRNAs against CYLD. The data are presented as mean ±SD of three independent experiments.

production (Figures S4E and S4F). Taken together, these results are consistent with a pathway in which STAT3-activated miR-21 directly inhibits PTEN, thereby resulting in increased Akt activity and subsequent activation of NF-κB that is required for the positive feedback loop that maintains the epigenetically stable transformed state.

miR-181b-1 Transforms Cells via Direct Targeting of the CYLD Tumor Suppressor Gene

Unlike the case for miR-21, direct targets and potential pathways for miR-181b-1 are unknown. Interestingly, bioinformatic analysis reveals sequence complementarity between miR-181b-1 and the CYLD tumor suppressor gene 3′ UTR (Figure 6A). During

ER-Src transformation, miR-181b-1 and CYLD expression levels are inversely correlated (Figure 6B), and miR-181b-1 overexpression inhibits activity of a luciferase reporter construct containing the CYLD 3′ UTR (Figure 6C), as well as CYLD mRNA (Figure 6D) and protein expression (Figure 6E) levels. Conversely, suppression of miR-181b-1 expression results in upregulation of CYLD expression (Figure 6F), suggesting that miR-181b-1 regulates directly CYLD expression during transformation.

CYLD is a deubiquitinating enzyme that negatively regulates NF-κB activity (Brummelkamp et al., 2003; Trompouki et al., 2003). In ER-Src cells, inhibition of miR-181b-1 expression results in reduced NF-κB activity (Figure 6G) and reduced production of IL-6, a direct NF-κB target gene (Figure 6H). Importantly, this effect is blocked by concurrent inhibition of CYLD, suggesting that miR-181b-1 regulates NF-κB through CYLD. In accord with the previously characterized tumor suppressor function of CYLD, MCF10A cells acquire the ability to form colonies in soft agar after siRNA inhibition of CYLD expression (Figure 6I). Furthermore, CYLD inhibition results in increased NF-κB (Figure 6J) and IL-6 luciferase activity (Figure 6K) and mRNA expression (Figure S5A). Lastly, CYLD suppression increases the motility of ER-Src cells in a manner that depends on NF-κB (Figure S5B). Taken together, these observations are consistent with a pathway in which miR-181b-1 directly targets CYLD, leading to increased NF-κB activity and maintenance of the inflammatory feedback loop necessary for the transformed states.

STAT3-Regulated MicroRNAs Are Important for Tumorigenicity of Cancer Cell Lines of Diverse Developmental Origin

The results above indicate that STAT3-induced microRNAs (miR-21 and miR-181b-1) are important for transformation and tumorigenicity of breast cells. To generalize these observations beyond breast cells, we asked whether miR-21 and miR-181b-1 are important for tumorigenicity of cancer cell lines of diverse developmental origin (Figure 7A). Inhibition of miR-21 dramatically reduces colony formation in soft agar of colon (HCT-116, HT-29) cancer cells and, to a lesser extent, prostate (PC3), lung (A549), and hepatocellular (Hep3B) cancer cells. Inhibition of miR-181b-1 yields similar results, although the effects are often less pronounced. Neither microRNA affects colony formation in cervical (HeLa) or pancreatic (Panc1) cells. Thus, miR-21 and miR-181b-1 are important for tumorigenicity of many, but not all, developmentally unrelated cancer cell lines.

STAT3 and STAT3-Regulated MicroRNAs Are Important for Tumor Growth in Mouse Xenografts

To examine the ability of miR-21 and miR-181b-1 to regulate tumor growth in mouse xenografts, ER-Src-transformed cells were injected subcutaneously in nu/nu mice to generate tumors with a size of 60 mm³. The mice were randomly separated into nine groups and treated intraperitoneally (i.p.) with IL-6 antibodies, siRNA against STAT3, antisense RNAs against miR-21 and miR-181b-1, or appropriate negative controls for four cycles every 5 days (Figure 7B). Inhibition of miR-21 or (to a lesser extent) miR-181b-1 reduces tumor growth with respect to

control experiments, although tumors are not eliminated after 40 days. In contrast, the combined inhibition of miR21 and miR-181b-1 completely suppresses tumor growth, as is also observed upon inhibition of STAT3 or IL-6. As expected, tumors treated with IL-6 antibody or siRNA against STAT3 had much reduced levels of miR-21 and miR-181b-1 microRNAs (Figure 7C). These results indicate that STAT3 activation of both miR-21 and miR-181b-1 is important for tumor growth in mouse xenografts, with miR-21 having a more pronounced effect.

