

eventually expand the niche to an extent sufficient to support a maximal antibody response (Supplementary Figure S5 is available at *International Immunology* Online). Although medullary remodeling normalizes by 21 days post-immunization in a model of acute inflammation, the remodeled state would likely be sustained in the context of chronic inflammation. Given that medullary remodeling potentially regulates the size of the plasma cell pool in inflamed LNs, the mechanism for the maintenance and contraction of medullary remodeling presents as an attractive target for the suppression of undesirable antibody responses.

In conclusion, we have described a new role for non-cognate B cells in the humoral immune response, as potential regulator of the size of the plasma cell niche in inflamed LNs. Further understanding of the cellular and molecular mechanisms of the induction and normalization of medullary remodeling will pave the way for the design of novel therapeutic approaches against allergic and autoimmune diseases through modulation of the LN plasma cell niche.

Supplementary data

Supplementary data are available at *International Immunology* Online.

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Polycomb-Mediated Loss of miR-31 Activates NIK-Dependent NF- κ B Pathway in Adult T Cell Leukemia and Other Cancers

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SUMMARY

Constitutive NF- κ B activation has causative roles in adult T cell leukemia (ATL) caused by HTLV-1 and other cancers. Here, we report a pathway involving Polycomb-mediated miRNA silencing and NF- κ B activation. We determine the miRNA signatures and reveal miR-31 loss in primary ATL cells. MiR-31 negatively regulates the noncanonical NF- κ B pathway by targeting NF- κ B inducing kinase (NIK). Loss of miR-31 therefore triggers oncogenic signaling. In ATL cells, miR-31 level is epigenetically regulated, and aberrant upregulation of Polycomb proteins contribute to miR-31 downregulation in an epigenetic fashion, leading to activation of NF- κ B and apoptosis resistance. Furthermore, this emerging circuit operates in other cancers and receptor-initiated NF- κ B cascade. Our findings provide a perspective involving the epigenetic program, inflammatory responses, and oncogenic signaling.

INTRODUCTION

Adult T cell leukemia (ATL) is an aggressive T cell neoplasm with very poor prognosis (Yamaguchi and Watanabe, 2002). Human T cell leukemia virus type I (HTLV-I) is recognized as an etiological factor in T cell malignancy. Although mounting molecular evidence has contributed to our ability to cure several cancers and other diseases, the genetic background of ATL leukemogenesis is not yet fully understood. Thus, it is an urgent request to clarify the molecular mechanism of ATL development.

Constitutive activation of nuclear factor- κ B (NF- κ B) is observed in the ATL cell lines and primary isolated tumor cells from ATL patients, although the viral oncoprotein Tax, a powerful activator of NF- κ B, is not expressed in these malignant cells

(Hironaka et al., 2004; Watanabe et al., 2005). NF- κ B activation aberrantly contributes to cell propagation and anti-apoptotic responses in ATL and other cancers (Prasad et al., 2010). In our previous study, inhibition of NF- κ B activity with a specific inhibitor, DHMEQ, drastically impaired the levels of ATL cell growth and resistance to apoptosis (Watanabe et al., 2005), suggesting that the molecular background of aberrant NF- κ B activation may give us potential therapeutic targets. A recent report provided a new readout that NF- κ B-inducing kinase (NIK) has a causal role in tumor progression and the aggressive phenotypes of various cancers, including ATL (Saitoh et al., 2008). NIK plays a pivotal role in the noncanonical (alternative) NF- κ B pathway as a crucial kinase in receptor-initiating signaling, including signaling from CD40, LTBR, and BAFFR.

Significance

Here, we propose a molecular perspective of the onset of oncogenic signaling. NIK overexpression is a major driving force for constitutive NF- κ B activation in various types of cancers. Using ATL cells as a model of NF- κ B-addiction, we identified miR-31 as a suppressor of NIK that is completely silenced in ATL cells. Furthermore, an oncogenic function of a subset of Polycomb is implicated in NF- κ B signaling via miRNA regulation. This study introduces a fundamental link between the Polycomb-mediated epigenetic regulation and the NF- κ B signaling, allowing us to attribute the constitutive activation of NF- κ B to epigenetic reprogramming.

Several studies have recently implicated another functional significance of NIK protein in epithelial cell proliferation, inflammatory response, and oncogenic signaling (for review, see Thu and Richmond, 2010). Although the expression level of NIK is strictly maintained by proteasomal degradation in normal cells (Liao et al., 2004), increased level of NIK transcript are observed in some cancers, causing inappropriate accumulation of NIK protein without stimuli (Annunziata et al., 2007; Saitoh et al., 2008). Overexpression of NIK leads to aberrant phenotypes in several cell types; however, little is known about the abnormal accumulation of NIK in malignant cells.

