

# Genetic and Epigenetic Silencing of MicroRNA-203 Enhances *ABL1* and *BCR-ABL1* Oncogene Expression

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DOI 10.1016/j.ccr.2008.04.018

## SUMMARY

The mammalian genome contains several hundred microRNAs that regulate gene expression through modulation of target mRNAs. Here, we report a fragile chromosomal region lost in specific hematopoietic malignancies. This 7 Mb region encodes about 12% of all genomic microRNAs, including miR-203. This microRNA is additionally hypermethylated in several hematopoietic tumors, including chronic myelogenous leukemias and some acute lymphoblastic leukemias. A putative miR-203 target, *ABL1*, is specifically activated in these hematopoietic malignancies in some cases as a *BCR-ABL1* fusion protein (Philadelphia chromosome). Re-expression of miR-203 reduces *ABL1* and *BCR-ABL1* fusion protein levels and inhibits tumor cell proliferation in an *ABL1*-dependent manner. Thus, miR-203 functions as a tumor suppressor, and re-expression of this microRNA might have therapeutic benefits in specific hematopoietic malignancies.

## INTRODUCTION

MicroRNAs (miRNAs) are noncoding RNAs 18–25 nt in length that regulate a variety of biological processes by silencing specific target genes (Ambros, 2004). miRNAs are well conserved during evolution, and it has been estimated that about 250–600 miRNAs have been evolutionarily conserved in vertebrates (Bentwich et al., 2005). Additional nonconserved miRNAs have also been characterized in primates, and humans are reported to contain about 800–1000 miRNAs (Bentwich et al., 2005; Zamore and Haley, 2005). Little is known regarding how miRNA expression is regulated in mammalian cells. Primary miRNA transcripts are generated by polymerases II and III, and they are usually capped and polyadenylated (Borchert et al., 2006; Kim and Nam, 2006). Some miRNAs are clustered and transcribed as multicistronic primary transcripts, but many others are not clus-

tered and are transcribed independently (Ambros, 2004; He and Hannon, 2004; Landgraf et al., 2007). These miRNAs can down-regulate various gene products by translational repression when partially complementary sequences are present in the 3' untranslated regions (3'UTR) of the target mRNAs or by directing mRNA degradation. Using these posttranscriptional control mechanisms, mammalian miRNAs appear to target a diversity of cellular functions, including cell proliferation and differentiation (He and Hannon, 2004).

In the last few years, it has become evident that miRNA expression is deregulated in human cancer, resulting in specific oncogenic events reviewed in Calin and Croce (2006) and Esquela-Kerscher and Slack (2006). Specific over- or underexpression has been shown to correlate with particular tumor types (Lu et al., 2005; Volinia et al., 2006). These changes in expression might modulate known oncogenes or tumor suppressors. For

## SIGNIFICANCE

We describe here a tumor suppressor microRNA, miR-203, that is inactivated in human tumors by both genetic and epigenetic mechanisms. This microRNA controls the expression levels of *ABL1*, a classic oncogene extensively characterized in hematopoietic malignancies. Interestingly, miR-203 also controls the expression levels of the *BCR-ABL1* translocation protein produced by the Philadelphia chromosome, a hallmark of chronic myelogenous leukemia (CML) and some B cell leukemias in children. Re-expression of miR-203 dramatically reduces the proliferation of tumor cells in an *ABL1*-dependent manner. These results demonstrate that microRNAs are able to modulate the expression of tumor-specific translocation proteins. In addition, these data suggest complementary therapeutic opportunities in Philadelphia+ malignancies and *ABL1*-overexpressing tumors, including some acute lymphoblastic lymphomas or leukemias.

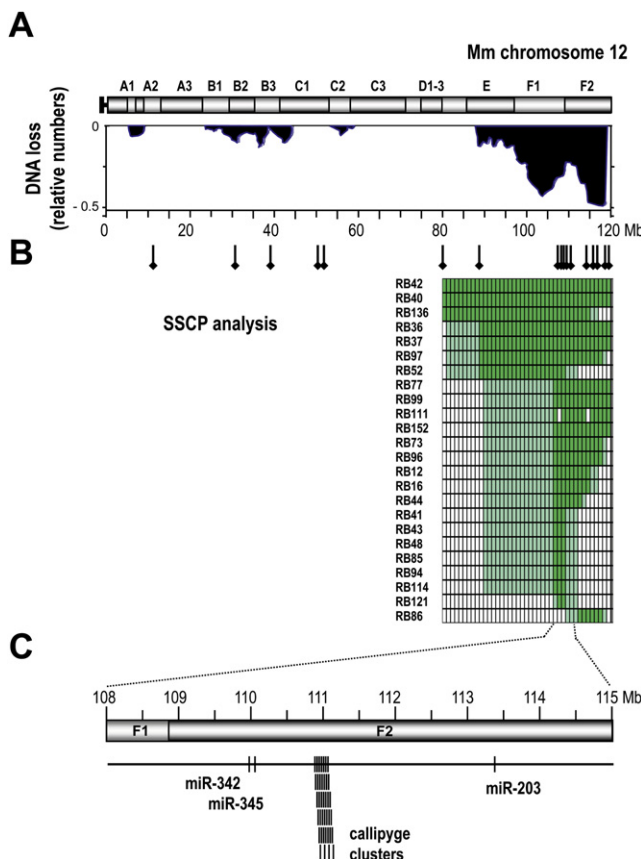
example, let-7, a microRNA that inhibits RAS expression, is downregulated in lung cancer, leading to increased RAS protein levels (Johnson et al., 2005). On the other hand, miR-17-5p and miR-20a modulate the balance between cell death and proliferation in response to the c-Myc oncogene (O'Donnell et al., 2005). In some cases, it has been demonstrated that miRNAs are inactivated by specific genetic or epigenetic alterations. Thus, miR-15a and miR-16-1 are inactivated by deletions or specific translocations in their chromosomal regions (Calin et al., 2002). In fact, microRNAs exhibit a high frequency of DNA copy changes that correlate with altered levels of expression in various human malignancies (Zhang et al., 2006). Other microRNAs are inactivated by epigenetic mechanisms, and their re-expression by epigenetic drugs can lead to downregulation of target oncogenes (Brueckner et al., 2007; Fazi et al., 2007; Lujambio et al., 2007; Saito et al., 2006). Altered expression of miRNAs, moreover, can predict clinical outcome (Calin et al., 2005; Takamizawa et al., 2004; Yanaihara et al., 2006), suggesting that miRNAs play an important role in human cancer.

In this study, we have identified a region of mouse chromosome 12 (F2 region, conserved in human chromosome 14q32) that is frequently lost in T cell malignancies. This chromosomal region is especially rich in microRNAs, since it contains several clusters of microRNAs and expresses 52 mature microRNAs (about 12% of the mammalian miRNA genome: miRBase v 10.0). We have narrowed down the fragile site to a region of about 7 Mb that contains 51 out of these 52 miRNAs. After miRNA expression profiling, we have detected significant silencing of one of these miRNAs, miR-203. Since affected tumors usually lose only one copy of this DNA region, we have investigated whether miR-203 expression is also downregulated by epigenetic mechanisms. We demonstrate here that miR-203 is silenced by the loss of one allele and promoter CpG hypermethylation in the remaining DNA copy. We have further identified *ABL1* as one of the targets of miR-203, indicating that both genetic and epigenetic mechanisms participate in transcriptional silencing of miR-203, leading to upregulation of the *ABL1* and *BCR-ABL1* oncogenes in various murine and human hematopoietic malignancies. Restoration of miR-203 by exogenous transfection or epigenetic drugs results in *ABL1* and *BCR-ABL1* downregulation (similar to that elicited with RNA interference) and decreased proliferation of tumor cells. This antiproliferative effect is partially rescued by overexpression of *BCR-ABL1* and fully rescued by a *BCR-ABL1* cDNA without the endogenous 3'UTR, suggesting that *ABL1* is a crucial target of the tumor suppressor activity of miR-203.

