2014 124: 84-95
doi:10.1182/blood-2013-09-527234 originally published online May 1, 2014

miR-150 influences B-cell receptor signaling in chronic lymphocytic leukemia by regulating expression of GAB1 and FOXP1

Marek Mraz, Liguang Chen, Laura Z. Rassenti, Emanuela M. Ghia, Hongying Li, Kristen Jepsen, Erin N. Smith, Karen Messer, Kelly A. Frazer and Thomas J. Kipps

Blood (print ISSN 0006-4971, online ISSN 1528-0020), is published weekly by the American Society of Hematology, 2021 L St, NW, Suite 900, Washington DC 20036.

LYMPHOID NEOPLASIA

miR-150 influences B-cell receptor signaling in chronic lymphocytic leukemia by regulating expression of GAB1 and FOXP1

Marek Mraz,[1,2,3] Liguang Chen,[1] Laura Z. Rassenti,[1] Emanuela M. Ghia,[1] Hongying Li,[1] Kristen Jepsen,[1] Erin N. Smith,[1] Karen Messer,[1] Kelly A. Frazer,[1] and Thomas J. Kipps[1]

[1]University of California-San Diego, Moores Cancer Center, Department of Medicine, La Jolla, CA; [2]Central European Institute of Technology, Masaryk University, Brno, Czech Republic; and [3]Department of Internal Medicine, Hematology and Oncology, University Hospital Brno, Brno, Czech Republic

Key Points

- The most abundant miRNA in CLL, *miR-150*, is expressed at lower levels in cases with unfavorable clinicobiological markers and worse prognosis.
- *miR-150* regulates expression of genes encoding proteins that modulate BCR signaling in CLL.

We examined the microRNAs (miRNAs) expressed in chronic lymphocytic leukemia (CLL) and identified *miR-150* as the most abundant, but with leukemia cell expression levels that varied among patients. CLL cells that expressed ζ-chain–associated protein of 70 kDa (ZAP-70) or that used unmutated immunoglobulin heavy chain variable (IGHV) genes, each had a median expression level of *miR-150* that was significantly lower than that of ZAP-70–negative CLL cells or those that used mutated IGHV genes. In samples stratified for expression of *miR-150*, CLL cells with low-level *miR-150* expressed relatively higher levels of forkhead box P1 (FOXP1) and GRB2-associated binding protein 1 (GAB1), genes with 3′ untranslated regions having evolutionary-conserved binding sites for *miR-150*. High-level expression of *miR-150* could repress expression of these genes, which encode proteins that enhance B-cell receptor signaling, a putative CLL-growth/survival signal. Also, high-level expression of *miR-150* was a significant independent predictor of longer treatment-free survival or overall survival, whereas an inverse association was observed for high-level expression of *GAB1* or *FOXP1* for overall survival. This study demonstrates that expression of *miR-150* can influence the relative expression of *GAB1* and *FOXP1* and the signaling potential of the B-cell receptor, thereby possibly accounting for the noted association of expression of *miR-150* and disease outcome. (*Blood*. 2014;124(1):84-95)

Introduction

Chronic lymphocytic leukemia (CLL) is the most common leukemia among adults in the Western world. The clinical course of CLL patients is heterogeneous, ranging from indolent to highly aggressive. Several prognostic markers have been described in CLL that can reliably segregate patients into subgroups that differ in treatment-free survival (TFS) or overall survival (OS).[1-3] Some of these markers, such as the immunoglobulin heavy chain variable (IGHV) gene mutation status or expression of ζ-chain–associated protein of 70 kDa (ZAP-70) or CD38, are associated with the B-cell receptor (BCR) signaling pathway.[4-6] This suggests that BCR signaling may be involved in the pathogenesis and/or progression of CLL.

The intensity of BCR signaling varies between CLL cells of different patients, which in turn might account for some of the heterogeneity observed in the proclivity for disease progression (reviewed in Kipps[7]). Some CLL cells are more responsive to ligation of surface immunoglobulin, particularly CLL cells that express ZAP-70, the expression of which is associated with more aggressive disease.[1,7] Similarly, there might be differences in other BCR-associated kinases, phosphatases, and their adaptor molecules between the CLL cells of different patients that also could modulate BCR signaling and potentially contribute to differences in the tendency for disease progression.[8] As such, understanding the factors that modulate BCR signaling intensity in CLL cells may identify other features that are associated with prognosis and/or response to newly defined inhibitors of BCR signaling, which are found to have clinical activity in patients with this disease.[9]

Factors that might regulate expression of genes encoding proteins involved in BCR-signaling are microRNAs (miRNAs).[10] These short noncoding RNAs each can regulate expression of a variety of different genes at the posttranscriptional level. miRNAs can regulate the stability and translation of a large number of target messenger RNAs (mRNAs) and thus "fine tune" essential cell functions.[11-14] In lymphoid cells, such gene-dose regulation is needed for survival and proper maturation of B and T cells, immunoglobulin production by B cells, and relative proficiency of T-cell receptor signaling in T lymphocytes.[10,12,15-19] The miRNAs that regulate essential pathways in immune cells generally are abundantly expressed and evolutionarily conserved.[12,20-23] Aberrations in such miRNA-mediated regulation

Submitted September 18, 2013; accepted April 17, 2014. Prepublished online as *Blood* First Edition paper, May 1, 2014; DOI 10.1182/blood-2013-09-527234.

The data reported in this article have been deposited in the Gene Expression Omnibus database (accession number GSE49896).

The online version of this article contains a data supplement.

There is an Inside *Blood* Commentary on this article in this issue.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

were directly implicated in cancer pathogenesis (reviewed in O'Connell and Baltimore[12]). This is particularly the case for CLL, the first human disease in which deregulation of miRNAs was linked to pathogenesis.[20,24]

In CLL deletion of *miR-15a/miR-16-1*, located at 13q14, is implicated in the pathogenesis of about 50% of all cases.[20,24] Aberrant expression of miRNAs also is observed in other B-cell malignancies.[25] Comparison of miRNA expression in CLL cases with favorable vs unfavorable prognosis have revealed other miRNAs that are associated with more aggressive disease or unfavorable cytogenetics (reviewed in Mraz et al[26] and Mraz and Pospisilova[27]).

We and others have observed differential expression of approximately 20 miRNAs between CLL cells of patients with progressive disease vs those from patients with indolent disease.[17,28-33] Several of these miRNAs contribute to the deregulation of anti-apoptotic molecules, such as bcl-2 (*miR-15a/16-1*),[34] mcl-1 (*miR-29*),[35] or tcl-1 (*miR-29*, *miR-181*),[36] molecules that play important roles in the resistance to apoptosis or growth of neoplastic B cells. Lack of functional p53 also can affect expression of miRNAs that might influence apoptosis (eg, *miR-34a*).[32] Additionally, the levels of certain miRNAs, such as *miR-181b*, can decrease with disease progression, whereas others, such as *miR-125b*, can influence the metabolic adaptation of B cells to their malignant phenotype.[33,37] However, the pathways regulated by most of the miRNAs expressed in CLL cells and their contributions to CLL-cell biology remain unknown.

We hypothesized that miRNAs that are abundantly expressed in CLL may regulate expression of genes encoding proteins involved in key molecular pathways, such as those involved in BCR signaling. We identified the most abundant miRNA in CLL to be *miR-150*, which we found expressed at different levels in CLL cells of different patients. We examined for genes that are differentially expressed between CLL cells that have relatively high vs low levels of *miR-150*, allowing us to discover the regulatory activity of *miR-150* on 2 genes encoding proteins that can modulate the intensity of BCR signaling and potentially contribute to the heterogeneity noted in disease progression of patients with CLL.

Methods

CLL cohort

Blood samples were collected from patients (n = 168) at the University of California-San Diego Moores Cancer Center who satisfied diagnostic and immunophenotypic criteria for common CLL after providing written informed consent in compliance with the Declaration of Helsinki and the institutional review board of University of California-San Diego. Peripheral blood mononuclear cells were isolated from CLL patients using density centrifugation with Ficoll-Hypaque (GE Healthcare; obtained purity of ≥95% of $CD5^+19^+$ cells). The basic clinicobiological characteristics of this patient cohort are summarized in Table 1.

Gene expression microarray analysis and quantitative real-time polymerase chain reaction

Total RNA was isolated, labeled, and hybridized to Affymetrix HG-U133+2 GeneChips according to the manufacturer's protocol, as described previously.[38] Expression of individual protein-coding genes/*miR-150* (TaqMan Assays; Applied Biosystems) and miRNA expression data (TaqMan Array MicroRNA Cards; Applied Biosystems) were obtained and normalized according to the manufacturer's protocol, as described previously[39] (see supplemental Methods on the *Blood* Web site).

Table 1. Cohort characteristics (n = 168)

	N	%
Median age at diagnosis (range), y	56.5 (34-79)	
Median follow up (y)	8.3	
Median survival (y)	19.8	
Median time from diagnosis to blood collection (y)	2.6	
Treated during follow-up*	105	63.0
Gender		
Male	107	63.7
Female	61	36.3
Rai stage		
0	54	42.6
I	43	32.4
II	25	18.4
III-IV	9	6.6
Not determined	37	
IGHV/ZAP-70/CD38 status		
unmut IGHV/mut IGHV	72/96	42.9/57.1
$ZAP\text{-}70^+/ZAP\text{-}70^-$	73/95	43.5/56.5
$ZAP\text{-}70^-$ AND mut IGHV	83	49.4
$ZAP\text{-}70^+$ AND unmut IGHV	60	35.7
$CD38^+/CD38^-$	49/119	29.2/70.8
Hierarchical cytogenetics (FISH)		
del 17p13	12	10.4
del 11q23	12	10.4
del 13q14	47	40.8
Trisomy 12	15	13.0
Normal karyotype	29	25.2
Not determined	53	

mut, mutated; unmut, unmutated.
*Details provided in supplemental Table 3.

