Reversal of microRNA-150 silencing disadvantages crizotinib-resistant NPM-ALK(+) cell growth

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The regulatory microRNA miR-150 is involved in the development of hemopathies and is downregulated in T-lymphomas, such as anaplastic large-cell lymphoma (ALCL) tumors. ALCL is defined by the presence or absence of translocations that activate the anaplastic lymphoma kinase (ALK), with nucleophosmin-ALK (NPM-ALK) fusions being the most common. Here, we compared samples of primary NPM-ALK(+) and NPM-ALK(−) ALCL to investigate the role of miR-150 downstream of NPM-ALK. Methylation of the MIR150 gene was substantially elevated in NPM-ALK(+) biopsies and correlated with reduced miR-150 expression. In NPM-ALK(+) cell lines, DNA hypermethylation-mediated miR-150 repression required ALK-dependent pathways, as ALK inhibition restored miR-150 expression. Moreover, epigenetic silencing of miR-150 was due to the activation of STAT3, a major downstream substrate of NPM-ALK, in cooperation with DNA methyltransferase 1 (DNMT1). Accordingly, miR-150 repression was turned off following treatment with the DNMT inhibitor, decitabine. In murine NPM-ALK(+) xenograft models, miR-150 upregulation induced antineoplastic activity. Treatment of crizotinib-resistant NPM-ALK(+) KARPAS-299- CR06 cells with decitabine or ectopic miR-150 expression reduced viability and growth. Altogether, our results suggest that hypomethylating drugs, alone or in combination with other agents, may benefit ALK(+) patients harboring tumors resistant to crizotinib and other anti-ALK tyrosine kinase inhibitors (TKIs). Moreover, these results support further work on miR-150 in these and other ALK(+) malignancies.

Introduction
Systemic anaplastic large-cell lymphoma (ALCL) is an aggressive subtype of peripheral T cell non-Hodgkin’s lymphoma derived from CD4 T cells (1, 2). WHO classification of lymphoid malignancies recognizes 2 systemic forms of ALCL, defined by the presence (+) or absence (−) of chromosomal translocations involving the anaplastic lymphoma kinase (ALK) gene at the 2p23 locus (3, 4).

In ALCL, the most common translocation is t(2;5)(p23;q35) that codes for the nucleophosmin-ALK (NPM-ALK) fusion protein. In addition to NPM, several other ALK translocation partners have been identified in ALK(+) ALCL. ALK fusion proteins have also been reported in other cancers, including a proportion of non-small-cell lung cancers (NSCLC) and inflammatory myofibroblastic tumors (IMTs). The ALK fusion partners induce homodimerization, leading to constitutive ALK kinase domain (KD) activation (5). Aberrant ALK activation triggers various pro-survival signaling pathways whose main target is the STAT3 pathway and subsequent oncogenesis (3, 5).

Systemic ALCL patients respond well to common first-line chemotherapy regimens such as CHOP (cyclophosphamide, hydroxy doxorubicin, Oncovin [vincristine], and prednisone). The majority of patients with NPM-ALK(+) ALCL are young and respond well to CHOP, with a long-term disease-free survival rate of over 70%. In contrast, patients with the NPM-ALK(−) disease have less than a 50% long-term disease-free survival rate with similar treatments. However, NPM-ALK(+) ALCL relapses after chemotherapy have a poor prognosis. Some patients with relapsed or refractory disease do not respond to combination therapy; for these patients, higher doses of chemotherapy followed by a stem cell transplant may be prescribed (4, 6, 7).