Given the importance of miR-21 and miR-181b-1 microRNAs for tumorigenicity in colon cancer cells in vitro (Figure 7A), we performed similar experiments in which HCT-116 and HT-29 colon cancer cells were injected subcutaneously into nu/nu mice. After the completion of the second cycle of treatments in HCT-116 cells and the third cycle of treatments in HT-29 cells, there was significant suppression of tumor growth in all treated mice (Figure 7D). As observed with transformed ER-Src cells, reduced growth of tumors generated by both colon cancer cell lines was more pronounced upon miR-21 inhibition than miR-181b-1 inhibition, and complete suppression of tumor growth occurred with simultaneous inhibition of both microRNAs or inhibition of STAT3.

STAT3, miR-21-PTEN, and miR-181b-1-CYLD RNA Levels in Human Cancer Tissues

To address whether the above observations in cancer cell lines are relevant to human cancer, we examined the relationship between STAT3 and miR-21 or miR-181b-1 expression levels in colon adenocarcinomas. There is a striking positive correlation between STAT3 and miR-21 expression levels (r = 0.9012) and STAT3 and miR-181b-1 expression levels (r = 0.768) in the same cancers (Figure 7E). These data suggest a mechanistic relationship between STAT3, miR-21, and miR-181b-1 expression levels in at least one type of human cancer. In addition, we found an inverse correlation between miR-21 and PTEN expression levels (r = −0.678) and miR-181b-1 and CYLD expression levels (r = −0.7) in colon adenocarcinomas (Figure 7F), strongly suggesting that both the miR-21/PTEN and miR-181b-1/CYLD pathways are relevant for human cancer.

DISCUSSION

Kinetic Profiling Identifies MicroRNAs that Are Important at Distinct Stages of Cellular Transformation and Are Linked to Human Cancer

Using an inducible model of cellular transformation involving breast epithelial cells, we perform a kinetic profile of microRNA expression and identify 29 microRNAs that are differentially regulated during this process (22 upregulated and 7 downregulated). In general, microRNAs whose expression is affected early in the transformation process are also more highly regulated (fold induction or fold repression) at later times. This progressive enhancement of differential microRNA expression suggests that the transition between nontransformed and transformed cells occurs in a continuous fashion rather than in a set of distinct stages. In a functional sense, these early- and highly regulated microRNAs are more important for cellular transformation than late- and weakly regulated microRNAs. Of particular
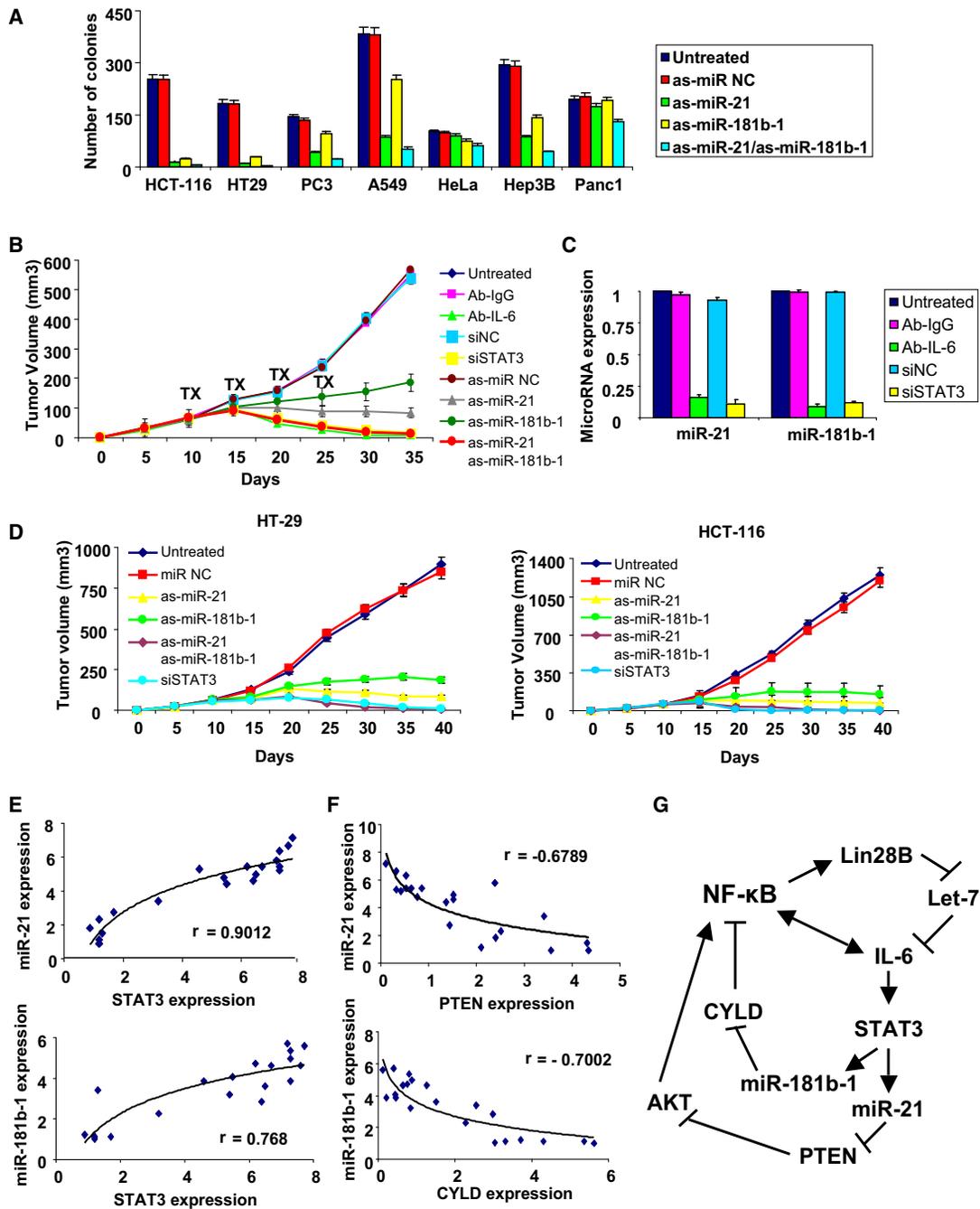
Figure 7. STAT3-Regulated MicroRNAs in Cancer Cells, Xenografts, and Cancer Patients