## RESULTS

### A Chromosomal Region Enriched in MicroRNAs Is Frequently Lost in Irradiated T Cell Lymphomas

In a search for genomic alterations throughout the tumor genome, we performed comparative genome hybridization (CGH) of 12  $\gamma$ -radiation-induced lymphomas versus wild-type tissues from C57BL/6J mice. We identified consistent DNA losses in the telomeric region of chromosome 12 (about 50% of samples;  $n = 12$ ). CGH analysis of this fragment (Figure 1A) did not provide resolution enough to analyze putative candidate tumor suppressor genes. We therefore further refined this region using specific



**Figure 1. Chromosomal Losses in Mouse Chromosome 12 by CGH and SSCP Analysis**

(A) DNA copy losses detected by CGH in the murine tumors analyzed. The profile shows the area most frequently lost (cytobands F1 and F2) in these murine T cell malignancies.

(B) Analysis of loss of heterozygosity by SSCP. The different markers used through the complete chromosome 12 are shown as vertical flags. DNA losses in cytobands D3 to F2 are shown by the intense colored boxes whereas less intense boxes indicate blind regions between the closer markers in their boundaries.

(C) The precise location of microRNAs located at the deleted region (108–115 Mb) is indicated by vertical lines. The “callipyge” clusters contain 44 additional microRNAs whose names have been omitted for clarity.

single-strand conformation polymorphism (SSCP) analysis in 44 additional tumor samples using F1 mice generated from crosses between pure C57BL/6J and RF/J inbred mice. These studies demonstrated that the DNA fragment most frequently lost in these tumors corresponds to the chromosome region between markers D12Mit132 and D12Mit18 (chromosome 12 positions 107.4 to 113.7 Mb) (Figure 1B). This region contains about 80 protein-coding genes, including *Bcl11b*, a potential tumor suppressor candidate for this region (Wakabayashi et al., 2003). Interestingly, in addition to these genes, this chromosomal region also contains 52 microRNAs (Figure 1C), accounting for about 12% of the known microRNAs in the mouse genome (Sanger miRBase repository, Release 10.0; August 2007) (Griffiths-Jones et al., 2006). These microRNAs include several clusters located at the callipyge locus, a chromosomal region known to be regulated by genomic imprinting (Davis et al., 2005) and other nonclustered microRNAs.

### Genetic and Epigenetic Alteration of Mouse and Human miR-203

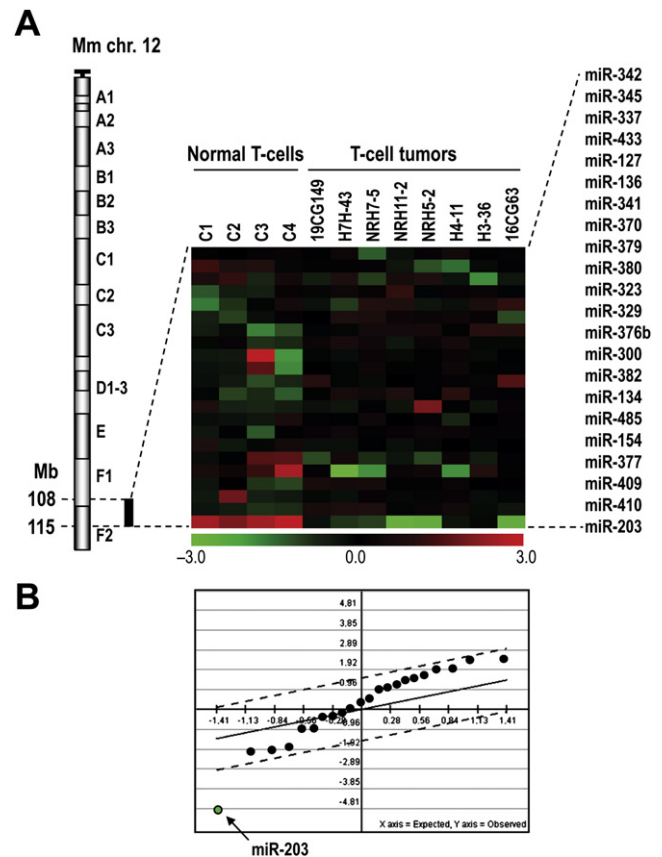
To analyze whether some of these miRNAs were silenced in T cell malignancies, we performed expression profiling using miRNA microarrays (Figure 2). High-stringency analysis of these tumors indicated that only one of these miRNAs, miR-203, was significantly silenced in murine T cell lymphomas (FDR < 0.001;  $\Delta = 1.4$ ; Figure 2B).

Since these murine T cell lymphomas lost expression of miR-203, despite maintaining a normal allele, we analyzed whether this miRNA could be silenced by epigenetic mechanisms. miR-203 presents a clear CpG island in its upstream chromosomal region flanking the transcriptional start site (TSS) similar to that present in many tumor suppressor genes (Figure 3). As a control, we used miR-345, since it also presents a clear CpG island in its upstream chromosomal region and both are located in the chromosome 12 region deleted in these T cell malignancies. After bisulfite conversion of genomic DNA from these samples, we could not detect any methylation at the miR-345 CpG island, in either normal lymphocytes or tumor samples (Figure S1A available online). Similarly to miR-345, miR-203 was not methylated in normal lymphocytes. However, it was significantly methylated in seven out of eight murine T cell lymphoma tumor samples (Figure 3). Detailed analysis by bisulfite sequencing indicated that most of the CpG dinucleotides in this CpG island were methylated in some tumors but not in normal lymphocytes (Figure 3C).

The chromosome 12 region identified in mouse T cell lymphomas is syntenic to a telomeric region in human chromosome 14. Interestingly, all microRNA clusters and genes located at this region in the mouse are conserved in the human genome. In fact, both the miR-203 pre-miR and its putative TSS are highly conserved between human and mouse genomes (Figure 3B). To test whether this epigenetic silencing of miR-203 was also present in human tumors, we analyzed four human T cell tumor cell lines (KARPAS-45, PEER, JURKAT and MOLT-4) and three additional primary peripheral T cell leukemias by methylation-specific PCR (MS-PCR) analysis. In all these human samples, miR-203 was dramatically hypermethylated as detected by MS-PCR (Figure S1B) and bisulfite sequencing (Figure 3D), whereas this sequence was not methylated in human normal T-lymphocytes.

### miR-203 Directly Regulates *ABL1* Expression

Computational prediction of both human and mouse miRNA targets (TargetScanS and PicTar [Rajewsky, 2006]) suggests that miR-203 can modulate more than 300 genes. To further select a functional miR-203 target, we performed massive mRNA expression analysis of mouse T cell lymphomas with silenced miR-203 to identify possible targets upregulated in these tumors. Twenty-two of the putative miR-203 targets are significantly overexpressed in these T cell lymphomas (Figure 4A), including *Abl1*, the murine homolog of *ABL1*, a known oncogene in hematopoietic malignancies (Ren, 2005). In fact, *Abl1* is overexpressed in these T cell tumors as detected by protein analysis, indicating a correlation between *Abl1* overexpression (Figure 4B) and loss of miR-203 in these primary tumors (Figure 4B and Figure S2). Both the murine and human 3'UTR of *ABL1* genes contain miR-203 target sequences with a computed free energy of  $-26.8$  kcal/mol and  $-21.0$  kcal/mol, respectively (Figure 4C). This target site is well conserved in other vertebrates (Figure S3),