The dual ALK/MET inhibitor crizotinib, also known as PF2341066 or Xalkori, revolutionized perspectives on ALK-related disease management. Crizotinib was recently approved for the treatment of metastatic and late-stage ALK-rearranged NSCLC and is currently undergoing clinical trials for use in other ALK-related diseases (8, 9). Crizotinib blocks the kinase activity of ALK by binding to the ATP-binding pocket and therefore prevents the recruitment of ATP molecules. As predicted from clinical experience with other tyrosine kinase inhibitors (TKIs), mutations conferring resistance to crizotinib have already been described in ALK-associated NSCLC and in one IMT patient who had been...
submitted to continuous crizotinib treatment (10). A leucine–
methionine substitution at residue 1196 (L1196M) has been shown to
mediate resistance to crizotinib by impairing inhibitor binding
due to steric hindrance. Leu1196 is located in the gatekeeper posi-
tion at the bottom of the ATP-binding pocket. Recently, Gamba-
corti-Passerini and coworkers reported on the compassionate
use of crizotinib administered as monotherapy to 11 ALK(+) lymph-
oma patients who were resistant/refractory to cytotoxic therapy
(11). Crizotinib exerted a potent antitumor activity with durable
responses in advanced, heavily pretreated ALK(+) lymphoma
patients, with a benign safety profile. Only 2 patients progressed
under short-term course continuous crizotinib treatment (11).
Deep sequencing of ALK KD of these 2 patients revealed the pres-
ence of ALK mutations (11). However, no study into the long-term
effects of crizotinib in ALK(+) lymphomas has been conducted.
Consequently, based on the data obtained from NSCLC and IMT
patients treated with crizotinib, it is highly probable that resistance
would also emerge in ALCL patients, thereby limiting any further
clinical benefit of crizotinib-based therapy (7, 10, 12, 13).

Recently, it has emerged that microRNAs (miRNAs) can play a role
in determining drug sensitivity/resistance (14). miRNAs are endogenous small (20–23 nucleotides long) noncoding RNAs
that negatively regulate gene expression at the posttranscriptional
level through base-pairing interactions with their messenger RNA
targets (mRNA targets). miRNAs have been found to play impor-
tant roles in the regulation of the lineage differentiation fate of
hematopoietic cells by modulating the expression of oncogenes or
tumor suppressors. Thus, the deregulation of miRNA expression
has been shown to be involved in multistep hematological malig-
nancies and has rapidly emerged as a therapeutic target (15–19).
Given that some epigenetically silenced miRNAs appear to have
tumor-suppressive potential, the restoration of their expression
may be an effective strategy for treating cancer (15–20). The meth-
ylation of cytosine residues within a CpG dinucleotide context by 3
members of the DNA methyltransferase (DNMT) family, DNMT1,
DNMT3a, and DNMT3b, is an important epigenetic mark that
mediates the repression of miRNA gene transcription (21). DNMT
inhibitors such as 5-aza-2′-deoxycytidine (hereafter referred to
as decitabine) cause the reexpression of methylation-silenced
miRNA genes. Decitabine is a chemical cytidine analog that inhib-
its DNA methylation by trapping the DNMTs (22). It has been
demonstrated experimentally that decitabine treatment leads to
the downregulation of microRNA-target oncogenes and suppress-
ion of tumor growth (23). In addition, numerous studies have con-
irmed the antitumoral effects of ectopic miRNA expression using
short double-stranded RNA mimics or miRNA expression vectors
(24). Resistance to chemotherapy remains a major obstacle in
effective anticancer treatment and results in relapse and progress-
ion in most malignant tumors. This chemoresistance may be due
to drug efflux by transporters, drug inactivation by detoxification
enzymes, an altered expression of proapoptosis proteins, changes
in tumor-suppressor gene expression, or an increased activity of
dna repair mechanisms (25). Recent evidence has demonstrated
that miRNAs play a role in all of these processes (26, 27).

Recent studies have identified a number of miRNAs aberr-
antly expressed in systemic ALCL patients and have suggested the involvement of miR-101, miR-29a, miR-135b, and miR-16 in
mediating oncogenic NPM-ALK signaling (28–31). We have previ-
ously reported that the repression of miR-29a is dependent on the
activity of both NPM-ALK and STAT3, and we demonstrated for
the first time in ALCL that repression of miR-29a is likely medi-
ated by epigenetic silencing (29). Importantly, the role of miRNAs
in ALCL drug sensitivity/resistance has never been reported. We
therefore sought to investigate the potential role of miRNAs in the
crizotinib-response of NPM-ALK(+) ALCL, and focused our atten-
dion on miR-150.