(A) Colony formation assay in colon (HCT-116, HT-29), prostate (PC3), lung (A549), cervical (HeLa), hepatocellular (Hep3B), and pancreatic (Panc1) cancer cell lines treated with antisense microRNA negative control, antisense-miR-21, antisense-miR-181b-1, or their combination. The data are presented as mean ±SD of three independent experiments.

(B) Tumor growth (mean ±SD) of ER-Src cells after i.p. treatment (days 10, 15, 20, and 25) with Ab-IgG, Ab-IL-6, siRNA negative control, siRNA against STAT3, antisense miR-NC, antisense-miR-21, and/or antisense-miR-181b-1.

(C) miR-21 and miR-181b-1 expression levels from tumors derived from the experiment described above.

(D) Tumor growth (mean ±SD) of HCT-116 and HT-29 colon cancer cells after i.p. treatment (days 10, 15, 20, and 25) with microRNA negative control, antisense-miR-21, antisense-miR-181b-1, or siRNA against STAT3.

(E) miR-21, miR-181b-1, and STAT3 mRNA expression levels in colon adenocarcinomas, with each data point representing an individual sample and correlation coefficients (r) indicated.

(F) miR-21, miR-181b-1, PTEN, and CYLD mRNA expression levels in colon adenocarcinomas.

(G) Model of the inflammatory positive feedback loop that mediates the epigenetic switch between nontransformed and transformed cells.

interest are microRNAs that are rapidly and dramatically lost (let-7 family and miR-335) or induced (miR-21, miR-181b-1, and miR-210) during cellular transformation.

MicroRNAs that are rapidly and dramatically regulated during the process of cellular transformation in our ER-Src model have been previously linked to cancer. Let-7 family members are downregulated in multiple types of cancer, and they directly target oncogenes such as HMGA2, KRAS, and IL-6 (Johnson et al., 2005; Mayr et al., 2007; Iliopoulos et al., 2009). In our model, the let-7 family is a key component of the epigenetic switch that mediates transformation, and let-7 downregulation is due to the rapid induction of the Lin28 processing factor, which is a direct target of NF-κB (Iliopoulos et al., 2009). Similar to the let-7 family, miR-335 is rapidly and dramatically downregulated during transformation, although it does not appear that Lin28 is involved. This tumor-suppressor role of miR-335 during the early stages of tumorigenesis is unexpected, as this microRNA was previously characterized as being downregulated in metastatic tumors (Tavazoie et al., 2008).

Important microRNAs induced early during the transformation process in ER-Src cells (miR-21, miR-181b-1, and mir-210) are also associated with human cancer. miR-21 has been characterized as an antiapoptotic microRNA that directly regulates PDCD4 and PTEN expression and is upregulated in many cancer types (Meng et al., 2007; Frankel et al., 2008; Yan et al., 2008). Overexpression of miR-21 and miR-181b-1 is associated with the progression of leukoplakia to oral carcinoma (Cervigne et al., 2009) and prognosis and therapeutic outcome in colon cancer (Schetter et al., 2008), although downregulation of miR-181b-1 occurs in other types of cancer (Shi et al., 2008; Conti et al., 2009). miR-210, a key player in the response to hypoxia, directly inhibits the Myc antagonist MNT, and it is upregulated in a variety of solid tumors, including breast cancer (Zhang et al., 2009). The observation that microRNAs critical for transformation in ER-Src cells are relevant for cancer provides additional validation for our experimental model, and it permits mechanistic analysis of these microRNAs in a well-defined system.