Cell transfection

B-cell lines MEC-1 and Raji were obtained from American Type Culture Collection and cultured in RPMI-1640 supplemented with 10% fetal bovine serum in 5% CO_2 at 37°C. Cell lines or CLL cells were respectively suspended at 2×10^6 per mL or 1×10^7 per mL in transfection medium for transfection using the DharmaFECT Duo Transfection Reagent (Dharmacon; Thermo Scientific) with a short artificial *miR-150* (MISSION microRNA Mimic, 100 nM; Sigma-Aldrich), control RNA (MISSION microRNA Mimic Negative Control, 100 nM), short interfering RNA (siRNA) (ON-TARGET plus siRNA-SMARTpool, 100 nM; Thermo Scientific), or fluorochrome-labeled short RNA (siGLO; Thermo Scientific).[17] Raji and MEC-1 cell lines were used for the transfection experiments because of their relatively low-level expression of *miR-150*, resilience to transfection with high cell viability (viability > 90%), and, in the case of Raji cells, high transfection efficiency (>85%; supplemental Figure 1A-B). Raji and MEC-1 cell lines both express *miR-150* at ~100-fold lower levels than the average level expressed by CLL cells. The immunoblot analysis of transfected cells is described in supplemental Methods.

Luciferase assay

Luciferase reporter assay was performed using the LightSwitch Luciferase Assay System (SwitchGear Genomics), according to the manufacturer's protocol, as described previously[40] (see supplemental Methods).

BCR crosslinking and measurement of intracellular calcium flux

BCR signaling induced by BCR ligation was evaluated by flow cytometry to measure changes in intracellular calcium flux induced by treatment with goat F(ab')2 anti-human immunoglobulin M (IgM; anti-μ, Southern Biotechnology; final concentration 10 μg/mL), as described elsewhere[4,5,41] (see supplemental Methods).

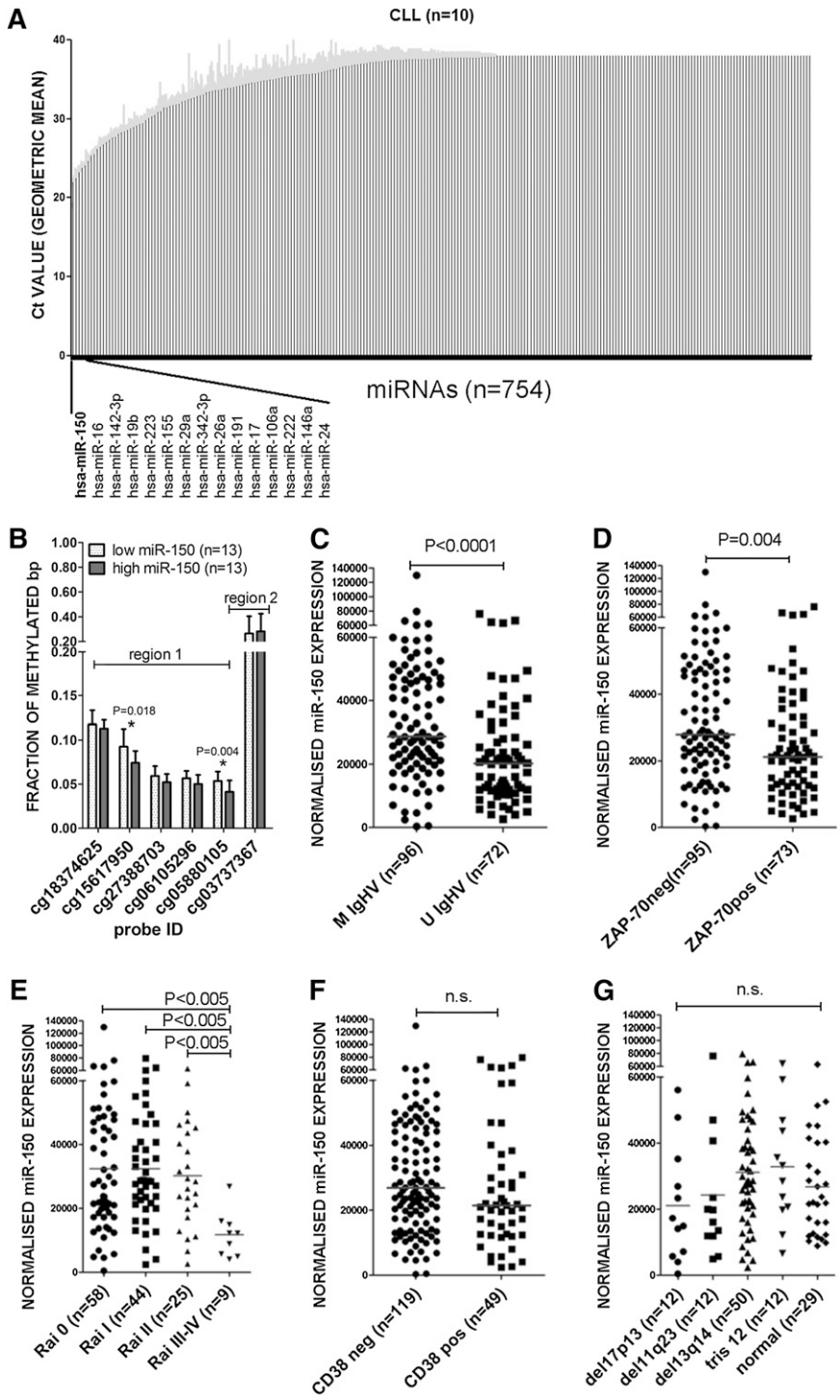

Figure 1. Expression of *miR-150* and its relationship with clinicobiological features. (A) The expression of 754 human miRNAs (TaqMan Array MicroRNA Cards; ABI) was screened in 10 purified CLL samples (>95% of $CD5^+19^+$ cells purified using RosseteSep Human B Cell Enrichment Cocktail, StemCell Technologies; RNA Integrity number >8). Results are visualized as geometric mean (black bars) and standard deviation (gray error bars). The lower Ct value corresponds to higher expression; Ct values >38 were considered as nondetectable miRNAs (n = 312). (B) The methylation levels in the region upstream of *miR-150* in CLL cases stratified as low *miR-150* expression (<median, n = 13) and high *miR-150* (>median, n = 13). The prediction of transcription start sides for *miR-150* in regions 1 and 2 is described in supplemental Figure 3. Analyzed region 1 contained 5 probes and region 2 contained 1 probe (region is defined by a size of ~1000 nt). The methylation levels were compared using the nonparametric Mann-Whitney *U* test. The error bars represent standard deviation. (C-G) *miR-150* expression was quantified in a cohort of 168 CLL patients (cohort characteristics in Table 1) and correlated to the clinicobiological characteristics of CLL cells such as IGHV mutation status (C), ZAP-70 expression (D), Rai stage (E), CD38 expression (F), and hierarchical classification of FISH abnormalities (G). The differences in expression were compared using the nonparametric Mann-Whitney *U* test. U IgHV, unmutated IGHV; M IgHV, mutated IGHV.

Methylation arrays

We obtained data on the methylation of the *miR-150* promoter region using an Illumina Human Methylation 450 BeadChip, following the manufacturer's protocol (see supplemental Methods).

Statistical analysis

Univariate and multivariate analysis of OS and TFS were computed using R package (see supplemental Methods). All other statistical analyses were performed with GraphPad Prism Software, v. 5.0 (GraphPad Software). All statistical tests were 2-sided and *P* values <.05 were considered significant.

Results

Expression of *miR-150* and its relationship with clinicobiological features of CLL

We examined for the expression of 754 human miRNAs in CLL cells isolated from 10 different patients (cohort characteristics are listed in supplemental Table 1). Altogether, we detected 271 different miRNAs (threshold cycle [Ct] value <38) in at least 5 CLL samples, and another 171 different miRNAs in 1 to 4 samples. We