Recent advances have led to deeper understanding of a new aspect of posttranscriptional gene regulation, i.e., regulation by a class of noncoding RNAs. MicroRNAs (miRNAs) are functional RNAs with 18–25 nt in length that contribute to a class of cellular functions by negatively controlling targeted gene expression via base-pairing to 3' untranslated region (3' UTR). A single miRNA regulates the expression of multiple genes, and the functions of miRNAs therefore need to be orchestrated for cellular homeostasis (Ventura and Jacks, 2009). In the context of cancer pathology, many studies have provided evidences that miRNAs can act as either oncogenes or tumor suppressors. Although the relationship between miRNA deregulation and oncogenes has been clarified in several cancer cells, there has been no integrated analysis of gene expression in ATL. Since miRNAs have important functions in living cells, miRNA expression needs to be tightly regulated. Our knowledge about the regulatory mechanisms of miRNA expression is very inadequate because research effort has focused mainly on the role of miRNAs, which remains one of the most intriguing questions. miRNA regulation involves multiple steps. miRNA maturation has been identified as an important step, and its deregulation leads to progression and development of cancer (Davis et al., 2008; Trabucchi et al., 2009). Genetic deletion in cancer cells has also been reported to account for specific miRNA defect (Varambally et al., 2008). In addition, miRNA expression seems to be epigenetically programmed. DNA methylation and histone modification are strong candidates for miRNA regulation and their abnormalities, therefore, have causal roles in cancer initiation, development, and progression. In particular, Polycomb group proteins have central functions in cellular development and regeneration by controlling histone methylation, especially at histone H3 Lys27 (H3K27), which induces chromatin compaction (Simon and Kingston, 2009). Recent studies have revealed that the amount of Polycomb family is closely associated with cancer phenotypes and malignancy in breast cancer, prostate cancer, and other neoplasms (Sparmann and van Lohuizen, 2006). However, the substantial status of Polycomb family and their epigenetic impact in ATL cells have not been documented. Furthermore, the general roles of Polycomb proteins in miRNA regulation are mostly unknown. As described above, since miRNAs are multifunctional molecules in gene regulation, it is of pivotal importance to clarify the miRNAs functions and their regulatory circuit in order to formulate therapeutic strategies.

In the present study, we first performed global miRNA and mRNA profilings of the ATL cells derived from patients to precisely define the significance of miRNA expressions and functions.

RESULTS

miRNA Expression Signature in Primary ATL Cells

To characterize the miRNA expression signature in the primary ATL cells, we first performed an miRNA expression microarray analysis. For results with physiological significance, we used total RNA prepared from clinical ATL samples ($n = 40$, Table S1 available online) and control CD4+ T cells from healthy donors ($n = 22$) aged 50–70 years. A strict threshold ($p < 1 \times 10^{-5}$) and two-dimensional hierarchical clustering analysis revealed 61 miRNAs that showed significantly altered levels of expression in ATL cells compared with those of control CD4+ T cells (Figure 1A). It is noteworthy that 59 miRNAs out of 61 (96.7%) showed decreased expression in the primary ATL cells. Among them, we identified miR-31 as one of the most profoundly repressed miRNAs in all ATL individuals (fold change, 0.00403; Figure 1B). miR-31 was recently reported as a tumor suppressor and/or metastasis-associated miRNA in metastatic breast cancer. However, the biological functions of miR-31 in lymphocytes have not been studied. We therefore focused on the biological significance and regulatory mechanisms of miR-31 expression in T cells as well as in solid cancers.

miR-31 Negatively Regulates NF- κ B Signaling via NIK Expression

To study the functional significance of miR-31 loss, we attempted to identify the target genes of miR-31 using four computational algorithms. We also performed gene expression microarray analysis of the primary ATL cells ($n = 52$, Table S1) and normal CD4+ T cells ($n = 21$) in order to detect aberrations in gene expression. Selected putative target genes are known to be involved in cell-cycle regulation and T cell development (Table S2). To experimentally identify the target genes, we performed reporter-based screens as described below. Luciferase-3' UTR reporter assays demonstrated a remarkable negative effect against upstream gene expression by the *MAP3K14* 3' UTR sequence (Figure S1B), which is consistent with an initial cloning report (Malinin et al., 1997). MAP3K14, also known as NIK, has a central role in noncanonical NF- κ B signaling by phosphorylation of IKK α . A previous report (Saitoh et al., 2008) and the present results (Table S2) show that NIK is overexpressed in ATL cells, leading to constitutive NF- κ B activation. As shown in Figure 2A, treatment with a miR-31 inhibitor increased *NIK* 3' UTR reporter activity, suggesting the involvement of endogenous miR-31 in NIK downregulation. A computational search predicted one site each of miR-31 and miR-31 antisense (miR-31*) binding sites in the *NIK* 3' UTR (Figure 2B). To identify the regulatory sequence in 3' UTR of *NIK*, we established additional two reporters with mutated sequence in each potential seed region (Figure 2C; Figure S1C). Mutant 1, which contains mutated sequence in the miR-31 seed region, partially canceled the negative effect of endogenous miR-31 (Figure S1D) and prevented the effect of Anti-miR-31 treatment (Figure 2D). On the contrary, our results suggest that miR-31* does not participate in NIK regulation. miR-31-mediated reporter regulation was also observed in T cell lines (Figure S1E). To confirm the results, we repeated the experiment to examine whether miR-31 could inhibit NIK expression through seed sequence. We made expression plasmid vectors carrying NIK, NIK-3'