Identification of Transcription Factor Binding Sites Associated with Differentially Regulated MicroRNAs during Transformation

MicroRNAs and transcription factors play critical regulatory roles in oncogenesis, but there are few examples of transcription factors directly regulating the expression of microRNAs that are relevant for this process. As a first step to identify such transcription factor-microRNA interactions on a more global basis, we performed a computational analysis to identify transcription factor binding site motifs that are both overrepresented and evolutionarily conserved in the putative promoter regions of differentially regulated microRNAs. Many individual motifs and pairwise combinations of motifs meet these criteria at varying levels of stringency, and binding of the cognate transcription factor to some of these sites in vivo was validated by ChIP. Furthermore, in some cases, the transcription factors associated with the microRNA promoters are important for regulating the expression of the microRNAs.

The above analysis identifies many potential transcription factors, individually or in combination, that may be important

for cellular transformation as well as potential regulatory sites through which they act. While our identification of Myc and STAT3 was not surprising given their well-known roles in transformation, the combination of Max and RXRα was unexpected. However, as the computational analysis only identifies candidate protein-DNA interactions, it is critical to establish their physiological relevance through ChIP and transcription measurements. In addition, as many motifs are recognized by a family of transcription factors, experimental validation is necessary to determine which family member(s) are physiologically relevant. Lastly, it is likely that transcription factors identified here via regulation of microRNAs will also be important for regulation of mRNAs during transformation.

STAT3 Activation of miR-21 and miR-181b-1 Is Important for Cellular Transformation and Associated with Some Human Cancers

While transcription factors and microRNAs are associated with human cancer, regulatory pathways directly connecting them are largely unknown. Previously, we described a regulatory circuit in which the transcription factor NF-κB is required for inhibition of the let-7 microRNA family, but this regulation is indirect and involves Lin28 as an intermediary protein (Iliopoulos et al., 2009). In addition, we and others have identified STAT3 as an oncogenic transcription factor activated by inflammatory responses (Iliopoulos et al., 2009; Bromberg et al., 1999; Grivennikov et al., 2009), but microRNA targets are unknown.

Here, we provide strong evidence that STAT3 activation of miR-21 and miR-181b-1 is important for cellular transformation. First, in a transformation-specific manner, STAT3 directly binds multiple sites in the miR-21 and miR-181b-1 promoter regions and is required for transcriptional induction of these microRNAs. Second, STAT3, miR-21, and miR-181b-1 are required for cellular transformation, tumorigenicity, and maintenance of the transformed state in vitro as well as tumor growth in vivo. Third, overexpression of either miR-21 or miR-181b-1 is sufficient to transform cells, indicating that these microRNAs can act as oncogenes. While overexpression of some microRNAs is correlated with transformed or tumor cells, to our knowledge, these are the first examples in which transient expression of oncogenic microRNAs can convert nontransformed cells to transformed cells. Taken together, our observations strongly suggest that STAT3 oncogenic function depends on miR-21 and miR-181b-1.

The importance of STAT3 activation of miR-21 and miR-181b-1 for cellular transformation is not limited to our experimental model or to breast cells. Indeed, miR-21 and (to a lesser extent) miR-181b-1 are important for tumorigenicity (colony formation in soft agar) of a variety of cell lines of diverse developmental origin. In addition, these microRNAs are important for growth of tumors in xenografts generated by colon cancer cells. However, this pathway does not appear to be involved in all types of cancer cells, as cervical and pancreatic cancer cell lines are unaffected by depletion of either microRNAs.

In accordance with previous work, RNA levels of STAT3 (Iliopoulos et al., 2009; Bromberg et al., 1999; Grivennikov et al., 2009), miR-21, and miR-181b-1 (Schetter et al., 2008; Yan et al., 2008; Cervigne et al., 2009) are often increased in cancer tissues relative to normal tissues. More importantly, we

observe a striking direct relationship between STAT3, miR-21, and miR-181b-1 levels in a set of colon adenocarcinomas, strongly arguing for a mechanistic relationship. Furthermore, in this same set of tumor samples, there is a striking inverse relationship between miR-21 and PTEN as well as between miR-181b-1 and CYLD. These observations indicate that the pathway in which STAT3 directly activates miR-21 and miR-181b-1, which in turn inhibit PTEN and CYLD, is relevant for at least some forms of human cancer.