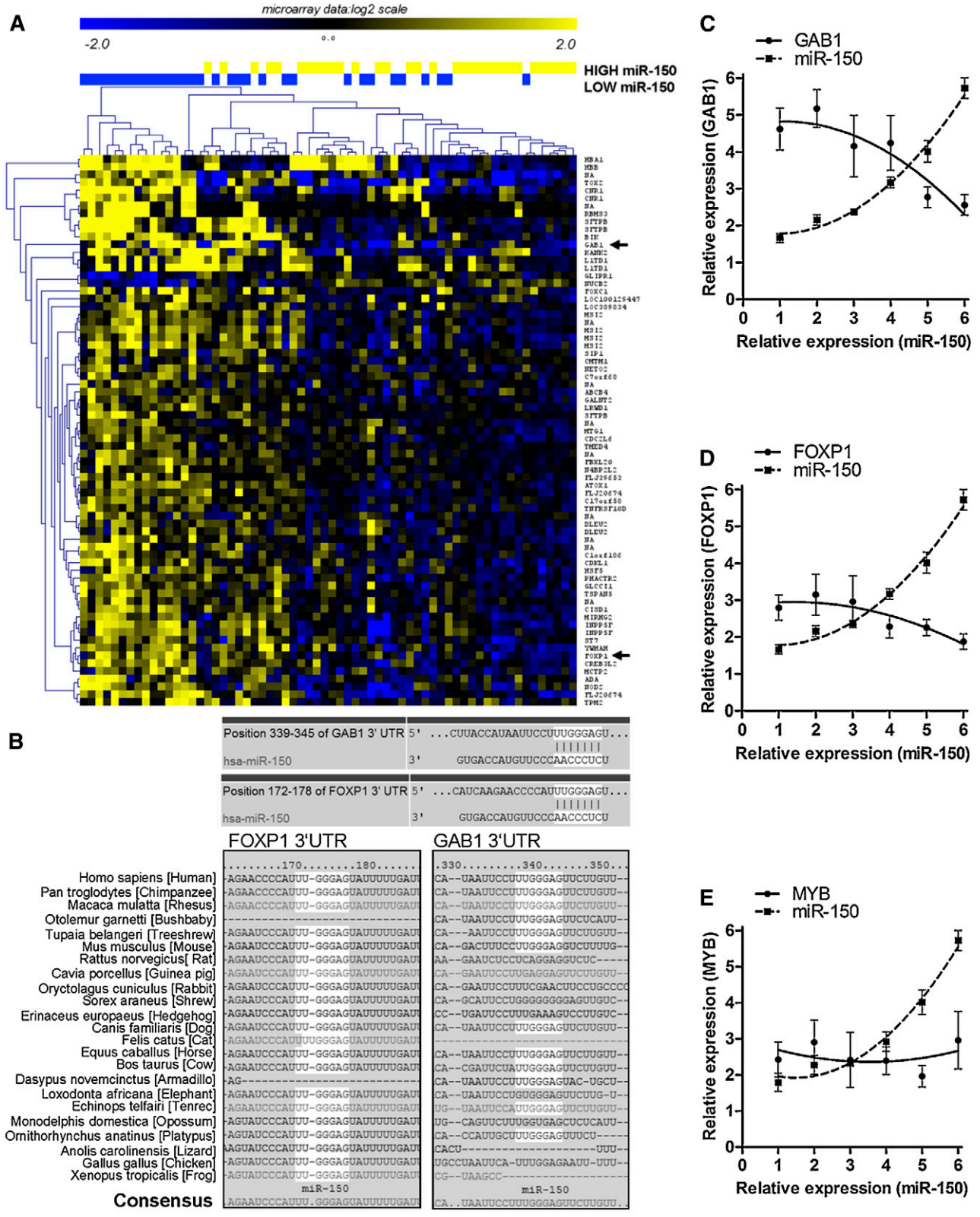

Figure 2. Identification of *miR-150* targets by gene expression analysis. (A) Microarray data for protein-coding gene expression (Affymetrix HG-U133+2 GeneChips) were compared in CLL samples stratified based on low (n = 32) vs high (n = 32) levels of *miR-150*. The samples from the lowest and highest tercile were compared (SAM in MeV, fold change >1.5, false discovery rate <0.1). This analysis identified differential expression of 58 genes (72 probes) between CLL cells expressing low vs high levels of *miR-150*. *GAB1* and *FOXP1* are marked with a black arrow. (B) The evolutionary conserved binding sides for *miR-150* in *GAB1* and *FOXP1* 3′UTR (TargetScan). (C-E) The association between *miR-150* levels and the expression of mRNAs for *GAB1* (C), *FOXP1* (D), or *c-MYB* (E) in a validation cohort of 60 CLL samples. Each point represents the average value for gene expression from 10 CLL samples. The standard deviation about the mean is indicated by error bars.

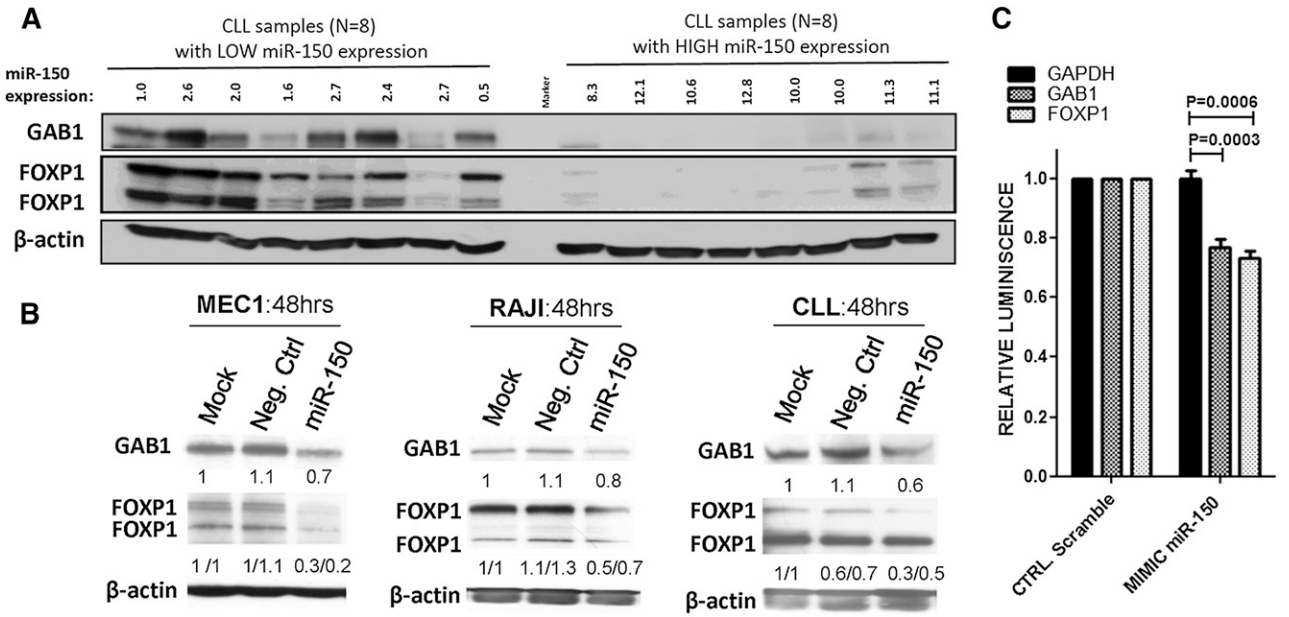

Figure 3. Validation of newly defined *miR-150* targets. (A) GAB1 or FOXP1 immunoblot analysis in CLL samples of 16 patients selected for low-level (n = 8) vs high-level expression of *miR-150* (n = 8). The relative levels of *miR-150* expression are indicated (first sample set as 1). The samples were solely chosen based on the differences in *miR-150* expression and partially overlapped with samples used in the microarray analysis (7 of 16 samples). The double bands identified by the anti-FOXP1 antibody (JC12 clone) represent 2 splicing variants of FOXP1 as described previously.[53] (B) A representative immunoblot for GAB1 or FOXP1 protein in MEC-1 and Raji, primary CLL cells transfected (48 hours) with artificial *miR-150* (*miR-150*, 100 nM), and control miRNA (negative control [Neg. Ctrl], 100 nM). MOCK represents cells treated only with the electroporation pulse. The Blot images were quantified with ImageJ 1.42q (National Institutes of Health), and the GAB1/β-actin and FOXP1/β-actin ratio in MOCK was arbitrarily set at 1. The FOXP1/β-actin rations for its 2 major splicing variants are provided (the ratio of the larger vs the smaller isoform is separated by a slash). (C) The luciferase activity in 293FT cells cotransfected with *miR-150* mimic (MIMIC *miR-150*, 100 nM) or luciferase reporter construct (100 ng) containing the 3′UTR region of *GAB1*, *FOXP1* or *GAPDH* (*GAPDH* as control). As a negative control, we used a control miRNA mimic (CTRL Scramble, 100 nM). Luciferase activity was measured 24 hours posttransfection and compared by paired Student *t* test.

did not detect expression of 312 different miRNAs in any of the samples. Of the 15 most abundantly expressed miRNAs, 11 had previously been found to vary between patients with a low risk vs a high risk for early disease progression (Figure 1A).[26,27] All 15 of these abundantly expressed miRNAs were evolutionarily conserved,[42] including *miR-150*, which was the most abundantly expressed miRNA in CLL (Figure 1A; supplemental Figure 2). CLL cells expressed higher median levels of *miR-150* than normal blood B cells or tonsillar B cells (3.5-fold and 6.5-fold, respectively, $P < .05$) (supplemental Figure 3A).

Despite expressing relatively high levels of *miR-150,* CLL cells of different patients (n = 168) varied in their relative expression of this miRNA (~12-fold difference between cases in lower 10th percentile vs those in the 90th percentile). The distribution in expression of *miR-150* among patients is provided in supplemental Figure 4. Two regions in the putative *miR-150* promoter were significantly less methylated in cases with high-level *miR-150* than in cases with low-level *miR-150*, suggesting epigenetic differences influence expression of this miRNA (Figure 1B; supplemental Figure 5). In our cohort of patients (Table 1), *miR-150* levels were significantly lower in cases that used unmutated IGHV or that expressed ZAP-70 (Figure 1C-D). Discordant cases that did not express ZAP-70, but used unmutated IGHV, expressed lower levels of *miR-150* than cases that lacked expression of ZAP-70 or that expressed mutated IGHV (supplemental Figure 6A). There were wide variations in the expression levels of *miR-150* among samples collected from patients who had Rai stage 0-II disease. However, those patients in our cohort that had Rai stage III-IV disease had CLL cells that expressed relatively low levels of *miR-150* ($P < .005$, Figure 1E). The expression levels of *miR-150* did not have any discernible relationship with the expression levels of CD38, or with specific chromosomal abnormalities, as detected by fluorescence in situ hybridization (FISH) (Figure 1F-G; supplemental Figure 6B).

Identification of *miR-150* targets by gene expression analysis

Several genes have been identified as being potentially regulated by *miR-150* in various cell types.[43-47] However, the genes targeted by a certain miRNA can vary depending upon the cellular context.[12,48-50] Because an miRNA can influence the relative stability of its target mRNA,[11-14] evaluating for differences in gene expression between cells that express high vs low levels of a given miRNA potentially could identify mRNA(s) that are regulated by that miRNA.[11-14] As such, we performed array-based transcriptome analyses of 100 CLL samples to examine for differences in gene expression between CLL cells that expressed high vs low levels of *miR-150.* For this purpose, we divided the samples into 3 groups based upon their relative expression levels of *miR-150.* We examined for gene expression differences between samples from the third of the cases having the lowest levels of *miR-150* (n = 32) with the third of the cases having the highest expression levels of *miR-150* (n = 32), identifying genes with a significance analysis of microarray (SAM) method (MeV) with a fold change of more than 1.5 (false discovery rate <0.1). This analysis identified 58 genes (with 72 probes) that differed in their relative expression levels between these 2 groups of samples (Figure 2A). Most of these genes (55/58) were expressed at relatively lower levels in samples with the high-level expression of *miR-150* (Figure 2A; supplemental Table 2). Thirteen of the 58 identified genes (22%) were predicted targets of *miR-150* using TargetScan software (supplemental Table 2). We noted that 2 of the 13 genes had evolutionary conserved binding sides for *miR-150*, namely *GAB1* and *FOXP1* (Figure 2B). Moreover, *FOXP1* and *GAB1* were predicted