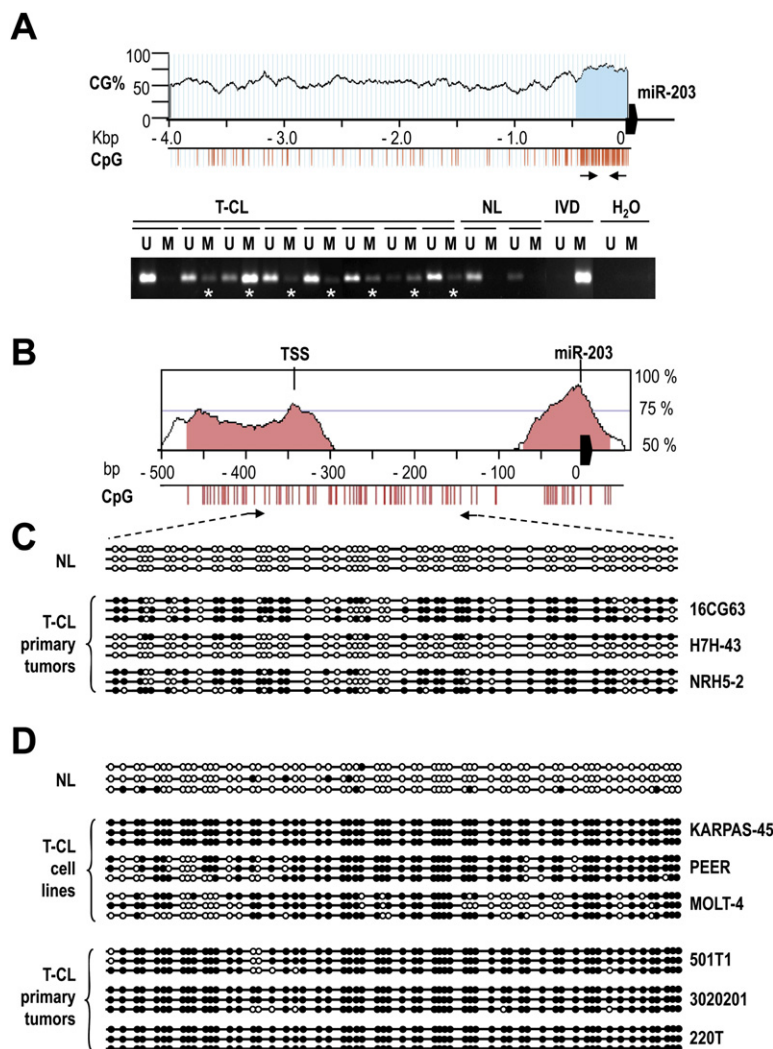
**Figure 2. Microarray Analysis of Chromosome 12 MicroRNA Expression**

(A) Heat map showing the expression of miRNAs located at the deleted region in mouse chromosome 12 (between position 108 Mb and 115 Mb). (B) Significance analysis of microarrays in these samples point to miR-203 as the only miRNA significantly downregulated (FDR < 0.0001) in tumor versus normal samples.

suggesting that miR-203 may control *ABL1* levels in a variety of organisms.

To experimentally validate that miR-203 can target *ABL1*, we analyzed *ABL1* protein levels in human T cell tumor cell lines after re-expression of miR-203. KARPAS-45 or PEER cells—both containing a methylated miR-203 promoter (see Figure 3D)—were infected with retroviral vectors expressing GFP or coexpressing GFP and miR-203. GFP-positive cells were isolated by cytometry and analyzed by immunoblot. As indicated in Figure 5, re-expression of miR-203 results in decelerated growth (doubling time of about 56 hr in both cell lines), compared to control cells infected with the empty retrovirus (doubling time of about 24 hr in both control cultures). This antiproliferative response is accompanied by a dramatic reduction (of about 70%) in *ABL1* levels in these tumor lymphocytes in both KARPAS-45 and PEER cell lines (Figure 5 and Figure S4). Interestingly, the antiproliferative effect elicited by miR-203 is similar to that observed after expressing a pool of four different small-hairpin interfering RNAs specific for the human *ABL1* gene (Figure 5B and Figure S4A), suggesting a specific requirement for *ABL1* in these T cell lymphomas.





**Figure 3. DNA Methylation Analysis of miR-203 in Mouse and Human T Cell Lymphomas and Leukemias**

(A) Methylation-specific (MS-PCR) analysis of the miR-203 upstream region in mouse chromosome 12. The percentage of C+G nucleotides (CG%) and the density of CpG dinucleotides are shown for a region spanning 4 kbp upstream of miR-203. Specific primers for this CpG island were designed (convergent arrows) and used to amplify these DNA fragments in normal and tumor samples. A MS-PCR protocol was followed to specifically amplify unmethylated (U) or methylated (M) DNA. Methylation of miR-203 upstream regions is patent in most tumor samples (asterisks) but not in normal cells. T-CL, T cell lymphomas; NL, lymphocytes from normal thymuses; IVD, DNA methylated in vitro. H<sub>2</sub>O indicates amplification where H<sub>2</sub>O was used instead of lymphocyte DNA.

(B) VISTA plot showing the homology between the mouse and human region upstream miR-203. The two regions with higher homology correspond to the position of miR-203 and the transcriptional start site (TSS) as identified by computer analysis (Saini et al., 2007) and paired-end ditag identification (Ng et al., 2005). Pink areas in VISTA plot indicate the CpG island. The density of individual CpG dinucleotides is also shown in red.

(C) Bisulfite sequencing of the miR-203 upstream region in mouse T cell lymphomas (T-CL) and normal lymphocytes (NL). No CpG dinucleotides are methylated in control samples, whereas some tumors such as 16CG63 and NRH5-2 are heavily methylated.

(D) Similar analysis in human T cell leukemia (T-CL) cell lines or primary tumors. Nonmethylated CpG dinucleotides are shown by an empty circle, whereas methyl-CpG is shown by filled circles. All CpG dinucleotides in a region of about 350 bp upstream of miR-203 are shown. Three representative tumor samples are shown for each group, and three independent clones were sequenced per sample.

Next, we subcloned the *ABL1* 3'UTR downstream of a luciferase reporter vector to analyze whether miR-203 directly targets *ABL1* as predicted from the alignment. In 293 cells, miR-203, but not GFP, is able to reduce luciferase activity in this construct by 30% (Figure 5C) indicating that this microRNA may directly target the *ABL1* 3'UTR. This reduction is abolished by a 3'UTR that contains specific point mutations in the seed region of the miR-203 target sequence (Figure 5C), indicating that miR-203 can directly influence *ABL1* protein levels through specific binding to its 3'UTR.

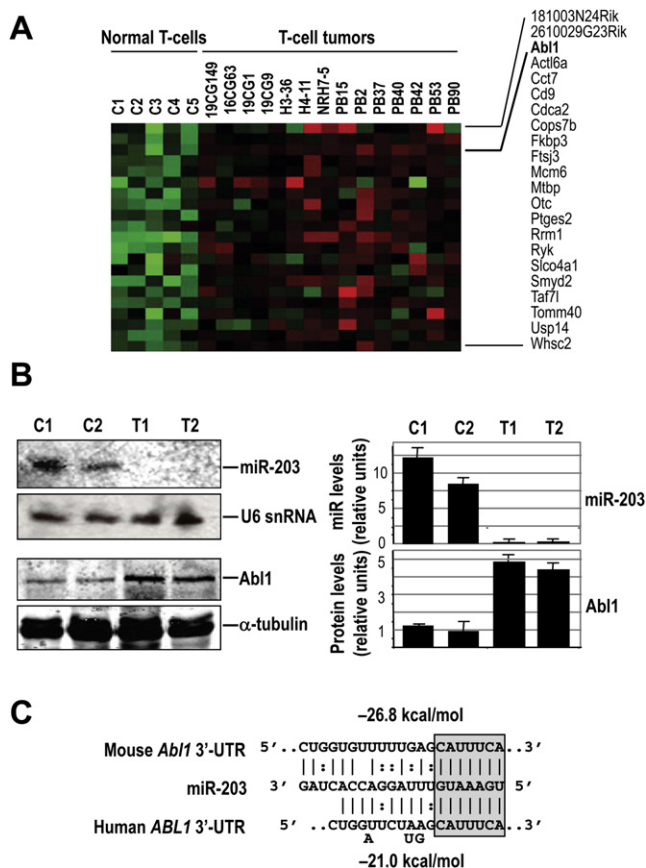
#### Specific Methylation of miR-203 in Philadelphia-Positive Human Leukemias

To further investigate the relevance of miR-203 in the oncogenic activity of *ABL1*, we next analyzed the epigenetic silencing of miR-203 in human tumors carrying a Philadelphia (Ph) chromosome. This alteration produces a *BCR-ABL1* fusion protein that drives tumor development in several malignancies including some B cell acute lymphoblastic leukemias (B cell ALL) in children and chronic myelogenous leukemias (CMLs). As shown in Figure 6 (and Figure S5) the upstream region of the human miR-203 is heavily hypermethylated in most Ph-positive tumors, including B

cell ALLs and both primary CMLs and cultured CML cell lines. This methylation correlates with decrease expression in these Ph-positive tumors (Figure S2B). Interestingly, the miR-203 promoter region is not methylated in other hematopoietic tumors that do not carry *ABL1* alterations. Thus, miR-203 is not significantly methylated in acute myelogenous leukemias (AMLs), chronic myeloproliferative diseases (CMPDs), or additional B cell ALLs that do not express *ABL1* fusion proteins (Figure 6 and Figure S5). These results suggest a specific pressure to downregulate miR-203 in Ph-positive tumors (CMLs and some B cell ALL) or tumors that overexpress *ABL1* (such as T cell lymphomas).