STAT3 Activation of miR-21 and miR-181b-1, via PTEN and CYLD, Is Part of the Epigenetic Switch from Nontransformed to Transformed Cells

In the ER-Src transformation model, a transient inflammatory signal (mediated by Src) activates a positive feedback loop involving NF-κB, Lin28, let-7 microRNAs, and IL-6 that is responsible for an epigenetic switch between stable nontransformed and transformed states (Iliopoulos et al., 2009). The epigenetic switch and hence transformation can be started by the appropriate manipulation of any component within the loop. In addition, IL-6 is required for activating the STAT3 pathway at later times during transformation, and we imagined that this function and the STAT3 pathway itself was required for transformation, but not part of the positive feedback loop necessary for the epigenetic switch (Iliopoulos et al., 2009).

In contrast to this view, our observation that overexpression of miR-21 or miR-181b-1 is sufficient for transformation strongly argues that STAT3 and these microRNAs are not simply downstream effectors, but are actually part of the regulatory circuit that constitutes the epigenetic switch (Figure 7G). To put it differently, miR-21 and miR-181b-1 are not merely upregulated in association with oncogenesis, but rather can act as oncogenic microRNAs when transiently overexpressed. Importantly, transformation by these transiently transfected microRNAs requires NF-κB and Lin28, and the transformed state is sufficiently stable for formation of colonies in soft agar and mammospheres, even though the introduced microRNAs are unlikely to be present. To achieve such a stable transformed state, STAT3 and its microRNA targets must feedback to induce NF-κB and start the inflammatory feedback loop. At a minimum, miR-21 directly targets PTEN, which acts through the Akt pathway, and miR-181b-1 targets CYLD, and each of these microRNA pathways is sufficient to induce NF-κB. Thus, STAT3 activation of miR-21 and miR-181b-1 induces two positive feedback loops that are part of the larger inflammatory feedback loop that constitutes the epigenetic change from nontransformed to transformed cells.

More generally, our results suggest that the link of inflammation to cancer is not a simple linear pathway, but rather a complex, self-reinforcing regulatory circuit. In this sense, the distinction between nontransformed and transformed cells is analogous to a switch in normal development in which a given stable state is converted to a different stable state. As discussed previously (Iliopoulos et al., 2009), activation of this inflammatory regulatory circuit is insufficient to trigger transformation of normal cells. Instead, this inflammatory circuit is likely to be relevant in "predisposed" cells that are genetically altered to be at an intermediate stage in the transition between a primary cell and a cancer cell. In such predisposed cells, anything that activates the positive factors (NF-κB, Lin28, IL-6, STAT3, miR-21, mir-181b-1) or inhibits the negative factors (let-7, PTEN, CYLD) will trigger the inflammatory feedback loop that induces and maintains the transformed state. As such, the triggering event does not have to be an inflammatory signal (e.g., Src) per se, but can be any genetic or environmental change that affects the activity of the factors in the inflammatory feedback loop.

EXPERIMENTAL PROCEDURES

Cell Culture and Transformation Assays

MCF10A cells containing the ER-Src fusion protein were grown in DMEM/F12 medium supplemented with 5% donor HS, 20 ng/ml epidermal growth factor (EGF), 10 μg/ml insulin, 100 μg/ml hydrocortisone, 1 ng/ml cholera toxin, and 50 units/ml pen/strep, with the addition of puromycin (Iliopoulos et al., 2009; Hirsch et al., 2010). To induce transformation, the Src oncogene was activated by the addition of 1 μM tamoxifen (Sigma, St. Louis) to confluent cell cultures. Cancer cell lines (HCT116, HT29, PC3, A549, HeLa, Hep3B, Panc1) were grown in DMEM, 10% FBS, and pen/strep.

MicroRNA Expression Analysis

Expression levels of 365 microRNAs at various times after TAM addition were evaluated with microRNA profiling assays (TLDA human miRNA v1.0) in the Dana Farber Molecular Diagnostics Facility. Validation of these results was performed using the mirVana qRT-PCR miRNA Detection Kit and qRT-PCR Primer Sets, according to the manufacturer's instructions (Ambion Inc.; Austin, TX). RNU48 expression was used as an internal control.

Morphological, Soft Agar Colony and Invasion Assays for Cellular Transformation

ER-Src cells were treated with 100 nM of microRNAs or antisense microRNAs for 24 hr, whereupon TAM was added and cellular transformation was assessed 36 hr later (total time 60 hr). Cell morphology was assessed by phase contrast microscopy (10X objective), and percentage of transformed ER-Src cells was calculated by evaluation of cell morphology by Metamorph v5.0 software. The soft agar colony and MATRIGEL invasion assays were performed as described previously (Iliopoulos et al., 2009; Hirsch et al., 2010). In all cases, experiments were repeated thrice, and the statistical significance was calculated using Student's t test.