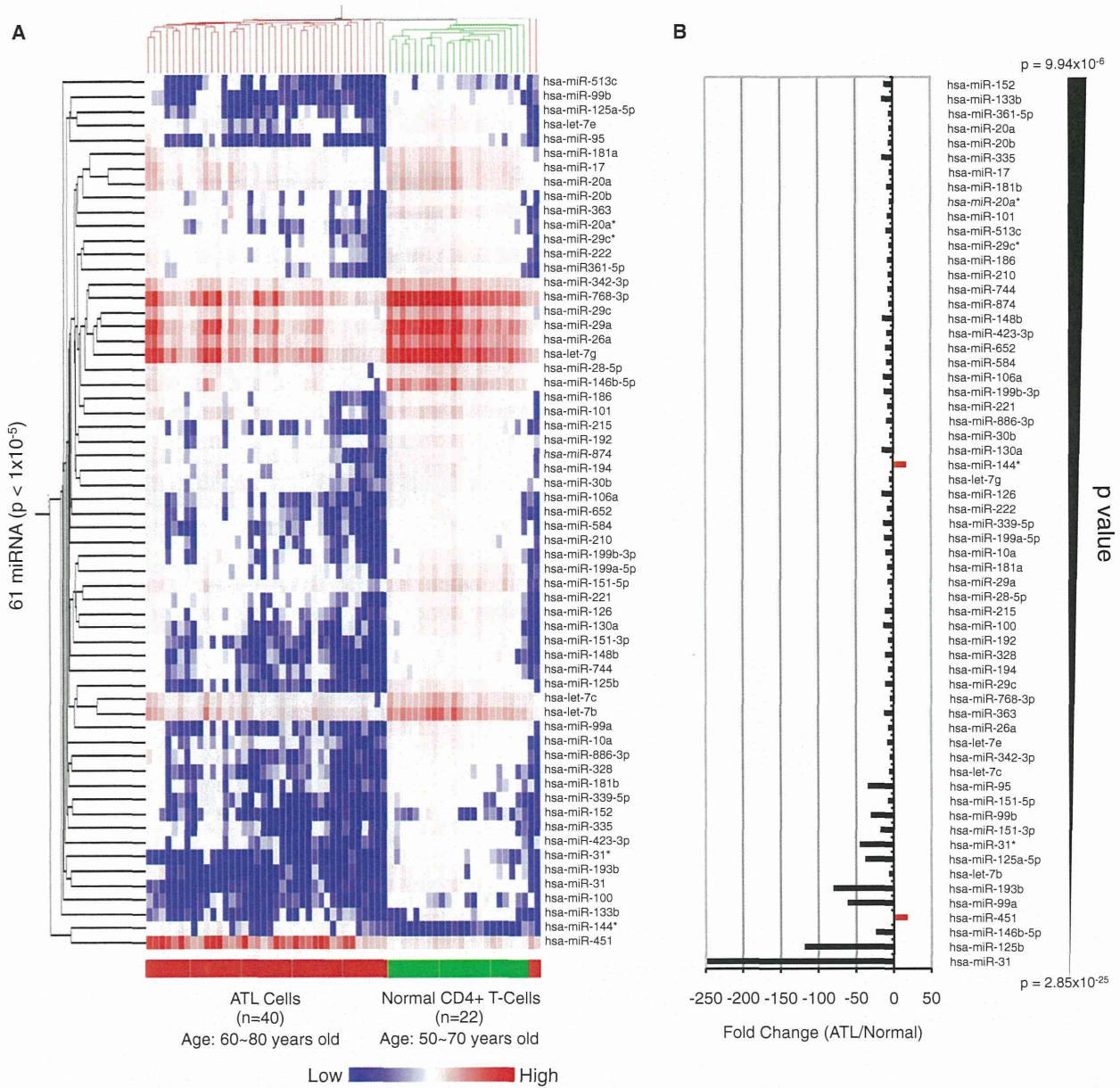


Figure 1. Global Profiling of Cellular miRNA on Primary ATL Cells

(A) Two-dimensional hierarchical clustering analysis and Pearson correlation as similarity measure on the miRNAs expressed at significantly different levels between the ATL (n = 40) and the control (n = 22) groups. Sixty-one miRNAs were identified ($p < 1 \times 10^{-5}$) and by filtering on more than 5-fold changes. A vertical branch shows the expression pattern of the selected miRNAs in each individual.