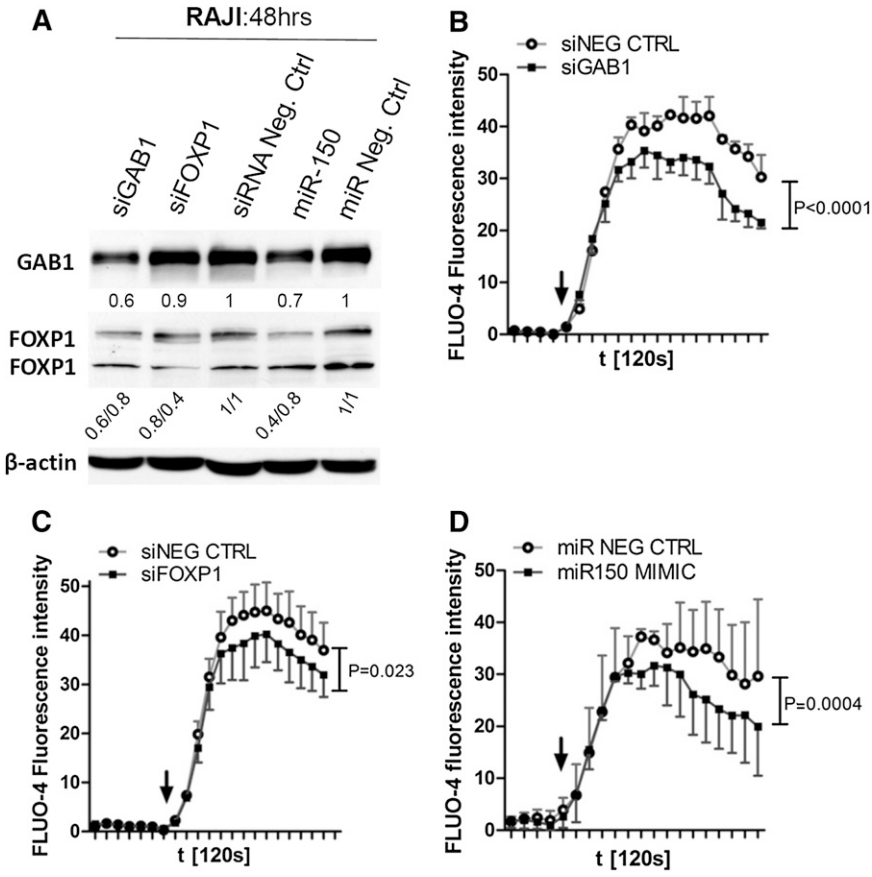

Figure 4. Expression of *miR-150* targets GAB1 or FOXP1 affects BCR signaling. (A) The Raji cell line was transfected with artificial *miR-150* (*miR-150*, 100 nM), control miRNA (miR negative control [Neg. Ctrl], 100 nM), siRNA against *GAB1* (siGAB1, 100 nM) or *FOXP1* (siFOXP1, 100 nM), and control siRNA (siRNA Neg. Ctrl, 100nM). The band intensities observed in immunoblot analysis were measured using ImageJ 1.42q, and the GAB1/β-actin and FOXP1/β-actin ratio in control was arbitrarily set at 1. The FOXP1/β-actin ratios for the 2 major isoforms of FOXP1 are provided (ratio for larger and smaller variant is separated by a slash). GAB1 and FOXP1 protein levels were analyzed 48 hours posttransfection and β-actin was used as a loading control (representative example of an immunoblot). (B-C) The effect of Raji cell transfection with siRNA against *GAB1* (siGAB1, 100 nM) or *FOXP1* (siFOXP1, 100 nM) or control siRNA (siNEG CTRL, 100 nM) on calcium influx after anti-μ stimulation (10 μg/mL; indicated by a black arrow). (D) The effect of Raji cell transfection with artificial *miR-150* (*miR-150* MIMIC, 100 nM) or control miRNA (miR NEG CTRL, 100 nM) on calcium influx after anti-μ stimulation (10 μg/mL; indicated by a black arrow). The calcium influx (FLUO-4 fluorescence intensity) in panels B-D were analyzed continuously for 120 seconds. The acquisition of background fluorescence (30 seconds) was followed by adding anti-μ (final concentration 10 μg/mL; indicated by a black arrow), and data at 6-second intervals were visualized as peak intensity. Error bars represent standard error of the mean of at least 2 independent experiments. Differences were statistically analyzed by 2-way analysis of variance.

targets of *miR-150* using other database tools (DIANAmT, miRnda, miRWalk, PICTAR5, or RNA hybrid). The 7-nucleotide binding site for *miR-150* (containing the whole seed sequence) in *GAB1* was identical to that found in *FOXP1* (Figure 2B).

We observed that the expression level of *miR-150* was inversely proportional to that of *GAB1* or *FOXP1*. This also was observed among CLL samples from a validation cohort of 60 patients (Figure 2C-D; supplemental Figure 7) and in normal peripheral $CD19^+$ B cells (supplemental Figure 3). On the other hand, a known target of *miR-150*, *c-MYB*,[47] was not inversely proportional to *miR-150* and expressed at very low levels (median Ct value 32.8), even in samples with low-level expression of *miR-150* (Figure 2A,E). The expression levels of *c-MYB* were close to the detection limit of our real-time polymerase chain reaction assay (median Ct value 32.8, detection limit Ct = ~35.0). Finally, the levels of *c-MYB* detected in CLL cells were 34-fold lower than those of *GAB1* (median Ct value 27.7) and 415-fold lower than those of *FOXP1* (median Ct value 24.1). Altogether, these data suggest that *GAB1* and *FOXP1*, rather than *c-MYB*, are potential targets of *miR-150* in the CLL transcriptome.

Validation of newly identified targets of *miR-150* in CLL

To study the relationship between expression of *miR-150* and proteins encoded by *GAB1* or *FOXP1*, we examined for GAB1 and FOXP1 protein in CLL cells of each of 16 patients that had relatively low- (n = 8) or high-level expression of *miR-150* (n = 8; ~5- to 10-fold difference in *miR-150* levels). We found that cases with relatively low *miR-150* had higher levels of GAB1 or FOXP1 ($P < .005$, fold change >10.0) than cases with high-level expression of *miR-150* by immunoblot analysis (Figure 3A). Transfection of the B-cell lines MEC-1 or Raji, or primary CLL cells, with a synthetic *miR-150*, reduced the levels of both GAB1 and FOXP1 by 28% to 44% and 46% to 80%, respectively ($P < .05$) (Figure 3B). Moreover, transfection of cells with *miR-150* down-modulated either splicing variant of FOXP1, but to varying degrees. In contrast, the levels of GAB1 or FOXP1 did not change in cells transfected with an irrelevant control miRNA (Figure 3B).

To test for interaction of *miR-150* with the 3′ untranslated region (UTR) of *GAB1* or *FOXP1*, we cotransfected 293FT cells with *miR-150*, or a control miRNA, and a luciferase reporter–construct containing the 3′ UTR of *GAB1* or *FOXP1* (Figure 3C). Cotransfection of *miR-150* with this luciferase construct decreased the luciferase activity of the transfected cells by about 25% relative to that of cells cotransfected with the luciferase construct and control miRNA (Figure 3C). Such differences are similar to those of luciferase constructs containing the 3′ UTR of other genes that are targeted by a cotransfected miRNA.[40] On the other hand, the *miR-150* mimic had no effect on the luciferase activity of cells cotransfected with a luciferase-reporter construct containing the 3′ UTR of GAPDH, which contained no predicted binding sites for *miR-150*. We conclude that *miR-150* interacts with the 3′UTR of either *GAB1* or *FOXP1* mRNA.

Relationship between expression of *miR-150*, *GAB1*, or *FOXP1* and relative sensitivity to BCR ligation

GAB1 is an adaptor molecule that can recruit phosphoinositide 3-kinase (PI3K) to the plasma membrane after surface immunoglobulin ligation and thereby enhance BCR signaling.[51] *FOXP1* is a transcription factor that is upregulated upon B-cell activation and

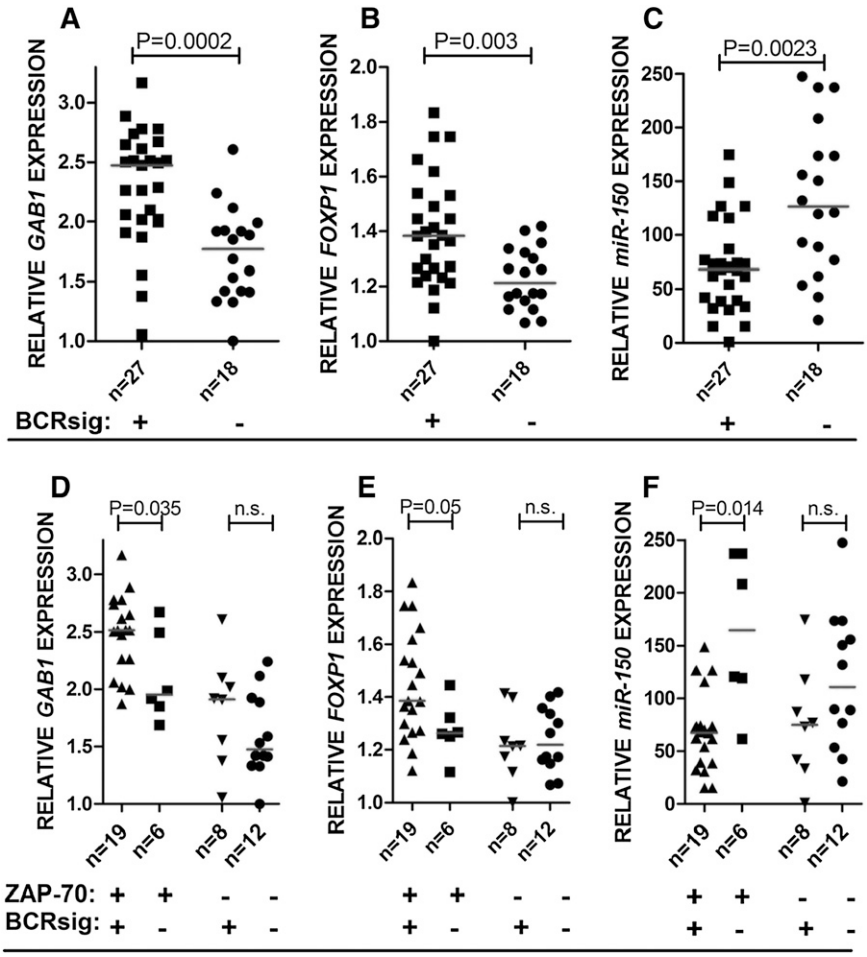

Figure 5. Relationship between expression of *miR-150*, *GAB1*, or *FOXP1* and the relative sensitivity to BCR ligation in CLL. The association between BCR responsiveness and *GAB1* (A), *FOXP1* (B), or *miR-150* (C) expression. Forty-five CLL samples were stimulated by anti-μ (10 μg/mL) and the calcium flux was assessed by flow cytometry. Below each panel is a "+" or a "−" in the row labeled "BCRsig" on the left to indicate samples that were high responders or low responders, respectively. (D-F) Another row is beneath the panels labeled on the left as "ZAP-70" to indicate the samples that were positive or negative for ZAP-70, as indicated by the "+" or "−", respectively. n.s., not significant; ZAP-70$^+$, ZAP-70 positive samples.

found associated with adverse prognosis and the activated B cell (ABC) phenotype of diffuse large B-cell lymphoma (DLBCL).[52,53] Because *miR-150* can target the genes encoding each of these factors, the expression level of *miR-150* could influence the intensity of BCR signaling induced by surface immunoglobulin ligation.