Lever and PhylCRM Analysis

The Lever and PhylCRM algorithms were described previously (Warner et al., 2008). MicroRNA promoters to be evaluated by Lever for each pair of transcription factor binding site motifs were rank-ordered according to their maximum PhylCRM window score. In PhylCRM analysis, we evaluated evolutionary conservation across 12 mammals—mouse, rat, human, rabbit, chimp, macaque, cow, dog, armadillo, tenrec, opossum, and elephant—using the MultiZ genome alignment as described in Supplemental Experimental Procedures. PBM data were for 104 TFs (transcription factors) from 22 DNA-binding domain structural classes (Badis et al., 2009) and for 168 additional homeodomains (Berger et al., 2008). Position weight matrix (PWM) representations of the PBM data derived using the "Seed-and-Wobble" algorithm (Berger et al., 2006) were used in the Lever analysis. The TRANSFAC PWMs were downloaded from the TRANSFAC human database. To annotate the TSS for the microRNAs, we incorporated the findings from Ozsolak et al. (2008) and Marson et al. (2008) and mapped the genome coordinates to the hg18 version of the human genome by using liftOver program from UCSC. We note that some of the identified promoters were more than 50 kb from the microRNA gene. For microRNAs whose predicted TSS locations differed substantially in the two studies, we used the ones identified by Ozsolak et al. (2008). For each microRNA gene, the Lever and PhylCRM analyses were applied to the sequence from 2 kb upstream to 7 kb downstream of the annotated TSS. See Supplemental Experimental Procedures for details.

ChIP

ChIP was carried out as described previously (Yang et al., 2006). Briefly, the chromatin fragments, derived from untreated and TAM-treated (36 hr) MCF10A ER-Src cells, were immunoprecipitated with 6 μg of antibody against STAT3 (9139, Cell Signaling; Beverly, MA), Myc (ab56, Abcam Inc.), Max (ab53570, Abcam Inc.), and RXRα (sc553, Santa Cruz Biotechnology Inc.; Santa Cruz, CA). DNA extraction was performed using QIAGEN Purification Kit. The samples were analyzed by quantitative PCR in real time using primers listed in Supplemental Experimental Procedures.

RNA Analysis

Equal amounts of purified RNA samples from untransformed and transformed MCF10A ER-Src cells were reverse transcribed to form cDNA, which was subjected to SYBR Green-based real-time PCR analysis as described previously (Iliopoulos et al., 2009; Hirsch et al., 2010). To analyze patient samples, RNAs from 21 colon adenocarcinomas (Origene and Biochain Inc.; Rockville, MD) were analyzed for levels of STAT3, miR-21, and miR-181b-1. Each sample was run in triplicate, and the data represent the mean ±SD. Correlation coefficient represents correlation between STAT3 and miR-21 or miR-181b-1 expression levels in these colon adenocarcinomas.

siRNA Experiments

MCF10A ER-Src cells were seeded in 6-well plates and transfected with different siRNAs (100 nM) from Ambion Inc. against STAT3 (s23967 and s23969), CYLD (s590), Myc (s9129 and s9130), Max (s8538 and s224031), and RXRα (s12385 and s12386) using siPORT *NeoFX* transfection agent. Transfection with 100 nM siRNA (AM4611, Ambion Inc.) was used as a control. No cell toxicity was detected due to the transfection agent. The resulting cells were analyzed for transformation or RNA levels as described above.

Xenograft Experiments

MCF10A-IL-6-transformed cells or HCT-116 and HT-29 colon cancer cells (5×10^6) were injected subcutaneously in the right flank of athymic nude mice (Charles River Laboratories; Wilmington, MA). Tumor growth was monitored every 5 days, and tumor volumes were calculated by the equation $V(mm^3) = a \times b^2/2$, where a is the largest diameter and b is the perpendicular diameter. When the tumors reached a size of ∼65 mm^3, mice were randomly distributed in six groups (four mice/group) and treated i.p. with Ab-IgG (2 μg/ml), Ab-IL-6 (2 μg/ml), siNC (100 nM), siSTAT3 (100 nM), as-miR NC (120 nM), as-miR-21 (120 nM), as-miR-181b-1 (120 nM), or combinations. These treatments were repeated in a weekly basis for four cycles (days 10, 15, 20, and 25). In addition, RNA was extracted from tumors treated with Ab-IgG, Ab-IL-6, siNC, and siSTAT3. Real-time PCR analysis was performed for miR-21 and miR-181b-1. Nude mice were maintained in accordance with Tufts Institutional Animal Care and Use Committee procedures and guidelines.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and seven tables and can be found with this article online at doi:10.1016/j.molcel.2010.07.023.