(B) Fold changes in the 61 miRNAs between ATL and Normal ($p < 10^{-5}$, fold change >5-fold). Selected miRNAs are arranged according to p values. See also Table S1.

UTRWT, or NIK-3' UTRMu1 and tested their expressions in 293T cells. Results demonstrated that expression of NIK-3' UTRWT was inhibited by simultaneous introduction of miR-31 (Figure 2E). miR-31 inhibition inversely rescued the NIK level, revealing that the cellular miR-31 level negatively affected that of the NIK protein through its 3' UTR sequence. These lines of evidence collectively demonstrated that miR-31 recognizes and regulates NIK mRNA through specific binding to its 3' UTR.

Transient introduction of the miR-31 precursor in TL-Om1 cells, which were established from an ATL patient, resulted in downregulation of NIK at the mRNA and protein levels, associated with downregulation of the phospho-IKK α/β level and NF- κ B activity (Figures S1F and S1G). In contrast, miR-31 inhibition resulted in accumulation of NIK mRNA and protein in HeLa cells (Figure 2F). Manipulation of the miR-31 level clearly indicated that the miR-31 level negatively correlates with cellular

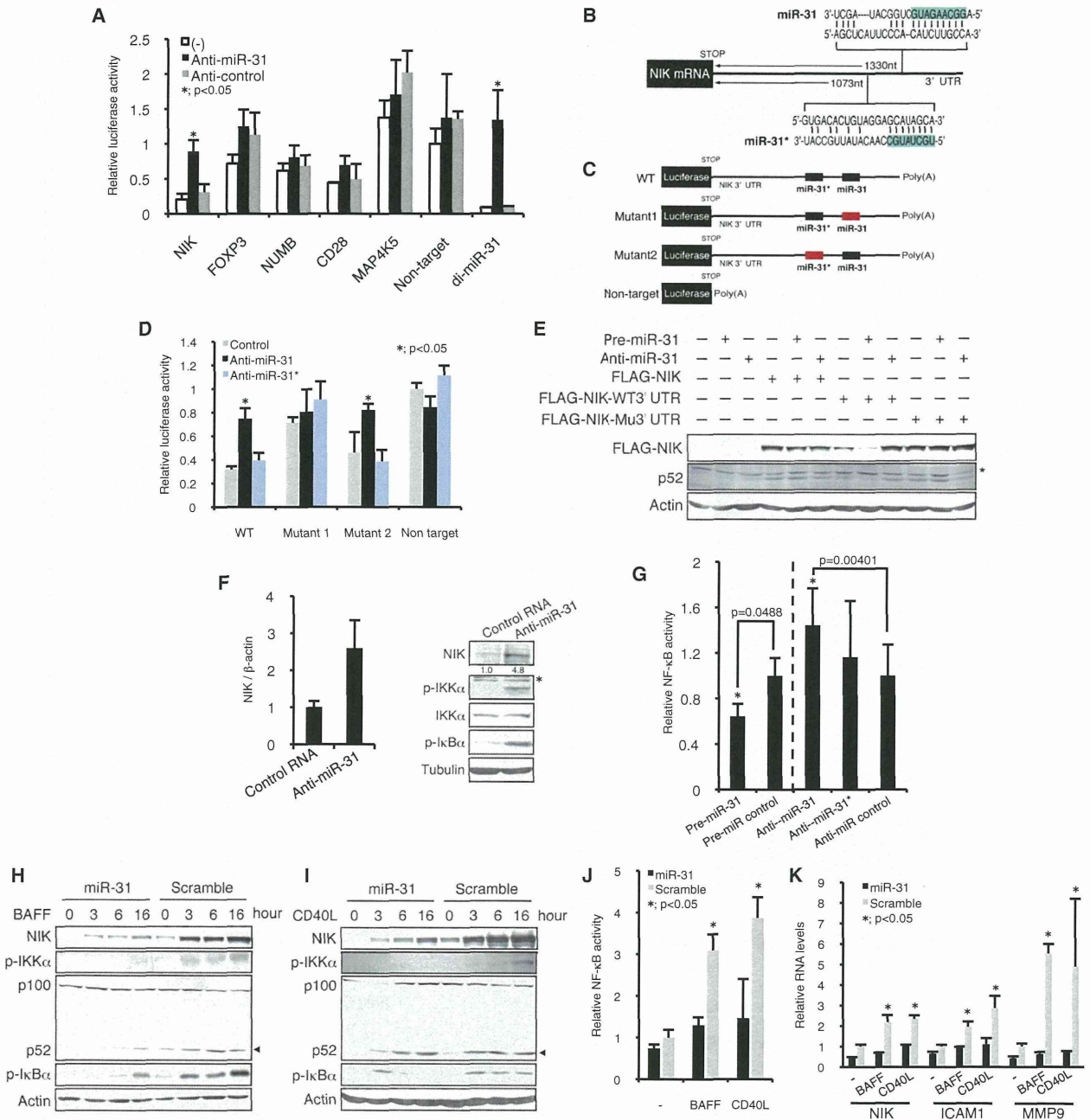


Figure 2. miR-31 Is a Negative Regulator of NF- κ B Pathway by Inhibiting NIK Expression

(A) Reporter-based miR-31's target gene screening. A series of 3' UTR-luciferase reporters was transfected in HeLa cells together with or without miR-31 specific inhibitory RNA (Anti-miR-31) or control RNA (Anti-control). Relative values of Dual-luciferase assay are presented. "Non-target" represents reporter without any 3' UTR. "di-miR-31" reporter contains two perfect match sequences. The data are presented as mean \pm SD of three independent experiments.