To examine for this, we transfected Raji cells with siRNA specific for *GAB1, FOXP1*, a synthetic *miR-150*, or control short RNA. We selected Raji cells because of their relatively low-level expression of *miR-150* and high transfection efficacy (>85%, supplemental Figure 1). Transfection with either *miR-150* or a siRNA specific for *GAB1* significantly reduced expression of GAB1 (Figure 4A), which was not affected by transfection with control short RNA. Similarly, transfection with either *miR-150* or siRNA for *FOXP1* reduced expression of FOXP1 relative to that noted in cells transfected with control siRNA (Figure 4A).

We monitored for changes in intracellular calcium induced with anti-μ treatment by flow cytometry. This demonstrated that the relative level of GAB1 or FOXP1 apparently influenced the magnitude of the calcium flux observed following BCR ligation (Figure 4B-C). The transfection with *miR-150* also significantly affected BCR responsiveness, but with higher variability (Figure 4D). The transfection with *miR-150* or *siGAB1* or *siFOXP1* did not have any significant effect on cell viability or the levels of calcium flux induced by treatment with ionomycin (supplemental Figure 1B; supplemental Figure 8). We noted that silencing FOXP1 downmodulated both the basal and anti-μ–induced levels of phosphorylated AKT, whereas silencing GAB1 reduced the levels of phospho-AKT induced by anti-μ (supplemental Figure 9).

To test the relevance of *GAB1* and *FOXP1* in BCR signaling in CLL, we treated 45 CLL samples with anti-μ and monitored the induced calcium flux by flow cytometry. This allowed us to examine for differences between samples in their response to BCR crosslinking, allowing us to stratify cases as relatively sensitive to BCR ligation (n = 27) or resistant to treatment with anti-μ (n = 18) (see supplemental methods). CLL samples that were more sensitive to BCR ligation had significantly higher levels of *GAB1* and *FOXP1* mRNAs (Figure 5A-B). On the other hand, cases with relatively high levels of *miR-150* were relatively insensitive to BCR ligation (Figure 5C). As expected,[4,5,41] most samples that were ZAP-70–positive responded well to treatment with anti-μ. However, there were cases that were ZAP-70–positive that were relatively insensitive to BCR ligation. Such samples had relatively low levels of *GAB1* or *FOXP1*, and relatively high levels of *miR-150* (Figure 5D-F). These data reveal that high-level expression of *GAB1* and *FOXP1* and low-level expression of *miR-150* associates with relatively high sensitivity to surface immunoglobulin ligation in CLL. Moreover, high expression of *miR-150* might account in part for the relatively low response to surface immunoglobulin ligation observed in some samples that express ZAP-70.

Prognostic significance of *miR-150*, *GAB1*, or *FOXP1*

The relationship between expression of *GAB1*, *FOXP1*, and *miR-150* with the sensitivity to BCR ligation prompted us to investigate the relationship between the relative expression of each of these

Table 2. The prognostic significance of *miR-150*, *GAB1*, or *FOXP1* in a univariate analysis

	Univariate Cox regression model of OS n = 154, number of events = 39*						Univariate Cox regression model of TFS n = 107, number of events = 58*					
	Nondelayed entry model			Delayed entry model			Nondelayed entry model			Delayed entry model		
Variable	HR	CI	*P*	HR	CI	*P*	HR	CI	*P*	HR	CI	*P*
miR-150 (≤ vs > median)	**2.8**	1.3-5.9	**.01**	**3.2**	1.5-6.7	**.003**	**1.7**	1-2.8	**.057**	**2.3**	1.4-4	**.002**
GAB1 (> vs ≤ median)	**2.3**	1.2-4.4	**.02**	**1.7**	0.9-3.3	**.12**	**2.1**	1.3-3.6	**.005**	**2.4**	1.4-4.1	**.002**
FOXP1 (> vs ≤ median)	**2.4**	1.2-4.8	**.02**	**2.9**	1.4-5.8	**.004**	**1.4**	0.8-2.3	**.3**	**1.3**	0.7-2.1	**.39**
IGHV (unmut. vs mut.)	12.2	4.9-30.5	<.001	7.5	3-18.9	<.001	2.9	2-4.4	<.001	2.5	1.4-4.2	.001
ZAP-70 (pos. vs neg.)	7.3	3.5-15.2	<.001	7.7	3.3-18	<.001	2.4	1.6-3.5	<.001	2.3	1.3-3.9	.002
CD38 (pos. vs neg.)	3.0	1.6-5.7	.001	2.7	1.4-5.2	.003	2.5	1.7-3.8	<.001	2.6	1.4-4.6	.002
Gender (male vs female)	1.3	0.7-2.5	.5	1.2	0.6-2.4	.56	1.4	1-2.2	.08	1.4	0.8-2.5	.2
Rai stage I vs 0	2.9	0.9-9.2	.07	2.3	0.7-7.5	.16	1.4	0.8-2.4	.2	0.8	0.4-1.7	.63
Rai stage II vs 0	4.4	1.2-15.7	.02	2.5	0.7-9.1	.15	3.2	1.8-5.8	<.001	1.2	0.5-2.6	.66
Rai stage ≥III vs 0	12.0	3.5-40.9	<.001	7.1	2.1-24	.002	5.2	2.4-11.1	<.001	3.2	0.7-14.4	.13
Age (> vs ≤ median)	3.3	1.7-6.6	<.001	2.7	1.3-5.7	.008	1.1	0.7-1.6	.75	0.9	0.5-1.6	.76
del17p13	2.9	1-8.8	.06	3.3	1.1-10	.04	1.9	1-3.8	.05	2.4	1-5.8	.05
dell11q23	2.6	0.9-8	.09	1.6	0.5-4.8	.43	2.4	1.3-4.4	.007	1.8	0.8-4	.13

CI, confidence interval (of 95%); HR, hazard ratio. The values in rows miR-150, GAB1, and FOXP1 are boldfaced to highlight results for these genes of interest.
*Analysis for miR-150, GAB1, and FOXP1 includes only samples obtained before the last day of follow-up for OS analysis (154/168) or before first therapy for TFS analysis (107/168); the others use all available samples.

genes and prognosis in a well-characterized cohort of patients with CLL (n = 168, Table 1). The median follow-up of patients was 8.3 years. During the follow-up period, 63% of patients required therapy (n = 105, median time to therapy 5.6 years) and 23% had died (n = 39, median survival 19.8 years). For the analysis of the relationship between relative gene expression and OS, we only used data from patients who had a stored blood sample available before the last day of follow-up (n = 154) or for TFS before first treatment (n = 107). We divided the cohort by median *miR-150* expression (cutoff point identification is depicted in supplemental Figure 10) and performed a univariate Cox regression (Table 2) and also Kaplan-Meier analysis. Compared with cases that had high-level expression of *miR-150*, the cases with low-level expression of *miR-150* had a significantly shorter median OS (13.9 years vs not reached, HR: 2.8 [CI 1.3-5.9]) and shorter median TFS (5.6 vs 8.1 years, HR: 1.7 [CI 1.0-2.8]; Figure 6 A-B; Table 2).

To determine if *miR-150* is an independent predictor of OS and TFS, we performed a multivariate analysis that included *miR-150* levels and 8 routinely used prognostic markers. Although low-level expression of *miR-150* was associated with the expression of ZAP-70 or use of unmutated IGHV (Figure 1C-D), low-level *miR-150* had a strong independent prognostic value for OS and TFS (OS HR: 9.6 [CI 1.6-57.8]; TFS HR: 2.4 [CI 1.2-4.7]; Table 3). As a sensitivity analysis, univariate and multivariate use of delayed-entry Cox models to account for the variable timing of the blood draws (see supplemental methods) provided similar results (Tables 2 and 3). In univariate analyses, high-level expression of *GAB1* and *FOXP1* (>median) each was associated with a significantly shorter OS; high-level *GAB1* also associated with shorter TFS (OS GAB1 HR: 2.3 [CI 1.2-4.4]; OS FOXP1 HR: 2.4 [CI 1.2-4.8], TFS GAB1 HR: 2.1 [CI 1.3-3.6]; Table 2; Figure 6C-F). The analysis performed with the use of delayed-entry models confirmed significant predictive association of *FOXP1* levels with OS, and *GAB1* levels with TFS (Table 2). Altogether, the adverse prognostic impact of low-level expression of *miR-150*, and the reverse trend for *GAB1* and *FOXP1* provides support for the relevance of these genes to the biology of neoplastic B cells.

Discussion

We identified *miR-150* as being the most abundantly expressed miRNA in CLL. However, we observed significant heterogeneity in the expression levels of this miRNA among CLL cells of different patients. Low-level expression of *miR-150* associated with unfavorable clinicobiological and prognostic markers, such as expression of ZAP-70 or use of unmutated IGHV ($P < .005$). Additionally, our data suggest that the levels of methylation of the upstream region of 1000 nt proximal to *miR-150* associate with its expression. We demonstrated that *GAB1* and *FOXP1* genes represent newly defined direct targets of *miR-150* in CLL cells. We also showed that high-level expression of *GAB1* and *FOXP1* associates with relatively high sensitivity of CLL cells to surface immunoglobulin ligation. High levels of *GAB1/FOXP1* and low levels of *miR-150* associate with a greater responsiveness to BCR ligation in CLL cells and more adverse clinical prognosis.