#### Modulation of *ABL1* and *BCR-ABL1* Expression by miR-203 and Epigenetic Drugs

Reintroduction of miR-203 in the CML cell lines K562 and KCL-22 results in restoration of miR-203 expression and concomitant reduction of *ABL1* protein levels (Figure 7 and Figure S4B). These cell lines also express the *BCR-ABL1* fusion protein resulting from the Ph chromosome translocation. Importantly, re-expression of miR-203 also results in reduced *BCR-ABL1* levels (Figure 7A and Figure S4B), likely due to the presence of the *ABL1* 3'UTR in the *BCR-ABL1* fusion transcript. This reduction in



**Figure 4. Correlation between miR-203 Inactivation and *Abl1* Overexpression in T Cell Lymphomas**

(A) Analysis of the expression of putative miR-203 targets by cDNA microarray analysis. Only 22 miR-203 predicted targets, including *Abl1*, are significantly upregulated in these T cell lymphomas as indicated by the significance analysis of microarrays (FDR < 0.001).

(B) Correlation between the downregulation of miR-203 and upregulation of *Abl1* in these tumors. miR-203 expression was detected by northern blot using U6snRNA as a control. *Abl1* expression was monitored at the protein level as detected by immunoblotting.  $\alpha$ -tubulin was used as a loading control. C indicates control (normal thymus) samples, whereas T indicates T cell lymphomas. These data were quantified and normalized with the corresponding controls. Histograms represent mean  $\pm$  SD.

(C) Predicted duplexes formed between miR-203 and the mouse and human 3' untranslated regions (UTR) of *ABL1*. The seed region is highlighted in a gray box. The prediction was calculated using the PicTar algorithm (Krek et al., 2005).

ABL1 and BCR-ABL1 protein levels is accompanied by a dramatic decrease in the proliferation rate in these CML cells (Figure 7B). As in T cells, downregulation of ABL1 and BCR-ABL1 by specific shRNAs results in a similar proliferation arrest. This arrest is accompanied by apoptotic cell death induced by both miR-203 and, more dramatically, ABL1 shRNAs (Figure 7C). To directly analyze whether the translocated ABL1 is a critical target of miR-203 in this antiproliferative activity in CML cells, we exogenously expressed the p210 *BCR-ABL1* fusion gene containing its endogenous 3'UTR downstream of the cDNA. This protein has little effect on K562 or KCL-22 cells (about 10% increase in cell numbers 3 days after transfection; data not shown) but

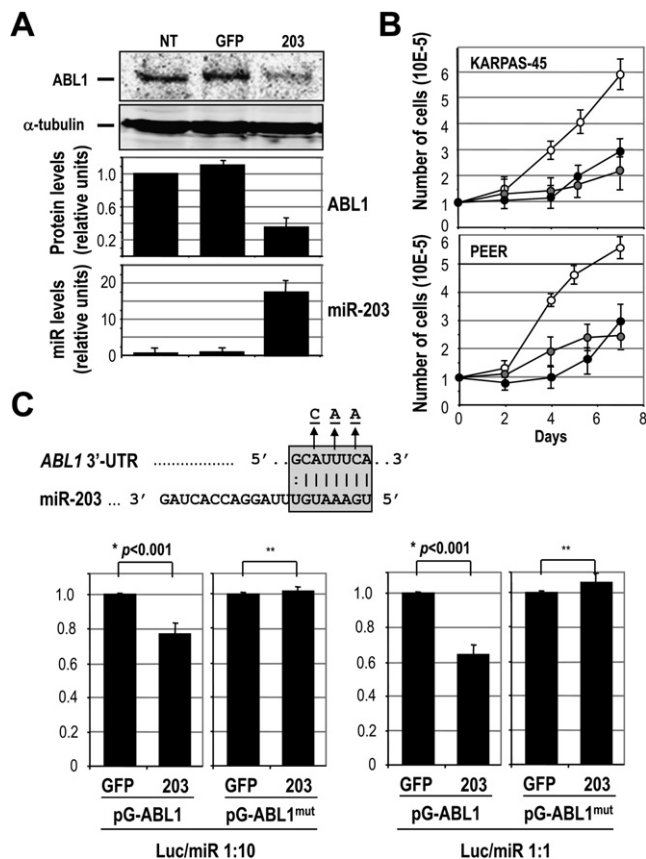
results in a significant rescue from miR-203-induced cell arrest (Figure 7D). As this exogenous *BCR-ABL1* 3'UTR construct contains the miR-203 target site, it probably competes with the endogenous *BCR-ABL1* transcript, reducing the titer of miR-203. We also tested two additional *BCR-ABL1* translocation variants (p210 and p190) devoid of their 3'UTR and therefore not containing miR-203 target sites. As shown in Figure 7D, expression of these miR-203-resistant *BCR-ABL1* cDNAs in K562 and KCL-22 tumor cells fully overcomes the antiproliferative effect of miR-203, resulting in a significant increase in cell proliferation.

Finally, since promoter methylation can be reversed using specific epigenetic drugs, we treated two T cell leukemias, KAR-PAS-45 and PEER, and two Ph-positive CML cell lines, K562 and KCL-22, with 5'-azacytidine (Aza), 4-phenylbutyrate (PBA), or a mixture of both drugs. Whereas single treatments with Aza alone or PBA alone have a minor effect on miR-203 demethylation in all these cell lines (Figures S6 and S7), the combined treatment results in a partial but efficient demethylation of the miR-203 upstream region both in T cell leukemias (Figure S6) and CML cell lines (Figure 8). This combined treatment restores miR-203 expression, and re-expression of this miRNA strongly correlates with a significant reduction in both ABL1 and BCR-ABL1 protein levels (Figure 8 and Figures S6 and S7). These results suggest that epigenetic drugs, in addition to re-expressing other methylated cDNAs, can result in oncogene downregulation mediated by the chemical restoration of miRNA function.

## DISCUSSION

It has been recently reported that miRNA loci are frequently located at fragile sites and suffer frequent genomic alterations in human cancer (Calin et al., 2004; Zhang et al., 2006). As we have described here, loss of heterozygosity (LOH) is relatively frequent in a 7 Mb region surrounding the callipyge locus in  $\gamma$ -radiation-induced T cell lymphomas, a model of neoplasia that resembles human peripheral T cell lymphomas (Melendez et al., 2003). This region, located at chromosome 12 in mice and chromosome 14 in humans, contains some imprinted genes involved in the regulation of muscle and fat biology (Georges et al., 2003). Apart from these genes, this region contains a surprisingly high density of miRNAs, including about 12% of the known mammalian miRNome. Most of these miRNAs are clustered into several transcripts, including six miRNAs at the *anti-Peg11* transcript that participate in the *trans* regulation of the *Rtl1/Peg11* gene (Davis et al., 2005). About 30 additional miRNAs are expressed in about four different transcripts downstream of the callipyge region, although all of these transcripts seem to be maternally expressed and controlled by genomic imprinting (Seitz et al., 2003, 2004). In fact, normal lymphocytes display diverse methylation of a CpG island flanking miR-134, likely as a consequence of the genomic imprinting at this locus (M.J.B. and M.M., unpublished data). The presence of this large number of miRNAs might confer specific susceptibility to chromosomal breaks, and large miRNA clusters have been previously suggested to produce fragile sites (Calin et al., 2004).