(B) Schematic of miR-31 target sites in the NIK 3' UTR.

(C) Mutation-induced reporters. Red box stands for mutated target region (see Figure S1C).

(D) miR-31 negatively regulates NIK 3' UTR analyzed by reporter assay (n = 4, mean \pm SD). Luciferase activities of reporter series were tested in a presence or absence of miR-31 inhibitor.

(E) FLAG-tagged NIK protein is negatively regulated through its 3' UTR and miR-31 binding. Plasmid vectors and miR-31 precursor or miR-31 inhibitor are cotransfected in 293T cells. Western blots showed levels of NIK and endogenous p52. Asterisk indicates nonspecific bands.

(F) NIK mRNA (left) and protein (right) levels in HeLa cells measured by quantitative RT-PCR (n = 3, mean \pm SD) and western blotting, respectively. Treatment of miR-31 inhibitor resulted in NIK accumulation. Result of densitometry is shown in the bottom panel. Asterisk indicates nonspecific bands.

(G) Cellular NF- κ B activity in HeLa cells (n = 5, mean \pm SD) in a presence or absence of miR-31 precursor or inhibitor.

NF- κ B activity (Figure 2G). Furthermore, enforced miR-31 expression in B cells attenuated both BAFF and CD40L-mediated NIK accumulation and the subsequent NF- κ B signaling (Figures 2H–2K). Consistent with previous reports (Ramakrishnan et al., 2004; Zarnegar et al., 2008b), we also found decreased levels of I κ B α phosphorylation. On the other hand, TNF- α -triggered canonical NF- κ B activation was not affected by miR-31 in Jurkat cells (Figures S1H–S1K). These results collectively show that miR-31 inhibits the basal and receptor-initiated activities of noncanonical NF- κ B pathway. With genetic evidence and an experimental approach, we further demonstrated that the function of miR-31 is well conserved among several classes of species (Figures S1L–S1O). Taking together all these results, miR-31, which is almost completely absent in primary ATL cells, appears to play a critical role in negative regulation of the NF- κ B pathway by manipulating the expression of NIK.

miR-31 Suppresses ATL Cell Growth and Promotes Apoptosis by Inhibiting NF- κ B

Although it was documented that abnormal NIK accumulation in ATL cells acts as a constitutive activator of the NF- κ B pathway, the mechanism underlying overproduction of NIK remains to be elucidated. The results described in the previous section indicated that the amount of miR-31 is linked to the level of NIK, and we therefore speculated that downregulation of miR-31 expression is at least partially responsible for the constitutive activation of NF- κ B in ATL cells. Quantitative RT-PCR revealed that *NIK* mRNA levels were negatively correlated with miR-31 levels in primary ATL cell samples (Figure 3A). To investigate the functional roles of NIK and miR-31, we established TL-Om1 cells stably expressing the miR-31 or NIK specific shRNA (shNIK) by retroviral vectors. RT-PCR and western blots showed that expression of miR-31 or shNIK reduced NIK at mRNA and protein levels as well as the levels of phospho-IKK α/β , p52, and I κ B α (Figures 3B and 3C). Decreased levels of nuclear RelA and RelB are considered to represent repressed activities of the canonical and noncanonical NF- κ B pathways, respectively (Figure 3D). EMSA and NF- κ B reporter assays also revealed the repressive function of miR-31 and shNIK on the NF- κ B activity (Figures 3E and 3F; Figures S2A, S2B, S5B, and S5C). Re-expression of NIK led to NF- κ B activation that was inhibited by miR-31, suggesting a reciprocal relationship between the level of miR-31 and that of NIK.

We and others previously showed that constitutive NF- κ B activation is a strong driver of ATL proliferation and pro-survival properties. Here, we examined the effects of miR-31 loss on ATL cell growth. We found that TL-Om1 cells expressing miR-31 or shNIK showed a significant attenuation of cell proliferation compared with control cells. In addition, serum starvation experiments showed greater sensitivity to induced cell death in NIK-repressed cells (Figure 3G). miR-31 expression showed the same phenotypic results in other ATL-derived cell lines

(Figures S2C, S2D, and S5E). Jurkat cells do not have significant basal activity of NF- κ B, and showed no significant difference in cell growth with or without induced expression of miR-31 (Figure S2E).