It was demonstrated that abundantly expressed, evolutionary-conserved miRNAs are important for immune-cell phenotypes and a can regulate stability of mRNAs (reviewed in O'Connell and Baltimore[12]). Several target genes have previously been described for *miR-150* in studies performed on various cell types.[43-47] To describe targets regulated by *miR-150* in CLL B cells, we performed array-based transcriptome profiling of 100 CLL samples stratified based on *miR-150* levels in combination with the computational prediction of its targets. This approach allows for identification of cell type–specific miR targets because of a dominant effect of miRNA on mRNA stability.[11-14,48,49] This approach obviated studies using forced expression of the studied miRNA that can lead to shifts in target mRNAs and off-target effects.[50,54,55] In our analysis, 95% of genes (55/58) were downregulated in cases with high-level expression of *miR-150* ($P < .001$), suggesting that the effects on mRNA stability of high-level expression of *miR-150* were not random. We found that out of the 58 genes, 2 genes (*GAB1* and *FOXP1*) contained evolutionary-conserved binding sides for *miR-150*. The expression of mRNAs for *GAB1* and *FOXP1* decreased with increase in *miR-150* levels. The higher mRNA levels of *GAB1* and *FOXP1* in CLL cases with low *miR-150* expression were also reflected in their higher protein

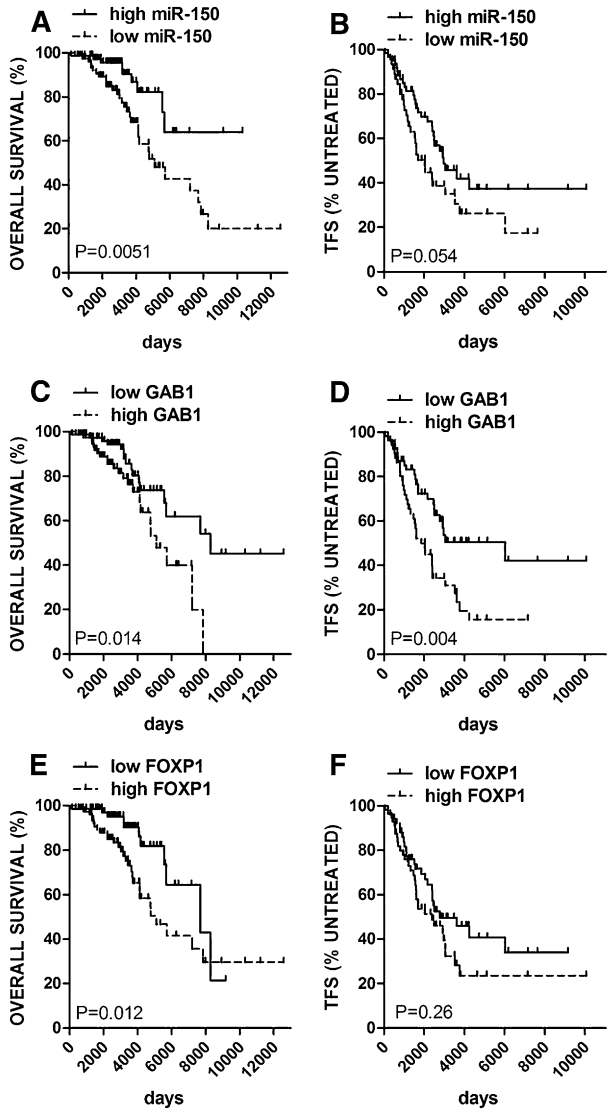

Figure 6. Prognostic significance of *miR-150*, *GAB1*, or *FOXP1*. The OS and TFS are depicted using the Kaplan-Meier curves (with log-rank test) in the CLL cohort divided by median *miR-150* expression (A-B), *GAB1* expression (C-D) and *FOXP1* expression (E-F). The cohort of CLL patients was divided by median expression of studied genes, and analysis included only samples obtained before the last day of follow-up for OS analysis (n = 154) or before first therapy for TFS analysis (n = 107).

levels, and transfection of B cells with synthetic *miR-150* resulted in their down-modulation. The cotransfection of *miR-150* with luciferase construct containing 3′UTR of *GAB1* or *FOXP1* resulted in a ~25% reduction in luciferase activity, confirming that these 2 genes represent newly defined evolutionary conserved targets for *miR-150.* However, we cannot exclude the possibility that some of the other identified differentially expressed genes represent targets of *miR-150* that evolved later in evolution and are not conserved in vertebrates. Additionally, it is possible that the spectrum of preferential targets regulated by *miR-150,* and the extent to which a target mRNA is regulated can vary with the global gene expression changes that are associated with events such as disease progression.[38] Nevertheless, neither *GAB1/FOXP1* nor *miR-150* previously had been identified in the screening of gene networks or miRNAs that change with disease progression.[33,38] In our study, the lower levels of *miR-150* in cases with higher Rai stage (III-IV) could indicate that *miR-150* decreases with disease progression, but more likely reflects that this subgroup of patients contained higher proportion (80%) of cases that used unmutated IGHV and expressed ZAP-70, which are each associated with lower level expression of *miR-150*.

None of the 58 genes identified by our microarray analysis of CLL cases with high vs low levels of *miR-150* overlapped with a known *miR-150* target, which illustrates the importance of identifying cell type–specific miRNA targets.[50] The only previously validated *miR-150* target in B cells is the *c-MYB* gene, which was identified in a mouse model in which the authors noticed that *miR-150* deficiency leads to a phenotype opposite to that caused by deleting *c-MYB*.[47] The B cell–specific deletion of the *c-MYB* gene leads to a block in B cell development at the pro- to pre-B transition.[56] *miR-150* regulates c-MYB levels during the transition of pro-B cell to pre-B cells; ectopic expression of *miR-150* in B cell progenitors could block B-cell development.[47] In our study, the expression of *c-MYB* was not inversely correlated with *miR-150* levels, which is not surprising because the original study had already described that *c-MYB* levels were not regulated by *miR-150* in mature resting B cells.[47] The c-MYB is expressed in pro- and pre-B cells; in mature B lymphocytes, its mRNA expression is kept low and controlled at the transcriptional level.[56,57] Interestingly, the phenotype of mice with manipulated *miR-150* expression supports the regulation of *FOXP1* by *miR-150* in B cells. The mice ectopically expressing *miR-150* in hematopoietic cells share comparable phenotype (pro-B to pre-B transition blockade) not only with *c-MYB* knockout animals, but also with *FOXP1* knockout mice.[47,52,56,58] Overall, our data demonstrate that in the context of peripheral blood CLL cell transcriptome, the *GAB1* and *FOXP1* genes are relevant targets of *miR-150*. However, it is likely that under circumstances that would activate expression of *c-MYB* mRNA in CLL, this mRNA could be targeted by *miR-150.*

The GAB1 and FOXP1 proteins are both known to have important functions in B cells. FOXP1 is a transcription factor that controls B-cell maturation, specifically the expression of genes required for rearranging immunoglobulin subgenes in immature B cells.[52] In malignant B cells, it was shown that its aberrant overexpression is present in cells with ABC in DLBCL and associated with worse survival in DLBCL, follicular lymphoma, or mucosa-associated lymphoid tissue lymphoma.[53,59-61] FOXP1 protein expression is used in some of the immunohistochemical algorithms that distinguish ABC vs germinal center phenotype in DLBCL.[62] In malignant B cells, *FOXP1* expression can be induced by activation of nuclear factor-κB or stimulation through the BCR,[53,59] and its levels have massive effect on the gene expression network in B cells.[63] However, the exact functions of this transcription factor in mature normal or malignant B cells are poorly understood. We found that silencing FOXP1 in B cells reduces their responsiveness to BCR stimulation and in CLL its higher levels are associated with higher responsiveness to BCR stimulation. Furthermore, we noted that silencing FOXP1 down-modulated both the basal and anti-μ–induced levels of phosphorylated AKT. It has been shown recently that other members of the forkhead-box family of proteins regulate basal and induced phospho-AKT levels.[64] Additionally, expression of FOXP1 has pleiotropic effects on multiple cell signaling pathways in B cells including BCR signaling and cell activation.[63] This could at least partially explain association of its high-level expression with poor prognosis in multiple B-cell malignancies.

The functions of GAB1 in modulating the sensitivity of B cells to anti-μ are better described.[51] GAB1 is an adaptor molecule recruiting the PI3K to the B-cell membrane, which allows for the amplification of BCR signaling through activation of AKT.[51] The GAB1 is also involved in other pathways where it serves as a

Table 3. The prognostic significance of *miR-150* in a multivariate analysis

		Multivariate Cox regression of OS n = 108, number of events = 22*						Multivariate Cox regression of TFS n = 76, number of events = 49*					
		Nondelayed entry model			Delayed entry model			Nondelayed entry model			Delayed entry model		
Variable		HR	CI	*P*	HR	CI	*P*	HR	CI	*P*	HR	CI	*P*
miR-150 (≤ vs > median)		**9.6**	1.6-57.8	**.01**	**6.0**	1.2-30.8	**.03**	**2.4**	1.2-4.7	**.014**	**3.6**	1.8-7.4	**.0004**
IGHV (unmut. vs mut.)		5.9	0.4-85.5	.19	2.4	0.1-40.0	.54	1.3	0.5-3.5	.64	0.3	0.1-1.2	.099
ZAP-70 (pos. vs neg.)		8.9	1.5-53.6	.02	28.5	2.1-78.3	.01	2.4	0.8-7.5	.11	5.5	1.6-19.2	.008
CD38 (pos. vs neg.)		1.7	0.5-5.5	.37	1.7	0.5-5.8	.41	3.2	1.4-7.1	.004	3.0	1.2-7.5	.02
Gender (male vs female)		2.6	0.7-9.5	.17	2.8	0.7-10.9	.14	2.1	0.9-4.6	.07	2.0	0.9-4.7	.1
Rai stage	I vs 0	5.2	0.8-35.8	.09	2.3	0.3-18.1	.42	2.9	1.3-6.6	.01	1.1	0.5-2.6	.79
	II vs 0	6.6	0.7-63.5	.10	5.0	0.4-55.2	.19	7.8	2.8-21.6	.0001	2.6	0.9-7.3	.07
	≥III vs 0	3.4	0.5-21.0	.19	2.5	0.4-15.7	.34	4.4	0.8-24.3	.09	2.2	0.4-12.6	.39
Age (> vs ≤ median)		2.4	0.6-9.2	.22	1.8	0.4-7.8	.46	0.5	0.3-1.0	.05	0.7	0.4-1.4	.28
del17p13		4.0	0.8-19.5	.09	3.0	0.5-16.3	.21	1.4	0.5-4.3	.52	3.2	1.1-9.5	.03
del11q23		1.2	0.3-4.4	.81	1.0	0.3-3.4	.97	1.2	0.5-2.9	.75	1.1	0.4-2.8	.9

CI, confidence interval (of 95%); HR, hazard ratio. The values in row miR-150 are boldfaced to highlight results for this gene of interest.