REFERENCES

Badis, G., Berger, M.F., Philippakis, A.A., Talukder, S., Gehrke, A.R., Jaeger, S.A., Chan, E.T., Metzler, G., Vedenko, A., Chen, X., et al. (2009). Diversity and complexity in DNA recognition by transcription factors. Science *324*, 1720–1723.

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

Berger, M.F., Philippakis, A.A., Qureshi, A.M., He, F.S., Estep, P.W., 3rd, and Bulyk, M.L. (2006). Compact, universal DNA microarrays to comprehensively determine transcription-factor binding site specificities. Nat. Biotechnol. *24*, 1429–1435.

Berger, M.F., Badis, G., Gehrke, A.R., Talukder, S., Philippakis, A.A., Peña-Castillo, L., Alleyne, T.M., Mnaimneh, S., Botvinnik, O.B., Chan, E.T., et al. (2008). Variation in homeodomain DNA binding revealed by high-resolution analysis of sequence preferences. Cell *133*, 1266–1276.

Bromberg, J.F., Wrzeszczynska, M.H., Devgan, G., Zhao, Y., Pestell, R.G., Albanese, C., and Darnell, J.E., Jr. (1999). Stat3 as an oncogene. Cell *98*, 295–303.

Brummelkamp, T.R., Nijman, S.M., Dirac, A.M., and Bernards, R. (2003). Loss of the cylindromatosis tumour suppressor inhibits apoptosis by activating NF-kappaB. Nature *424*, 797–801.

Cervigne, N.K., Reis, P.P., Machado, J., Sadikovic, B., Bradley, G., Galloni, N.N., Pintilie, M., Jurisica, I., Perez-Ordonez, B., Gilbert, R., et al. (2009). Identification of a microRNA signature associated with progression of leukoplakia to oral carcinoma. Hum. Mol. Genet. *18*, 4818–4829.

Chang, T.C., Yu, D., Lee, Y.S., Wentzel, E.A., Arking, D.E., West, K.M., Dang, C.V., Thomas-Tikhonenko, A., and Mendell, J.T. (2008). Widespread microRNA repression by Myc contributes to tumorigenesis. Nat. Genet. *40*, 43–50.

Conti, A., Aguennouz, M., La Torre, D., Tomasello, C., Cardali, S., Angileri, F.F., Maio, F., Cama, A., Germanò, A., Vita, G., and Tomasello, F. (2009). miR-21 and 221 upregulation and miR-181b downregulation in human grade II-IV astrocytic tumors. J. Neurooncol. *93*, 325–332.

Frank, D.A. (2007). STAT3 as a central mediator of neoplastic cellular transformation. Cancer Lett. *251*, 199–210.

Frankel, L.B., Christoffersen, N.R., Jacobsen, A., Lindow, M., Krogh, A., and Lund, A.H. (2008). Programmed cell death 4 (PDCD4) is an important functional target of the microRNA miR-21 in breast cancer cells. J. Biol. Chem. *283*, 1026–1033.

García, M.G., Alaniz, L.D., Cordo Russo, R.I., Alvarez, E., and Hajos, S.E. (2009). PI3K/Akt inhibition modulates multidrug resistance and activates NF-kappaB in murine lymphoma cell lines. Leuk. Res. *33*, 288–296.

Grivennikov, S., Karin, E., Terzic, J., Mucida, D., Yu, G.Y., Vallabhapurapu, S., Scheller, J., Rose-John, S., Cheroutre, H., Eckmann, L., and Karin, M. (2009). IL-6 and Stat3 are required for survival of intestinal epithelial cells and development of colitis-associated cancer. Cancer Cell *15*, 103–113.

Guigon, C.J., Zhao, L., Willingham, M.C., and Cheng, S.Y. (2009). PTEN deficiency accelerates tumour progression in a mouse model of thyroid cancer. Oncogene *28*, 509–517.

Hagan, J.P., Piskounova, E., and Gregory, R.I. (2009). Lin28 recruits the TUTase Zcchc11 to inhibit let-7 maturation in mouse embryonic stem cells. Nat. Struct. Mol. Biol. *16*, 1021–1025.

Hirsch, H.A., Iliopoulos, D., Joshi, A., Zhang, Y., Jaeger, S.A., Bulyk, M., Tsichlis, P.N., Shirley Liu, X., and Struhl, K. (2010). A transcriptional signature and common gene networks link cancer with lipid metabolism and diverse human diseases. Cancer Cell *17*, 348–361.

Iliopoulos, D., Hirsch, H.A., and Struhl, K. (2009). An epigenetic switch involving NF-kappaB, Lin28, Let-7 MicroRNA, and IL-6 links inflammation to cell transformation. Cell *139*, 693–706.

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.

Lin, C.H., Jackson, A.L., Guo, J., Linsley, P.S., and Eisenman, R.N. (2009). Myc-regulated microRNAs attenuate embryonic stem cell differentiation. EMBO J. *28*, 3157–3170.