Next, we hypothesized that miR-31-mediated NF- κ B modulation may affect cellular apoptosis, because numerous studies have demonstrated that NF- κ B activation is a strong antiapoptotic factor in ATL and other cancer cells. We found that repression of NIK by miR-31 or shNIK resulted in downregulation of a subset of genes involved in resistance to apoptosis such as BCL-XL, XIAP, and FLIP (Figure 3H), suggesting that miR-31 has a role in proapoptosis through inhibition of NF- κ B activity. To assess the biological function of miR-31 in apoptosis signals, we utilized a lentivirus gene transfer system for cell lines and freshly isolated tumor cells. The lentivirus vector is competent to infect nondividing cells and the infected cells can be monitored by the fluorescence of Venus. We found that lentivirus-mediated miR-31 expression promoted basal and Fas-directed apoptosis in TL-Om1 cells (Figure 3I). Venus-negative population showed no significant changes, demonstrating the specificity of miR-31 activity. To confirm the relationship among miR-31, NIK, and NF- κ B signaling, we also prepared another retroviral vector encoding NIK without its 3' UTR sequence. As results, re-expression of NIK reversed the miR-31-mediated apoptosis. In addition, miR-31 expression led to caspase 3 activation (Figure 3J). Collectively, these findings indicate that miR-31 mediates apoptosis through repression of NIK in ATL cell lines.

Tumor cells from ATL patients primarily represent the malignant characteristics. In fact, miR-31 loss is found from patient samples (Figures 1 and 3A). To demonstrate the responsibility of miR-31 for tumor cell survival, we tested whether lentivirus-mediated miR-31 expression has a killing effect against tumor cells. After establishment of lentivirus infection, the apoptotic cells were determined by flow cytometry. The results revealed that expression of miR-31 facilitated tumor cell death. Since NIK repression by shRNA lentivirus also showed a strong killing effect, NIK and NF- κ B activity are suggested as crucial players for survival in ATL tumor cells (Figure 3K). Strong toxicities were not observed in normal resting lymphocytes that express low levels of NIK. Taken together, these lines of experimental evidence, including data from cell lines and primary ATL cells, definitively support two notions that (1) miR-31 acts as a tumor suppressor in T cells, and (2) NIK-regulated NF- κ B has pivotal importance in cancer cell survival.

Loss of miR-31 Occurs in T Cells with Genetic and Epigenetic Abnormalities

The results described above together with previous publications indicate that regulation of miR-31 expression has profound impacts on multiple functions in human tumors as well as in normal cells. However, little is known about the regulatory mechanism of miR-31 expression. The human gene that encodes miR-31, *hsa-miR-31*, is located at 9p21.3, which is

(H–K) miR-31 attenuates signal-dependent NF- κ B activation in B cells. (H and I) BJAB cells expressing miR-31 or control RNA were treated with BAFF (0.2 μ g/ml) or CD40L (0.5 μ g/ml) for indicated time periods. The protein levels of NIK, phospho-IKK α/β , p100/p52 (arrowheads indicate active p52), and phospho-I κ B α were shown. Actin was detected as control. (J) NF- κ B activity (n = 5, mean \pm SD) evaluated by NF- κ B-luciferase reporter assay at 24 hr after cytokine treatments. (K) NF- κ B-dependent gene expressions were inhibited by miR-31 (n = 3, mean \pm SD). See also Table S2 and Figure S1.