*Analysis includes only samples with known FISH status and obtained before the last day of follow up for OS analysis (108/168) or before first therapy for TFS analysis (76/168). The multivariate analysis for samples including those with unknown FISH status obtained before the last day of follow-up for OS analysis (154/168) or before first therapy for TFS analysis (107/168) is provided in supplemental Table 4.

docking/scaffolding for PLCγ, Crk, and CrkL proteins (reviewed in Sármay et al[65]). In CLL cells, higher *GAB1* levels were associated with strong BCR responsiveness. Silencing of GAB1 in B-cell lines directly affected the amplitude of their response to BCR crosslinking. Importantly, the transfection of B cells with *miR-150* recapitulated this phenotype. Overall, this resembles miRNA's known role in balancing T-cell receptor signaling strength and PI3K signaling in T cells.[13,18,19]

The significance of *miR-150* in CLL B-cell biology is supported by the association of its expression and its targets with the magnitude of BCR signaling observed following surface immunoglobulin ligation. Additionally, lower *miR-150* levels associated with shorter OS and TFS (OS HR: 2.8 [CI 1.3-5.9]; TFS HR: 1.7 [CI 1.0-2.8]). The higher levels of *FOXP1* and *GAB1* in CLL cells associated with shorter OS of these patients (FOXP1 HR: 2.4 [CI 1.2-4.8]; GAB1 HR: 2.3 [CI 1.2-4.4]). This indicates that the regulatory connection between *miR-150* and the identified targets is an important one for the behavior of malignant B cells. However, it is possible that *miR-150* regulates other genes that also contribute to the biology of malignant B cells.

Conceivably inhibition of BCR signaling might also influence the expression of miRNAs (such as *miR-150*), which are involved in the

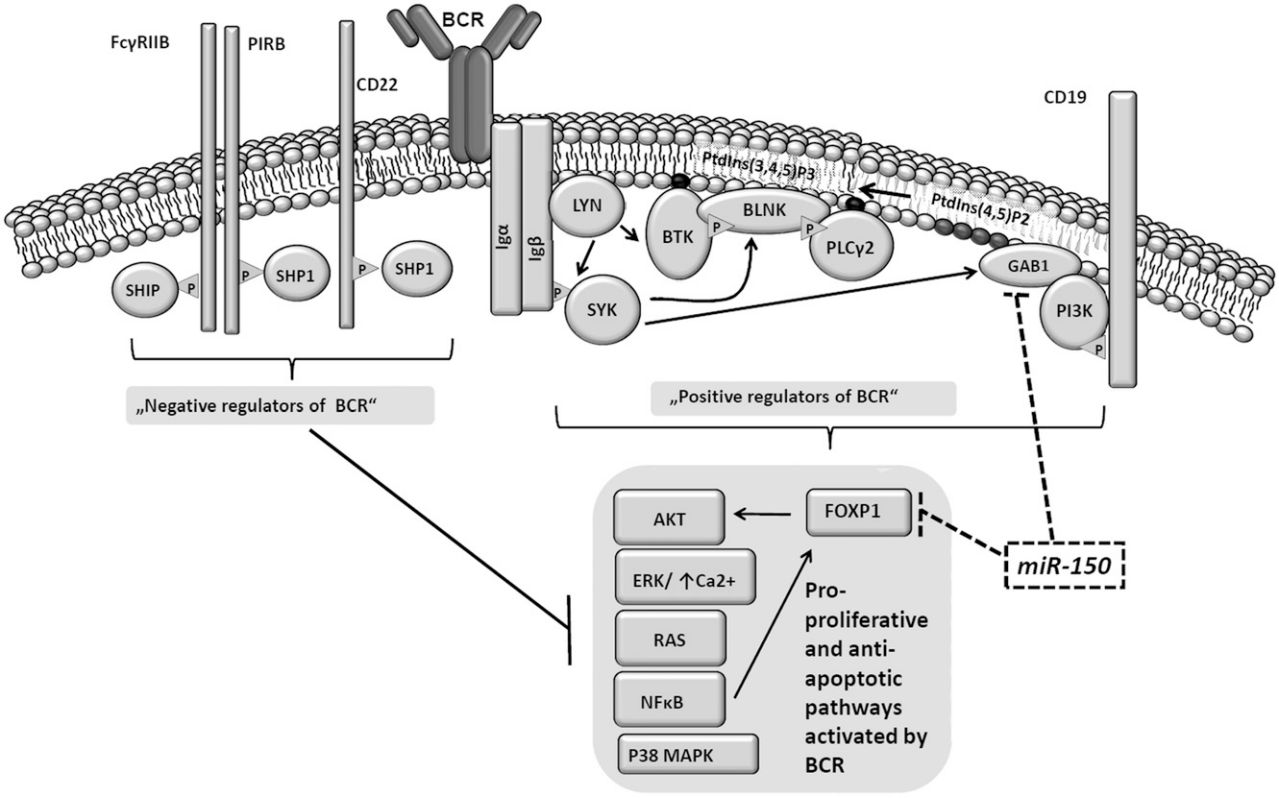

Figure 7. MicroRNA *miR-150* modulates the expression of GAB1 and FOXP1 that are involved in BCR-signaling pathway in CLL B cells.

regulation of BCR signaling. Also, the expression of such miRNAs may impact on the sensitivity of malignant B cells to inhibitors of BCR signaling. Examining CLL cells of patients obtained before and after initiation of therapy with such inhibitors could help address this hypothesis.

In summary, we have used integrated analysis of miRNA and gene-expression profiling to identify 2 novel targets regulated by *miR-150*. Regulation of *GAB1* and *FOXP1* by this miRNA contributes to the sensitivity to BCR ligation in CLL cells and associates with differences in disease prognosis. Our study represents a seminal example of how miRNA can modulate the expression of proteins that contribute to the competency of BCR signaling (Figure 7). It is likely that *miR-150* also will be found capable of influencing the expression levels of *GAB1* and *FOXP1* and BCR signaling, in other B-cell malignancies or normal B cells.

Acknowledgments

This work was supported by the National Institutes of Health Method to Extend Research in Time (grant R37 CA049870-23), National Institutes of Health CLL Research Consortium (grant 2P01 CA081534-12A1) , the University of California-San Diego Foundation Blood Cancer Research Fund, South Moravian Programme for Distinguished Researchers II (SoMoPro II) (cofinanced by the European Union and the South-Moravian Region), CZ.1.07/2.3.00/30.0009 (cofinanced by the European Union and Czech Republic), and the EHA Research Fellowship award granted by the European Hematology Association.

Authorship

Contribution: M.M. designed and performed research, analyzed data, and wrote the manuscript; L.C. performed and analyzed the anti-μ stimulation of CLL; L.Z.R. and E.M.G. provided clinical samples and collected the clinical data; H. L. and K.M. performed the statistical analysis of overall survival and treatment-free survival; K.J., E.N.S., and K.A.F. analyzed the methylation of *miR-150* upstream region; and T.J.K. designed research, analyzed data, and wrote the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: Thomas J. Kipps, University of California-San Diego, 3855 Health Sciences Dr, La Jolla, CA 92093; e-mail: tkipps@ucsd.edu.

References

1. Rassenti LZ, Huynh L, Toy TL, et al. ZAP-70 compared with immunoglobulin heavy-chain gene mutation status as a predictor of disease progression in chronic lymphocytic leukemia. *N Engl J Med.* 2004;351(9):893-901.
2. Döhner H, Stilgenbauer S, Benner A, et al. Genomic aberrations and survival in chronic lymphocytic leukemia. *N Engl J Med.* 2000; 343(26):1910-1916.
3. Damle RN, Wasil T, Fais F, et al. Ig V gene mutation status and CD38 expression as novel prognostic indicators in chronic lymphocytic leukemia. *Blood.* 1999;94(6):1840-1847.
4. Chen L, Apgar J, Huynh L, et al. ZAP-70 directly enhances IgM signaling in chronic lymphocytic leukemia. *Blood.* 2005;105(5):2036-2041.
5. Chen L, Widhopf G, Huynh L, et al. Expression of ZAP-70 is associated with increased B-cell receptor signaling in chronic lymphocytic leukemia. *Blood.* 2002;100(13):4609-4614.
6. Zupo S, Isnardi L, Megna M, et al. CD38 expression distinguishes two groups of B-cell chronic lymphocytic leukemias with different responses to anti-IgM antibodies and propensity to apoptosis. *Blood.* 1996;88(4):1365-1374.
7. Kipps TJ. The B-cell receptor and ZAP-70 in chronic lymphocytic leukemia. *Best Pract Res Clin Haematol.* 2007;20(3):415-424.
8. Stevenson FK, Krysov S, Davies AJ, Steele AJ, Packham G. B-cell receptor signaling in chronic lymphocytic leukemia. *Blood.* 2011;118(16): 4313-4320.
9. Wiestner A. Emerging role of kinase-targeted strategies in chronic lymphocytic leukemia. *Blood.* 2012;120(24):4684-4691.
10. Mraz M, Kipps TJ. MicroRNAs and B cell receptor signaling in chronic lymphocytic leukemia. *Leuk Lymphoma.* 2013;54(8):1836-1839.
11. Dorsett Y, McBride KM, Jankovic M, et al. MicroRNA-155 suppresses activation-induced cytidine deaminase-mediated Myc-Igh translocation. *Immunity.* 2008;28(5):630-638.
12. O'Connell RM, Baltimore D. MicroRNAs and hematopoietic cell development. *Curr Top Dev Biol.* 2012;99:145-174.
13. Henao-Mejia J, Williams A, Goff LA, et al. The microRNA miR-181 is a critical cellular metabolic rheostat essential for NKT cell ontogenesis and lymphocyte development and homeostasis. *Immunity.* 2013;38(5):984-997.
14. Guo H, Ingolia NT, Weissman JS, Bartel DP. Mammalian microRNAs predominantly act to decrease target mRNA levels. *Nature.* 2010; 466(7308):835-840.
15. Vigorito E, Perks KL, Abreu-Goodger C, et al. microRNA-155 regulates the generation of immunoglobulin class-switched plasma cells. *Immunity.* 2007;27(6):847-859.
16. Koralov SB, Muljo SA, Galler GR, et al. Dicer ablation affects antibody diversity and cell survival in the B lymphocyte lineage. *Cell.* 2008;132(5): 860-874.
17. Mraz M, Dolezalova D, Plevova K, et al. MicroRNA-650 expression is influenced by immunoglobulin gene rearrangement and affects the biology of chronic lymphocytic leukemia. *Blood.* 2012;119(9):2110-2113.
18. Ebert PJ, Jiang S, Xie J, Li QJ, Davis MM. An endogenous positively selecting peptide enhances mature T cell responses and becomes an autoantigen in the absence of microRNA miR-181a. *Nat Immunol.* 2009;10(11):1162-1169.
19. Li G, Yu M, Lee WW, et al. Decline in miR-181a expression with age impairs T cell receptor sensitivity by increasing DUSP6 activity. *Nat Med.* 2012;18(10):1518-1524.
20. Klein U, Lia M, Crespo M, et al. The DLEU2/miR-15a/16-1 cluster controls B cell proliferation and its deletion leads to chronic lymphocytic leukemia. *Cancer Cell.* 2010;17(1):28-40.
21. Lu LF, Boldin MP, Chaudhry A, et al. Function of miR-146a in controlling Treg cell-mediated regulation of Th1 responses. *Cell.* 2010;142(6): 914-929.
22. Ventura A, Young AG, Winslow MM, et al. Targeted deletion reveals essential and overlapping functions of the miR-17 through 92 family of miRNA clusters. *Cell.* 2008;132(5): 875-886.
23. Rodriguez A, Vigorito E, Clare S, et al. Requirement of bic/microRNA-155 for normal immune function. *Science.* 2007;316(5824): 608-611.
24. Calin GA, Dumitru CD, Shimizu M, et al. Frequent deletions and down-regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. *Proc Natl Acad Sci USA.* 2002;99(24):15524-15529.
25. Lawrie CH. MicroRNAs and lymphomagenesis: a functional review. *Br J Haematol.* 2013;160(5): 571-581.
26. Mraz M, Pospisilova S, Malinova K, Slapak I, Mayer J. MicroRNAs in chronic lymphocytic leukemia pathogenesis and disease subtypes. *Leuk Lymphoma.* 2009;50(3):506-509.
27. Mraz M, Pospisilova S. MicroRNAs in chronic lymphocytic leukemia: from causality to associations and back. *Expert Rev Hematol.* 2012;5(6):579-581.
28. Calin GA, Ferracin M, Cimmino A, et al. A MicroRNA signature associated with prognosis and progression in chronic lymphocytic leukemia. *N Engl J Med.* 2005;353(17):1793-1801.
29. Stamatopoulos B, Meuleman N, Haibe-Kains B, et al. microRNA-29c and microRNA-223 down-regulation has in vivo significance in chronic lymphocytic leukemia and improves disease risk stratification. *Blood.* 2009;113(21):5237-5245.
30. Rossi S, Shimizu M, Barbarotto E, et al. microRNA fingerprinting of CLL patients with chromosome 17p deletion identify a miR-21 score that stratifies early survival. *Blood.* 2010;116(6): 945-952.
31. Fulci V, Chiaretti S, Goldoni M, et al. Quantitative technologies establish a novel microRNA profile of chronic lymphocytic leukemia. *Blood.* 2007; 109(11):4944-4951.
32. Mraz M, Malinova K, Kotaskova J, et al. miR-34a, miR-29c and miR-17-5p are downregulated in CLL patients with TP53 abnormalities. *Leukemia.* 2009;23(6):1159-1163.
33. Visone R, Veronese A, Rassenti LZ, et al. miR-181b is a biomarker of disease progression