Among the miRNAs encoded in this area, miR-203 (located about 2 Mb downstream of the callipyge region) is the only sequence significantly downregulated in these T cell lymphomas. Recent results have indicated that some other microRNAs



**Figure 5. Direct Downregulation of ABL1 by miR-203**

(A) ABL1 protein levels in KARPAS-45 cells expressing GFP (GFP) or GFP + miR-203 (203), indicating a 3-fold reduction of ABL1 levels in the presence of miR-203.  $\alpha$ -tubulin was used as a loading control. ABL1 and  $\alpha$ -tubulin signals were quantified, and their ratio was normalized to that of noninfected cells (NT). miR-203 transcript levels were quantified by real-time quantitative PCR and normalized versus the expression levels in NT KARPAS-45 cells (relative value = 1).

(B) Growth rate of KARPAS-45 and PEER cells expressing miR-203 (filled circles), a pool of shRNAs against ABL1 (gray circles), or the empty vector (open circles).

(C) Luciferase activity in DNA constructs carrying the wild-type ABL1 3'-UTR or mutated versions (mutated residues are underlined) downstream of the luciferase reporter. 293 cells were cotransfected with pG-ABL1 (carrying the luciferase-ABL1 3'-UTR chimera) and a plasmid expressing GFP or miR-203. The reduction in luciferase activity induced by miR-203 expression is reversed after mutation of three specific residues in the seed region of the miR-203 target site. Luciferase activity was normalized for transfection levels as indicated in the experimental procedures. Data shown are representative of separate assays at two different luciferase/miRNA ratios as indicated. \*Significant differences at  $p < 0.001$ ; \*\*no significant differences found. Error bars represent SD.

in this area, such as miR-127, are also deregulated in human tumors by promoter methylation (Saito et al., 2006). We have demonstrated in this report that specific deletions in this region are selected in T cell tumor development where miR-203 is specifically silenced by epigenetics means. Thus, genetic and epigenetic mechanisms coordinately inactivate some specific miRNAs such as miR-203 in these neoplasias.

Expression of miR-203 seems to be restricted to specific cell types. Thus, miR-203 is significantly expressed in the epidermis

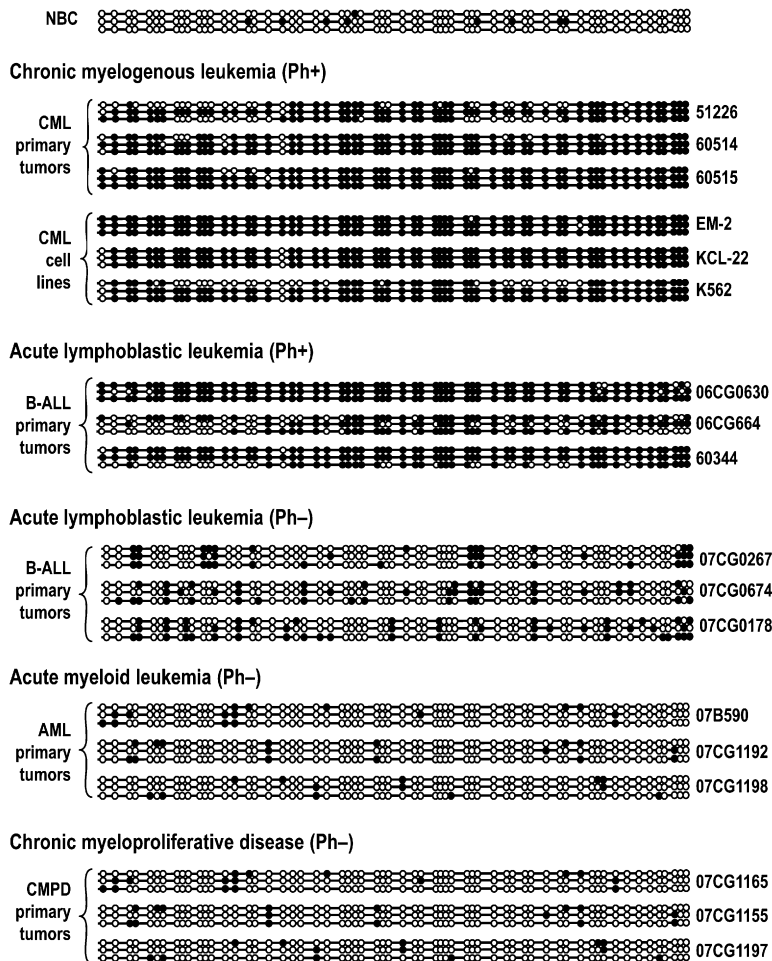
but not in the hair follicles of the skin (Yi et al., 2006). In these cells, conditional deletion of the miRNA processing enzyme Dicer results in decreased expression of miR-203 along with other miRNAs and provokes developmental perturbations in epidermal organization (Yi et al., 2006). miR-203 is not expressed either in primary fibroblasts or in many other established cell lines (M.J.B. and M.M., unpublished data). Moreover, inhibition of miR-203 expression induces increased cell proliferation (Cheng et al., 2005), suggesting an antiproliferative function for this miRNA.

Bioinformatics analyses predict that conserved vertebrate miRNAs target more than 100 to 400 regulatory genes. Among the putative targets of miR-203, we have validated ABL1 as a relevant target, at least in hematopoietic cells. ABL1 is a nonreceptor tyrosine kinase that is expressed in most tissues and participates in the transduction of signals from cell-surface growth factor and adhesion receptors to regulate cytoskeleton structure (Ren, 2005). ABL1 is involved in the development of the hematopoietic system, and its overexpression is associated with the development of diverse hematopoietic malignancies (Lin et al., 2006; Ren, 2005). ABL1 plays an important role in T cell signaling (Zipfel et al., 2004), mediates resistance to apoptosis in T-lymphocytes (Fuchs et al., 1995), and it is overexpressed in T cell tumors, in some cases as a result of chromosomal translocations with amplification as reported in the NUP214-ABL1 fusion (Graux et al., 2004, 2006). As reported in this article, RNAi-mediated downregulation of ABL1 in T cell leukemia cells reduces proliferation of these tumor cultures (Figure 5), suggesting putative therapeutic uses of ABL1 inhibitors in these malignancies.

The BCR-ABL1 fusion is a landmark of CML and it is also present in a percentage of B cell ALL with poor prognosis. All of these diseases, but not other Ph-negative leukemias, are accompanied by promoter methylation and decreased expression of miR-203 (Figures 6 and Supplemental Data), suggesting a proliferative advantage in silencing miR-203 in these tumor cells. It is also interesting to note that the 14q32 region of human chromosome 14 where this miRNA resides is frequently lost in lymphoid blast crisis of CML patients (Dastugue et al., 1986; Sercan et al., 2000). It is therefore tempting to speculate that acute transformation and progression of the disease may be accompanied by inactivation of tumor suppressor genes in this region, including miRNAs such as miR-203.

The recent success of some ABL1-targeted small-molecule kinase inhibitors in CML and other diseases (Baselga, 2006; Ren, 2005) has suggested that inhibition of ABL is critical for tumor treatment in these patients. In fact, CML patients with decreased levels of BCR-ABL1 transcripts have a significantly lower risk of disease progression (Druker et al., 2006). The fact that some miRNAs such as miR-203 can modulate ABL1 levels suggests that restoration of the expression of these miRNA can be beneficial to ALL or CML patients carrying the NUP214-ABL1 or BCR-ABL1 fusions. In addition, T cell lymphoma/leukemia patients with high ABL1 protein levels could also benefit from these treatments. Kinase inhibitors such as imatinib (Gleevec) do not completely eradicate ABL1- or BCR-ABL1-expressing cells from the body, and resistance in some cases emerges as a consequence of point mutations that render resistant isoforms of ABL1 or by amplification and overexpression of the BCR-ABL1 fusion (Shannon, 2002). Since the oncogenic transcripts in these translocations contain the target site for miR-203, our results suggest that restoration of miRNA function





might provide some beneficial effects for imatinib-resistant patients. Our data are also in agreement with the finding that epigenetic drugs such as decitabine display some therapeutic benefits, even in patients resistant to imatinib (Issa et al., 2005).