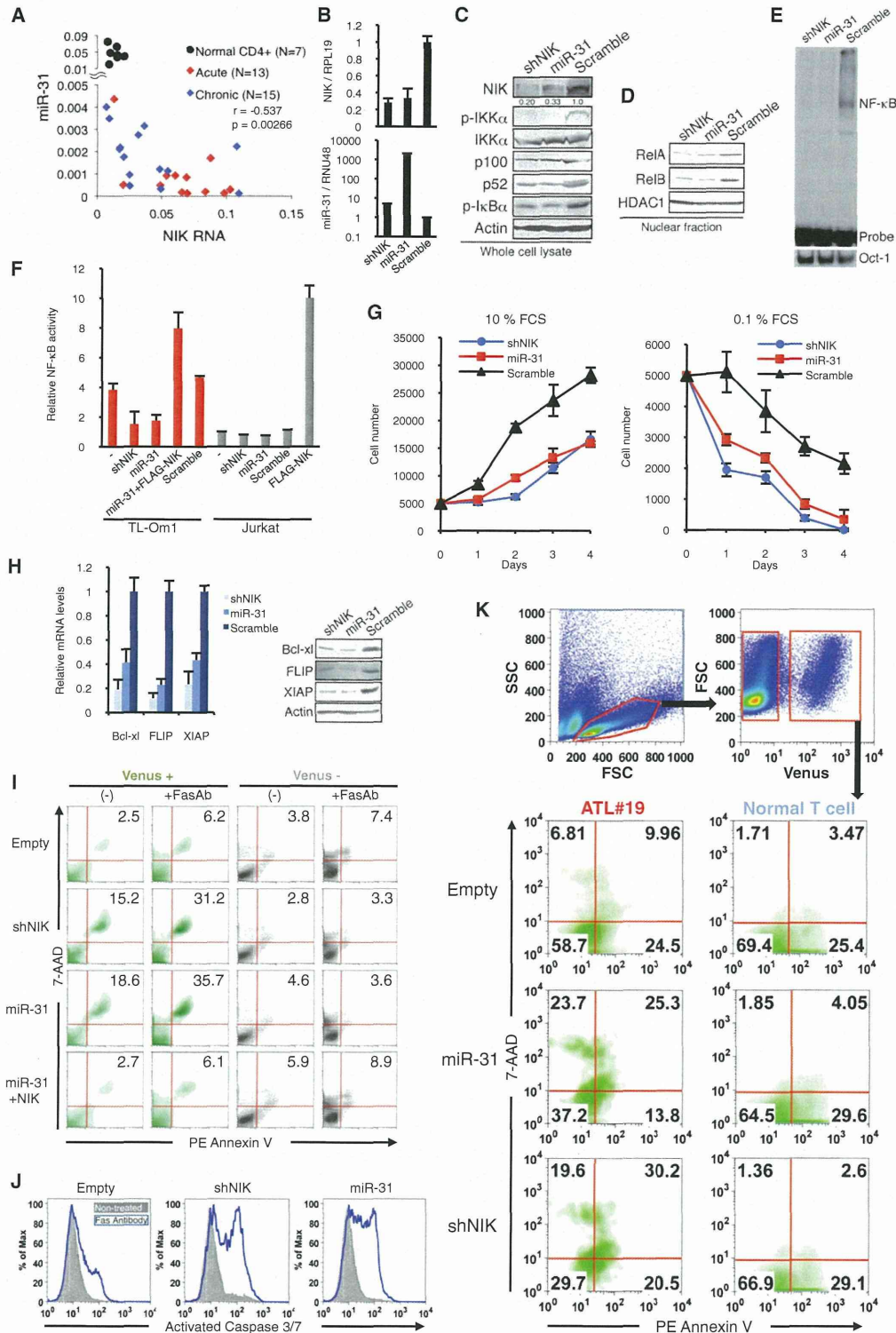


Figure 3. Loss of miR-31 Is Responsible for Constitutive NF- κ B Activation, Abnormal Cell Growth, and Resistance to Apoptotic Cell Death in ATL Cells

(A) Expression levels of miR-31 and *NIK* in individual ATL patients and normal controls using data set obtained by quantitative RT-PCR. Pearson's correlation coefficient within ATL samples was described in the graph.

adjacent to clusters of the *CDKN2* and *IFNA* families, and is a well-known hotspot of genomic loss in several types of human cancers. We performed genome-wide scans of genetic lesions in 168 ATL samples and demonstrated that 21 ATL cases (12.5%) had genomic deletion of 9p21.3 containing the *hsa-miR-31* coding region (Figure 4A; Figure S3A). All of these cases also have genomic defect in *CDKN2A* region. A major proportion of ATL cases that are without genetic deletion and somatic mutation in the *hsa-miR-31* region showed remarkable loss of miR-31 expression (Figure 4B). Detailed expression profiling revealed drastic downregulation of *Pri-miR-31* transcription in the primary ATL cells (Figure 4C). There was a strong correlation between the levels of mature miR-31 and primary transcript ($r = 0.9414$, $p = 5.45 \times 10^{-8}$). *hsa-miR-31* is located in intronic region of *LOC554202* gene. However, *LOC554202* mRNA levels were very low in primary T cells and there was no significant difference between ATL and normal cells, strongly suggesting that loss of miR-31 expression is due to specific transcriptional suppression in ATL cells. Using computational analysis, we identified a putative TATA box and transcriptional start site (TSS) 2500 bp upstream of the miR-31 coding region (Figure 4D). Although no CpG islands were found in this region, we unexpectedly discovered an assembly of YY1-binding motifs upstream of the miR-31 region in human and mouse (Figure 4D; Figure S3C). YY1 is a pivotal transcription factor and a recruiter of the Polycomb repressive complex (PRC) (Simon and Kingston, 2009). Convergence of the YY1 binding sequence, especially the repressive motif (Figure S3D), seems to be evolutionarily conserved, suggesting that YY1 is important in the regulation of miR-31 transcription. We further performed chromatin immunoprecipitation (ChIP) to evaluate repressive histone hallmarks, including di- and trimethylated H3K9 (H3K9me2 and H3K9me3) and trimethylated H3K27 (H3K27me3). The results showed higher levels of methylation at H3K9 and H3K27 in a broad area containing the miR-31 coding region (Figure 4E). As shown in Figures S3E–S3G, there was an inverse correlation between the levels of miR-31 expression and repressive histone methylation. These data allowed us to hypothesize that histone methylation, especially that of Polycomb family-dependent H3K27me3, may contribute to miR-31 repression. To confirm our hypothesis, we performed a YY1 knockdown experiment using a specific shRNA (Figures 4F–4I). As expected, knockdown of YY1 led to an increase in the levels of *Pri-miR-31* and mature miR-31 (Figures 4F and 4G). Furthermore, ChIP assays showed that