in chronic lymphocytic leukemia. *Blood.* 2011; 118(11):3072-3079.

34. Cimmino A, Calin GA, Fabbri M, et al. miR-15 and miR-16 induce apoptosis by targeting BCL2. *Proc Natl Acad Sci USA.* 2005;102(39):13944-13949.
35. Mott JL, Kobayashi S, Bronk SF, Gores GJ. mir-29 regulates Mcl-1 protein expression and apoptosis. *Oncogene.* 2007;26(42):6133-6140.
36. Pekarsky Y, Santanam U, Cimmino A, et al. Tcl1 expression in chronic lymphocytic leukemia is regulated by miR-29 and miR-181. *Cancer Res.* 2006;66(24):11590-11593.
37. Tili E, Michaille JJ, Luo Z, et al. The down-regulation of miR-125b in chronic lymphocytic leukemias leads to metabolic adaptation of cells to a transformed state. *Blood.* 2012;120(13): 2631-2638.
38. Chuang HY, Rassenti L, Salcedo M, et al. Subnetwork-based analysis of chronic lymphocytic leukemia identifies pathways that associate with disease progression. *Blood.* 2012; 120(13):2639-2649.
39. Mraz M, Malinova K, Mayer J, Pospisilova S. MicroRNA isolation and stability in stored RNA samples. *Biochem Biophys Res Commun.* 2009; 390(1):1-4.
40. Dolezalova D, Mraz M, Barta T, et al. MicroRNAs regulate p21(Waf1/Cip1) protein expression and the DNA damage response in human embryonic stem cells. *Stem Cells.* 2012;30(7):1362-1372.
41. Chen L, Huynh L, Apgar J, et al. ZAP-70 enhances IgM signaling independent of its kinase activity in chronic lymphocytic leukemia. *Blood.* 2008;111(5):2685-2692.
42. Friedman RC, Farh KK, Burge CB, Bartel DP. Most mammalian mRNAs are conserved targets of microRNAs. *Genome Res.* 2009;19(1):92-105.
43. Luo Z, Wen G, Wang G, et al. MicroRNA-200C and -150 play an important role in endothelial cell differentiation and vasculogenesis by targeting transcription repressor ZEB1. *Stem Cells.* 2013; 31(9):1749-1762.
44. Wu Q, Jin H, Yang Z, et al. MiR-150 promotes gastric cancer proliferation by negatively regulating the pro-apoptotic gene EGR2. *Biochem Biophys Res Commun.* 2010;392(3):340-345.
45. Yu ZY, Bai YN, Luo LX, Wu H, Zeng Y. Expression of microRNA-150 targeting vascular endothelial growth factor-A is downregulated under hypoxia during liver regeneration. *Mol Med Rep.* 2013;8(1):287-293.
46. Zhou H, Hasni SA, Perez P, et al. miR-150 promotes renal fibrosis in lupus nephritis by downregulating SOCS1. *J Am Soc Nephrol.* 2013; 24(7):1073-1087.
47. Xiao C, Calado DP, Galler G, et al. MiR-150 controls B cell differentiation by targeting the transcription factor c-Myb. *Cell.* 2007;131(1): 146-159.
48. Xiao C, Rajewsky K. MicroRNA control in the immune system: basic principles. *Cell.* 2009; 136(1):26-36.
49. Bartel DP. MicroRNAs: target recognition and regulatory functions. *Cell.* 2009;136(2):215-233.
50. Akbari Moqadam F, Pieters R, den Boer ML. The hunting of targets: challenge in miRNA research. *Leukemia.* 2013;27(1):16-23.
51. Ingham RJ, Santos L, Dang-Lawson M, et al. The Gab1 docking protein links the b cell antigen receptor to the phosphatidylinositol 3-kinase/Akt signaling pathway and to the SHP2 tyrosine phosphatase. *J Biol Chem.* 2001;276(15): 12257-12265.
52. Hu H, Wang B, Borde M, et al. Foxp1 is an essential transcriptional regulator of B cell development. *Nat Immunol.* 2006;7(8):819-826.
53. Brown PJ, Ashe SL, Leich E, et al. Potentially oncogenic B-cell activation-induced smaller isoforms of FOXP1 are highly expressed in the activated B cell-like subtype of DLBCL. *Blood.* 2008;111(5):2816-2824.
54. Bracken CP, Gregory PA, Kolesnikoff N, et al. A double-negative feedback loop between ZEB1-SIP1 and the microRNA-200 family regulates epithelial-mesenchymal transition. *Cancer Res.* 2008;68(19):7846-7854.
55. Arvey A, Larsson E, Sander C, Leslie CS, Marks DS. Target mRNA abundance dilutes microRNA and siRNA activity. *Mol Syst Biol.* 2010;6:363.
56. Thomas MD, Kremer CS, Ravichandran KS, Rajewsky K, Bender TP. c-Myb is critical for B cell development and maintenance of follicular B cells. *Immunity.* 2005;23(3):275-286.
57. Golay J, Cusmano G, Introna M. Independent regulation of c-myc, B-myb, and c-myb gene expression by inducers and inhibitors of proliferation in human B lymphocytes. *J Immunol.* 1992;149(1):300-308.
58. Zhou B, Wang S, Mayr C, Bartel DP, Lodish HF. miR-150, a microRNA expressed in mature B and T cells, blocks early B cell development when expressed prematurely. *Proc Natl Acad Sci USA.* 2007;104(17):7080-7085.
59. Green MR, Gandhi MK, Courtney MJ, Marlton P, Griffiths L. Relative abundance of full-length and truncated FOXP1 isoforms is associated with differential NFkappaB activity in Follicular Lymphoma. *Leuk Res.* 2009;33(12):1699-1702.
60. Sagaert X, de Paepe P, Libbrecht L, et al. Forkhead box protein P1 expression in mucosa-associated lymphoid tissue lymphomas predicts poor prognosis and transformation to diffuse large B-cell lymphoma. *J Clin Oncol.* 2006;24(16): 2490-2497.
61. Barrans SL, Fenton JA, Banham A, Owen RG, Jack AS. Strong expression of FOXP1 identifies a distinct subset of diffuse large B-cell lymphoma (DLBCL) patients with poor outcome. *Blood.* 2004;104(9):2933-2935.
62. Choi WW, Weisenburger DD, Greiner TC, et al. A new immunostain algorithm classifies diffuse large B-cell lymphoma into molecular subtypes with high accuracy. *Clin Cancer Res.* 2009; 15(17):5494-5502.
63. Sagardoy A, Martinez-Ferrandis JI, Roa S, et al. Downregulation of FOXP1 is required during germinal center B-cell function. *Blood.* 2013; 121(21):4311-4320.
64. Lin A, Piao HL, Zhuang L, Sarbassov DD, Ma L, Gan B. FoxO transcription factors promote AKT Ser473 phosphorylation and renal tumor growth in response to pharmacological inhibition of the PI3K-AKT pathway. *Cancer Res.* 2014;74(6): 1682-1693.
65. Sármay G, Angyal A, Kertész A, Maus M, Medgyesi D. The multiple function of Grb2 associated binder (Gab) adaptor/scaffolding protein in immune cell signaling. *Immunol Lett.* 2006;104(1-2):76-82.