## EXPERIMENTAL PROCEDURES

### Mouse Colony and Induction of Tumors

C57BL/6J and RF/J F1 hybrid mice and pure C57BL/6J animals were maintained in our animal facilities following the appropriate ethical recommendations from our institutions. For tumor induction, 4-week-old mice of both sexes were exposed to four weekly doses of 1.75 Gy/dose of ionizing gamma radiation (Guerrero et al., 1984) following the protocol approved by our Committee of Bioethics and Animal Care. These animals were observed in a daily basis, and sick mice were euthanized humanely in accordance with the Guidelines for Humane End Points for Animals used in biomedical research. Normal and tumoral tissues were processed for histological analysis (paraffin embedding and hematoxylin and eosin staining) following standard protocols. DNA, RNA, and proteins were isolated from these samples as described below.

### Comparative Genome Hybridization

For DNA preparation thymic lymphomas and normal tissues were cut, minced in small pieces, and placed in a microfuge tube containing 0.5 ml lysis buffer (20 mM Tris-HCl [pH 8.0], 25 mM EDTA, 0.5% SDS, 0.1 M NaCl). Proteinase K was added at a final concentration of 100 mg/ml and incubated at 55°C overnight. The lysate was extracted twice with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1, v/v) (GIBCO-BRL). DNA was precipitated by ad-

### Figure 6. Specific Methylation of miR-203 in Philadelphia-Positive Human Leukemias

Methylation map of the human miR-203 upstream region in Philadelphia-positive (Ph+) tumors, including chronic myelogenous leukemias (CML) and CML tumor cell lines, and B cell acute lymphoblastic leukemias (B cell ALL). Additional leukemias that do not contain rearranged *ABL1* loci such as Philadelphia-negative (Ph-) B cell ALL, acute myeloid leukemias (AML) and other chronic myeloproliferative diseases (CMPD) are also shown. In these Ph- tumors, miR-203 is not significantly methylated. Three representative tumor samples are shown for each group, and three independent clones were sequenced per tumor sample. Normal blood cells were used as a control (NBC).

dition of an equal volume of isopropanol and dissolved in TE buffer (10 mM Tris-HCl [pH 8.0] and 1 mM EDTA).

About 3000 mouse BAC clones with an average spacing of 1 Mb across mouse chromosomes 1 to 19 and the X chromosome (Chung et al., 2004) were printed on hydrophobic glass slide surfaces using an Omni Grid 100 array printer (BioRobotics). The average spacing between the linear map positions of this 3K collection of BAC clones (calculated as the distance between the midpoint positions of two consecutive BACs) is about 0.9 Mb (Chung et al., 2004). For comparative genome hybridization on these BAC microarrays, 1 µg of tumor or normal DNA was first amplified and labeled with aminoallyl-dUTP (Sigma, St Louis, USA) using the Bio-prime random priming kit (GIBCO) and then further labeled with Cy3 or Cy5 monoreactive NHS esters (Amersham, Piscataway, NJ). DNA samples from six nontreated C57BL/6J mice were pooled and used as a normal control. Tumor samples from male animals were hybridized to female pools and vice versa to have an internal control on DNA gains and losses in chromosomes X and Y. In all, 1 µg of labeled tumor DNA and an equal amount of control DNA labeled differentially were mixed with 200 µg mouse Cot I DNA (GIBCO), dis-

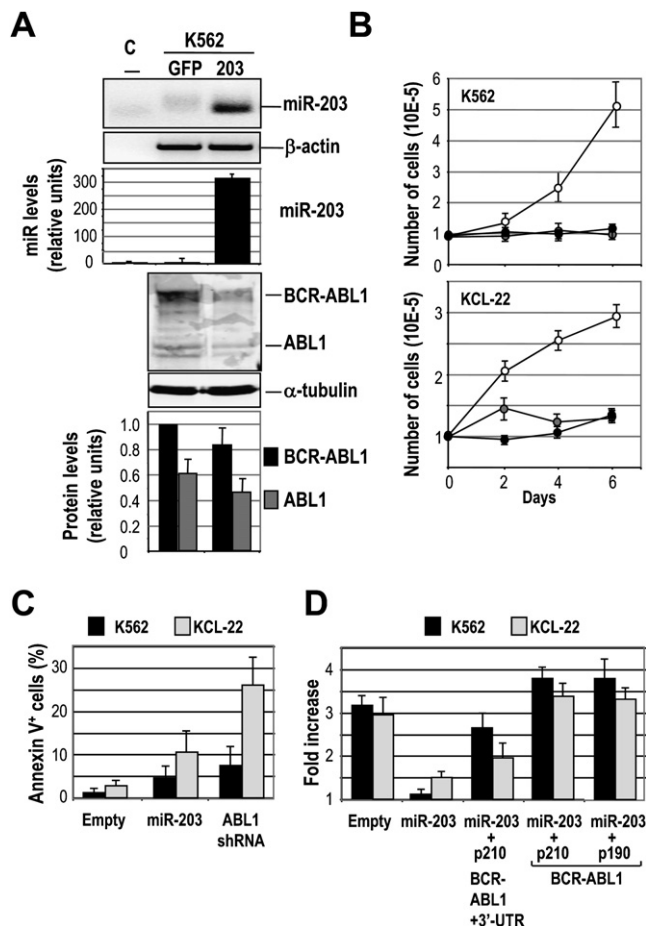
solved in a formamide-based hybridization buffer containing 50% formamide, 2× SSC, 2% SDS, and 10% dextran sulfate, heat denatured and annealed at 37°C to block repeats, and then hybridized to BAC arrays. Hybridization was carried out in a shaker incubator at 37°C for 16–20 hr. Arrays were washed in 2× SSC, 0.1% SDS at 45°C for 60 min and immediately scanned after rinsing with deionized water. Array images were quantified using DNA Microarray Scanner G2565BA (Agilent). Array data have been deposited in the Gene Expression Omnibus (GEO) database under accession number GSE10858.

### LOH Analysis

LOH analysis was carried out by comparing electrophoresis patterns of tumor and control DNA. The following chromosome 12 markers were used according to sequences reported in the Mouse Genome Database: D12Mit240 (position 11.2 Mb), D12Mit136 (30.8 Mb), D12Mit154 (39.8 Mb), D12Mit64 (51.5 Mb), D12Mit110 (52.9 Mb), D12Mit4 (80.0 Mb), D12Mit239 (89.8 Mb), D12Mit132 (108.2 Mb), D12Mit122 (108.4 Mb), D12Mit53 (108.6 Mb), D12Mit123 (110.0 Mb), D12Mit18 (114.4 Mb), D12Mit150 (117.1 Mb), and D12Mit144 (120.2 Mb; all positions refer to NCBI database m37; April 2007; ENSEMBL REL. 48, DEC. 2007). In addition, a specific polymorphism in the *Bcl11b* gene was used at position 109.2 Mb as well as two different SNPs in this region, rs3700933 (position 116.4 Mb) and rs3683037 (119.4 Mb). The specific sequences used are available from the authors per request. PCR products were separated on a 3% agarose, 10% polyacrylamide gel for SSCP analysis.