YY1 occupied the miR-31 region, especially in the upstream region of TSS, where there is an array of YY1 binding sites (Figures 4D and 4H). The results also demonstrated that decreased occupancy of YY1 and concomitant derecruitment of EZH2, a key component of PRC2, were induced by YY1 knockdown, indicating involvement of EZH2 in the repressive complex recruited to the miR-31 region (Figures 4H and 4I; Figure S3H). These results collectively suggest that YY1 regulates PRC2 localization and initiates miR-31 suppression. Indeed, we found significant escalation of methylated histone H3K9 and H3K27 at the miR-31 locus of peripheral blood lymphocytes of ATL patients (Figure 4J), indicating that aberrant abundance of suppressive histone methylation may be responsible for the loss of miR-31 in the primary ATL cells.

Overexpression of PRC2 Components Leads to miR-31 Repression

Given that Polycomb-mediated repressiveness affects miR-31 level, our findings imply that the amount of EZH2 is related to miR-31 expression (Figure 4I; Figures S3G and S4A). We found a significantly upregulated expression of PRC2 components, especially EZH2 and SUZ12, in the primary ATL cells (Figures 5A and 5B; Table S3). Quantitative RT-PCR revealed that miR-31 levels inversely correlated with both *EZH2* and *SUZ12*, respectively (Figure 5C). miR-101 and miR-26a, which are putative negative regulators of EZH2, seem to be associated with this relationship in ATL cells (Figures S4B–S4E). To further confirm our hypothetical mechanism linking the epigenetic machinery and miR-31 expression, we performed a “loss-of-PRC2-function” assay. Retroviral delivery of shSUZ12 and shEZH2 in the ATL cell lines resulted in a great increase in the levels of *Pri-miR-31* and its mature form (Figure 5D; Figure S4F). Knockdown of PRC2 induced histone demethylation at H3K27 in the miR-31 region, which is concomitant with the decrease in H3K9me3 levels, EZH2 occupancy, and HDAC1 recruitment (Figure 5E), suggesting that this multimeric complex leads to a completely closed chromatin architecture as a result of histone modifications in the miR-31 genomic region.

To further examine whether the proposed mechanism holds true in other human cancers, we analyzed a couple of carcinoma cell lines, including HeLa cells and nonmetastatic and metastatic breast carcinoma cell lines, MCF7 and MDA-MB-453 cells, respectively. qRT-PCR revealed that expression of *EZH2* and *SUZ12* inversely correlated with miR-31 levels (Figure S4G).

(B) miR-31 restoration by retroviral vector inhibits *NIK* RNA accumulation in TL-Om1 cells. The results of *NIK* and mature miR-31 quantifications are shown ($n = 3$, mean \pm SD).

(C) miR-31 or shNIK expression downregulates *NIK* protein expression and inhibits downstream pathway of noncanonical NF- κ B in TL-Om1 cells.

(D) Reduced nuclear translocation of RelA and RelB proteins in miR-31- or shNIK-expressing TL-Om1 cells.

(E) miR-31-dependent downregulation of NF- κ B activity in TL-Om1 cells examined by EMSA.

(F) NF- κ B-luciferase reporter assays ($n = 5$, mean \pm SD). FLAG-*NIK* plasmid was transiently introduced 48 hr prior to the assay.

(G) miR-31 level is relevant to proliferation of ATL cells. Cell proliferation curve of TL-Om1 cells were evaluated in two FCS conditions ($n = 3$, mean \pm SD).

(H) Apoptosis-related gene expression in TL-Om1 cells analyzed by qRT-PCR ($n = 3$, mean \pm SD) and western blots.

(I) Lentivirus-mediated *NIK* depletion promotes basal and Fas antibody-mediated apoptosis. Venus-positive population represented lentivirus-infected cells. Apoptotic cells were determined by PE-Annexin V / 7-AAD stainings ($n = 4$). Representative FACS analyses are shown.

(J) miR-31 activates Caspase 3/7 determined by FACS ($n = 3$).

(K) miR-31 expression and *NIK* depletion induce tumor cell death. Primary tumor cell from ATL patient and healthy CD3+ T cells were infected with lentivirus and analyzed by FACS. The apoptotic cells were defined by sequential gating beginning with FSC-SSC to select intact lymphocytes, subgating on the Venus-positive population, and calculating the PE-Annexin V and 7-AAD profilings. Representative result is shown and summarized data are presented in Figure 6J. See also Figure S2.