Löffler, D., Brocke-Heidrich, K., Pfeifer, G., Stocsits, C., Hackermüller, J., Kretzschmar, A.K., Burger, R., Gramatzki, M., Blumert, C., Bauer, K., et al. (2007). Interleukin-6 dependent survival of multiple myeloma cells involves the Stat3-mediated induction of microRNA-21 through a highly conserved enhancer. Blood *110*, 1330–1333.

Marson, A., Levine, S.S., Cole, M.F., Frampton, G.M., Brambrink, T., Johnstone, S., Guenther, M.G., Johnston, W.K., Wernig, M., Newman, J., et al. (2008). Connecting microRNA genes to the core transcriptional regulatory circuitry of embryonic stem cells. Cell *134*, 521–533.

Mayr, C., Hemann, M.T., and Bartel, D.P. (2007). Disrupting the pairing between let-7 and Hmga2 enhances oncogenic transformation. Science *315*, 1576–1579.

Meng, F., Henson, R., Wehbe-Janek, H., Ghoshal, K., Jacob, S.T., and Patel, T. (2007). MicroRNA-21 regulates expression of the PTEN tumor suppressor gene in human hepatocellular cancer. Gastroenterology *133*, 647–658.

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.

Ozsolak, F., Poling, L.L., Wang, Z., Liu, H., Liu, X.S., Roeder, R.G., Zhang, X., Song, J.S., and Fisher, D.E. (2008). Chromatin structure analyses identify miRNA promoters. Genes Dev. *22*, 3172–3183.

Schetter, A.J., Leung, S.Y., Sohn, J.J., Zanetti, K.A., Bowman, E.D., Yanaihara, N., Yuen, S.T., Chan, T.L., Kwong, D.L., Au, G.K., et al. (2008). MicroRNA expression profiles associated with prognosis and therapeutic outcome in colon adenocarcinoma. JAMA *299*, 425–436.

Shi, L., Cheng, Z., Zhang, J., Li, R., Zhao, P., Fu, Z., and You, Y. (2008). hsa-mir-181a and hsa-mir-181b function as tumor suppressors in human glioma cells. Brain Res. *1236*, 185–193.

Tavazoie, S.F., Alarcón, C., Oskarsson, T., Padua, D., Wang, Q., Bos, P.D., Gerald, W.L., and Massagué, J. (2008). Endogenous human microRNAs that suppress breast cancer metastasis. Nature *451*, 147–152.

Trompouki, E., Hatzivassiliou, E., Tsichritzis, T., Farmer, H., Ashworth, A., and Mosialos, G. (2003). CYLD is a deubiquitinating enzyme that negatively regulates NF-kappaB activation by TNFR family members. Nature *424*, 793–796.

Viswanathan, S.R., Daley, G.Q., and Gregory, R.I. (2008). Selective blockade of microRNA processing by Lin28. Science *320*, 97–100.

Warner, J.B., Philippakis, A.A., Jaeger, S.A., He, F.S., Lin, J., and Bulyk, M.L. (2008). Systematic identification of mammalian regulatory motifs' target genes and functions. Nat. Methods *5*, 347–353.

Yan, L.X., Huang, X.F., Shao, Q., Huang, M.Y., Deng, L., Wu, Q.L., Zeng, Y.X., and Shao, J.Y. (2008). MicroRNA miR-21 overexpression in human breast cancer is associated with advanced clinical stage, lymph node metastasis and patient poor prognosis. RNA *14*, 2348–2360.

Yang, A., Zhu, Z., Kapranov, P., McKeon, F., Church, G.M., Gingeras, T.R., and Struhl, K. (2006). Relationships between p63 binding, DNA sequence, transcription activity, and biological function in human cells. Mol. Cell *24*, 593–602.

Yu, H., Kortylewski, M., and Pardoll, D. (2007). Crosstalk between cancer and immune cells: role of STAT3 in the tumour microenvironment. Nat. Rev. Immunol. *7*, 41–51.

Yu, H., Pardoll, D., and Jove, R. (2009). STATs in cancer inflammation and immunity: a leading role for STAT3. Nat. Rev. Cancer *9*, 798–809.

Yuan, T.L., and Cantley, L.C. (2008). PI3K pathway alterations in cancer: variations on a theme. Oncogene *27*, 5497–5510.

Zhang, Z., Sun, H., Dai, H., Walsh, R.M., Imakura, M., Schelter, J., Burchard, J., Dai, X., Chang, A.N., Diaz, R.L., et al. (2009). MicroRNA miR-210 modulates cellular response to hypoxia through the MYC antagonist MNT. Cell Cycle *8*, 2756–2768.