### MicroRNA and cDNA Expression Analysis

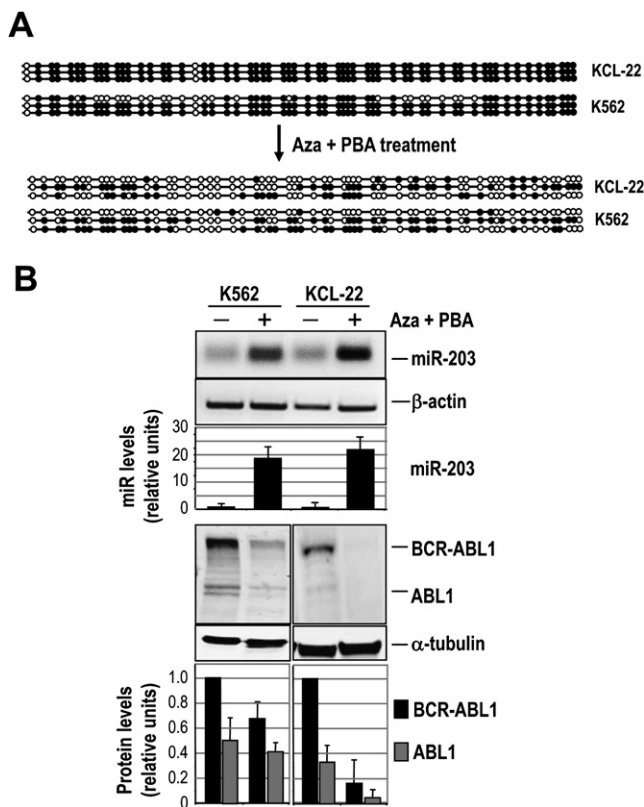
MicroRNA expression profiles were performed essentially as described previously (Liu et al., 2004). Briefly, tumor RNA was isolated using Trizol (Invitrogen),



**Figure 7. Restoration of miR-203 Expression in Ph-Positive Tumor Cells Downregulates ABL1 and BCR-ABL1**

(A) K562 cells were transfected with GFP or miR-203 vectors. Overexpression of miR-203 results in decreased ABL1 and BCR-ABL1 protein levels as detected by immunoblotting. miR-203 transcript levels were quantified by real-time quantitative PCR and normalized versus the expression levels in non-transfected K562 cells (relative value = 1).  $\alpha$ -tubulin was used as a protein loading control. ABL1, BCR-ABL1, and  $\alpha$ -tubulin signals were quantified, and their ratios were normalized to BCR-ABL1 protein levels in GFP-expressing cells. (B) Growth rate of K562 and KCL-22 cells expressing miR-203 (filled circles), a pool of four shRNAs against ABL1 (gray circles), or the empty vector (open circles). (C) Apoptotic cell death after transfection with miR-203 or ABL1 shRNAs in K562 or KCL-22 cell lines. These vectors were cotransfected with a GFP-expressing plasmid, and apoptosis was monitored with Annexin V staining 5 days after transfection. (D) K562 or KCL-22 cells were transfected with an empty vector, a vector expressing miR-203, or a combination of vectors expressing miR-203 and the BCR-ABL1-3'UTR construct or miR-203 and the p210 or p190 forms of BCR-ABL1 that do not contain the endogenous 3'UTR. The overexpression of BCR-ABL1-3'UTR partially rescues the effect of miR-203, whereas the exogenous expression of miR-203-resistant BCR-ABL1 cDNAs fully rescues miR-203-mediated cell proliferation arrest. Three days after transfection, the number of cells was scored and normalized to the original number of cells transfected (fold increase). Error bars represent SD.

and 5  $\mu$ g of RNA was used for hybridization. MicroRNA microarrays have been described previously (Volinia et al., 2006). These microarrays were hybridized in 6 $\times$  SSPE (0.9 M NaCl/60 mM Na<sub>2</sub>HPO<sub>4</sub>·H<sub>2</sub>O/8 mM EDTA [pH 7.4])/30% formamide at 25°C for 18 hr, washed in 0.75 $\times$  TNT (Tris-HCl/NaCl/Tween



**Figure 8. Restoration of miR-203 Expression and Downregulation of BCR and BCR-ABL1 by Epigenetic Drugs**

(A) The CML cell lines K562 and KCL-22 were treated with the epigenetic drugs Aza + PBA, resulting in significant demethylation of the miR-203 upstream region. Three independent clones were sequenced per treated cell line.

(B) Upregulation of miR-203 and concomitant downregulation of ABL1 and BCR-ABL1 protein levels after 5-aza + PBA treatment in K562 and KCL-22 cells. miR-203 transcript levels were quantified by real-time quantitative PCR and normalized versus the expression levels in nontreated K562 or KCL-22 cells (relative value in untreated cells = 1). ABL1, BCR-ABL1, and  $\alpha$ -tubulin signals were quantified, and their ratios were normalized to BCR-ABL1 protein levels in nontreated cells. Histograms in (B) represent mean  $\pm$  SD.

20) at 37°C for 40 min, and processed by using a method of direct detection of the biotin-containing transcripts by streptavidin-Alexa Fluor 647 conjugate. Processed slides were scanned using a microarray scanner, with the laser set to 635 nm, at fixed PMT setting, and a scan resolution of 10  $\mu$ m. The SAM (Statistical Analysis of Microarray) tool included in the TM4 package (<http://www.tm4.org/mev.html>) was used to analyze the statistical significance of microRNA expression data. MicroRNA array data have been deposited in the GEO database under accession number GSE10891.

cDNA microarray experiments were performed using the CNIO RatonChip 23K (v3). This chip contains both the NIA15K and the 7.4K clone sets from the National Institute on Aging (<http://lgsun.grc.nia.nih.gov/cDNA/cDNA.html>), plus 800 additional clones specifically associated with cancer, angiogenesis, apoptosis, signal transduction, and stress processes obtained from either the Fantom1 collection (RIKEN) or the I.M.A.G.E. Consortium (Research Genetics, Huntsville, AL). About 3000 clones are duplicated to reach a total of 27,648 spots, which included 166 spots containing nonmurine DNA as negative controls. Total RNA was extracted by combination of Trizol reagent (Invitrogen) and purification using the RNeasy Protect kit (QIAGEN, Hilden). The quality of the RNA was evaluated using the BioAnalyzer system (Agilent). Twenty-five micrograms of the test or reference amplified RNAs were labeled with fluorescent Cy5 and Cy3 (Amersham, Sunnyvale), respectively, using the SuperScript II RNase H Reverse Transcriptase Kit (Invitrogen, USA).



Hybridizations were performed at 55°C for 17 hr using the SlideHyb Microarray Hybridization buffer (Ambion). In all microarray experiments, each sample was cohybridized with a pool of amplified RNAs obtained from the Universal Mouse RNA (Stratagene, La Jolla, CA), used as a reference. After washing, the slides were scanned in an Agilent G2565BA scanner. Images were then analyzed with GenePix 5.1 (Axon Instruments, Inc., Union City, CA) and the TM4 software. Microarray data have been deposited in the GEO database under accession number GSE10859, and the entire set of T cell lymphoma arrays can be accessed under GSE10861.

To validate expression of specific transcripts, total RNA from tumors and cell lines was isolated using Trizol (Invitrogen). Expression of miR-203 in mouse tumors was analyzed by northern (NorthernMax, Ambion) or RNA protection (mirVana, Ambion) assays using a miR-203-specific probe (see [Supplemental Experimental Procedures](#)). 6UsnRNA and miR-16+4 were used as controls in northern hybridizations and RNA protection assays, respectively. The expression levels of individual microRNAs were quantified using the mirVana qRT-PCR miRNA detection kit following the manufacturer's recommendations (Ambion). GAPDH served as normalization control. In addition, real-time quantitative amplification of miR-203 was performed in triplicate with the TaqMan MicroRNA assays kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions in an Applied Biosystems 7900HT Fast Real-Time PCR apparatus. Amplification of RNU19 was used for normalization. The data analysis was done using the SDS (Sequence Detection Systems) 2.2.2 program (Applied Biosystems, Foster City, CA).

#### DNA Methylation Analysis

The following human cell lines were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ): JURKAT, MOLT-4, KARPAS-45, and PEER (T cell acute lymphoblastic leukemias; T cell ALL); K562, KCL22, and EM-2 (CMLs). Primary human T cell ALLs (501T1, 3020201, and 220T), CMLs (51226, 60514, 60515, 07CG1139, 07CG1187, 07CG1212, 07CG1257, and 08B0061), B cell ALLs (06CG664, 06CG630, 60344, 07CG0144, 07CG0178, 07CG0223, 07CG0267, 07CG0331, 07CG0354, 07CG0356, 07CG0407, 07CG0674, 07CG0689, 07CG0750/0806, 07CG0751, 07CG0758, 07CG0912, 07CG0956, 07CG0995, 07CG1001, 07CG1035, 07CG1092, 07CG1214, 08CG0100, and 08CG0115), acute myelogenous leukemias (AMLs: 07B1590, 07CG1192, and 07CG1198), or chronic myeloproliferative diseases (CMPDs: 07CG1165, 07CG1155, and 07CG1197) were obtained from our repository or were kindly provided by J. Benitez, R. Villuendas, or M.A. Piris (CNIO, Madrid). Leukemic bone marrow samples were obtained by the Cytogenetics Diagnostics Laboratory at our Center under our approved protocol that complies with the recommendations included in the Spanish Law for Clinical Diagnostic Laboratories. All used samples had previously informed consent, corresponded to the remaining exceeding material after diagnosis, and were anonymously coded. For methylation analysis, specific oligonucleotides were designed using the MethPrimer ([Li and Dahiya, 2002](#)) and Methyl Primer Express Software v1.0 (Applied Biosystems) to amplify methylated or unmethylated mouse or human microRNA promoters ([Table S1](#)). Analysis of DNA methylation by MS-PCR or bisulfite sequencing was performed essentially as described previously ([Saito et al., 2006](#)). Briefly, genomic DNA (1 µg) was converted with sodium bisulfite as previously reported ([Frommer et al., 1992](#)). After amplification of the bisulfite-converted DNA, methylation levels were analyzed by sequencing of the bisulfite-modified promoter regions.

For demethylation studies, cells were seeded at  $4 \times 10^5$  cells per 10 cm dish 24 hr prior to the treatment with 5 µM 5'-azacytidine (Aza; Sigma-Aldrich, St. Louis, MO), 3 mM 4-phenylbutyrate (PBA; Sigma-Aldrich), or a combination of both drugs. Aza was given at the indicated doses continuously for 72 hr. After 24 hr of recovery, PBA was given at the indicated doses for 2 days in the combined protocol.

#### DNA Transfection and Retroviral Infection

Cultured cells were maintained at logarithmic growth in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, and 100 units/ml each of penicillin G and streptomycin. To analyze the effect of DNA demethylation, cells were incubated for 3 days with 1–5 µM 5-aza-2'-deoxycytidine (5-aza; Sigma-Aldrich, St. Louis, MO) and/or 1–3 mM 4-phenylbutyric acid (PBA; Sigma-Aldrich). To re-express miR-203 in T cells, we used a GFP-expressing MSCV-driven retroviral vector (a gift from S. Gon-

zález and M. Serrano; CNIO, Madrid) or a pBabe-puro-derivative vector containing a 400 bp genomic region around the hsa-miR-203 locus. These vectors were transfected into 293T cells, and the resulting viruses were incubated on KARPAS-45 and PEER cells. Three days after infection, GFP-positive cells were isolated by FACS (Becton Dickinson), and the selected cells were used for cell proliferation assays or immunoblotting on protein lysates. K562 and KCL-22 cells were transfected with pBabe-puromycin vectors using a nucleofector apparatus (Amaxa Biosystems, USA) following the manufacturer's recommendations. p-Babe-puromycin empty vector was used as a control. Twenty-four hours after nucleofection, cells were selected with 2 µg/ml of puromycin during 5 days. After selection cells were maintained in 1 µg/ml of puromycin and were used for cell proliferation assays. To rescue the antiproliferative effect of miR-203, cultured cells were cotransfected with EGFP-expressing MSCV-driven retroviral vectors that express the BCR-ABL1 fusion proteins (p210 and p190) (a gift from Veronika Sexl, Medical University of Vienna; and Isidro Sánchez-García, CIC-Salamanca) and with a pBabe-puromycin vector expressing miR-203 using nucleofector technology. Cells were maintained in puromycin selection during 5 days, and then EGFP-positive cells isolated by FACS were used to determine the cell growth rate. For RNA interference, four different vectors that express small-hairpin RNAs against the human *ABL1* were used (see [Supplemental Experimental Procedures](#) and [Table S2](#)). Apoptotic cell death was monitored using an Annexin V staining kit (PharMingen BD Biosciences) following the manufacturer's recommendations.

#### Protein Analysis and Luciferase Assays

Protein lysates were obtained from tissues or cells using RIPA Cell Lysis buffer (150 mM NaCl, 50 mM [pH 8] Tris HCl, 0.5% deoxycholate sodium, 0.1% sodium dodecyl sulfate, 1% Triton X-100, 1 mM EDTA, and 1 mM EGTA) and the recommended amounts of Protease Inhibitor Cocktail, Phosphatase Inhibitor Cocktail 2 (Sigma, St. Louis, MO), and Benzamide (Novagen, Darmstadt, Germany). Samples were spun at  $20,000 \times g$  at 4°C for 15 min, and the supernatant was stored at –80°C or immediately quantified using a protein assay (Bio-Rad). Seventy micrograms of protein lysates were loaded onto 8% polyacrylamide-SDS gels. Proteins were transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA) and probed with ABL1 antibodies (Calbiochem, Darmstadt, Germany). In addition, anti- $\alpha$ -tubulin antibody (Sigma, St. Louis, MO) was used as a loading control. After washing, blots were incubated with the appropriate secondary antibodies coupled to Alexa Fluor 680 or 800 (Invitrogen). Subsequently, the membrane was scanned using the Odyssey Infrared Imaging System (Li-Cor Biosciences).

Luciferase constructs were made after amplification of the ABL1 3'UTR and subcloning in a modified pGL3-Control vector (Promega). 293 or HeLa cells were transfected with 0.3 µg of firefly luciferase reporter vector containing the ABL1 3'UTR and 0.3 µg of the control vector containing Renilla luciferase pRL-CMV (Promega), using Effectene (QIAGEN). Three micrograms of pMSCV-203 or the empty vector were used to analyze the effect of microRNA expression on luciferase signal. Luciferase assays were performed 48 hr after transfection using the Dual Luciferase Reporter Assay System (Promega). Firefly luciferase was normalized to Renilla luciferase activity.

#### ACCESSION NUMBERS

Array data have been deposited in the Gene Expression Omnibus (GEO) database (<http://www.ncbi.nlm.nih.gov/geo/>) under accession number GSE10858. MicroRNA array data have been deposited in the GEO database under accession number GSE10891. Microarray data have been deposited in the GEO database under accession number GSE10859, and the entire set of T cell lymphoma arrays can be accessed under GSE10861.

#### SUPPLEMENTAL DATA

The Supplemental Data include Supplemental Experimental Procedures, seven supplemental figures, and two supplemental tables and can be found with this article online at <http://www.cancer-cell.org/cgi/content/full/13/6/496/DC1/>.

## ACKNOWLEDGMENTS

We thank Susana Temiño for excellent technical assistance, M. Guerrero for CGH analysis, and Amaia Lujambio for help with expression assays. We also thank I. Sánchez-García, J. Benítez, R. Villuendas, M.A. Piris, V. Sexl, S. González, and M. Serrano for providing reagents and samples. M.J.B. was supported by a fellowship from the Spanish Ministry of Education and Science. I.P.d.C. is supported by a Ramón y Cajal contract. This work was funded by grants from the Association International for Cancer Research (AICR #08-0188) to M.M.; the European Commission (EURATOM 2003, F16R-CT-2003-508842 to J.S.); the Human Factor, Mobility, and Marie Curie activities of the European Commission (017448-IRG7 to I.P.d.C.); the Foundation Ramón Areces (to I.P.d.C.); the Foundation of the Asociación Española contra el Cáncer (AECC; to M.M.); Foundation Mutua Madrileña Automovilista (to M.M.); and the Ministry of Education and Science (MEC; SAF2006-09437 to J.F.-P., SAF2004-07459 and SAF2007-64571 to I.P.d.C., SAF2005-04340 to J.C.C., and SAF2006-05186 to M.M.). The Cell Division and Cancer Group of the CNIO is supported by the OncoCycle Programme (S-BIO-0283-2006) from the Comunidad de Madrid, and the OncoBIO Consolider-Ingenio 2010 Programme (CSD2007-00017) from the MEC, Madrid.

Received: August 31, 2006

Revised: January 22, 2008

Accepted: April 2, 2008

Published: June 9, 2008

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