Among the hematopoietic cell types, miR-150 is predomi-
nantly expressed in spleen and lymph nodes (32, 33). miR-150 is
strongly upregulated during the differentiation of mature T cells
and B cells, indicating that it might participate in B cell lympho-
poiesis, T cell lymphopoiesis, or both (32, 33). In normal lymph-
cocytes, miR-150 is thought to have a regulatory role, and the
expression of miR-150 in naive cells has been shown to be rapidly
downregulated after stimulation (34). miR-150 has also proven to
be involved in the development of hematological malignancies
(35, 36); for example, miR-150 repression has been demonstrated
in an aggressive form of cutaneous T cell lymphoma (37, 38). Thus,
there is now accumulating evidence that miR-150 plays essential
regulatory roles in both normal and malignant hematopoiesis and
holds great potential as a therapeutic target for treating various
types of hematopoietic malignancies (35, 39). Honda and collab-
orators showed that miR-150 downregulation in scleroderma
dermal fibroblasts is caused by DNA methylation and therefore can
be reexpressed by decitabine treatment (40). In addition, Hassler and
coworkers reported that a low dose of decitabine resulted in high
antineoplastic activity in vitro and in vivo in NPM-ALK(+) KAR-
RAS-299 ALCL cell lines and xenografted tumors (41). Thus, these
data suggest that decitabine could be highly effective in treating
NPM-ALK(+) ALCL and warrants further clinical evaluation for
future therapeutic use. Furthermore, an aberrantly low expression
of miR-150 has been identified in cutaneous T lymphoma Sezary
syndrome and in NK/T cell lymphoma. The reexpression of miR-
150 in these NK/T cell lymphoma cells reduces cell proliferation,
suggesting that miR-150 functions as a tumor suppressor in these
cells (37). In addition, Merkel and coworkers reported that miR
150 is downregulated in various NPM-ALK(+) lymphoma
models, suggesting that this miRNA may play important roles in the
pathogenesis of NPM-ALK(+) ALCL (28). However, the impact of
miR-150 deregulation on the oncogenic potency of NPM-ALK(+)–
expressing cells has not yet been investigated. In that context, we
hypothesized that repression of miR-150, caused by DNA meth-
ylation, plays a central role in the pathogenesis of T cell lympho-
omagenesis. To test that idea, we screened for miR-150 expression
in NPM-ALK(+) human and murine lymphoma models (human
NPM-ALK(+) cell lines and primary tumors, and NPM-ALK con-
ditional transgenic mice). We show here that miR-150 expression
is significantly diminished in all NPM-ALK(+) models tested.
Furthermore, we show that NPM-ALK activity is responsible for miR-
150 silencing in the NPM-ALK(+) ALCL cell lines. This repression
is likely mediated by the epigenetic silencing of miR-150 by a
DNMT1-dependent activity. In addition, DNMT1 is upregulated
at the transcriptional level as a consequence of activation of the
STAT3 protein, the major downstream executor of the NPM-ALK
chimeric oncogenic kinase (42–44). Increasing miR-150 levels
The expression of miR-150 is downregulated in human ALCL cell lines and biopsies. (A) miRNA-specific qPCR analysis of miR-150 in both PBMC and isolated CD4 lymphocytes S or NS with PHA, in 3 NPM-ALK(+) ALCL cell lines (KARPAS-299, SU-DHL-1, and COST) and 2 NPM-ALK(–) ALCL cell lines (FE-PD and Mac-2a). RNU24 was used as an internal control. Relative miR-150 expression was expressed as the 2–ΔΔCt relative to RNU24. **P < 0.001, and ***P < 0.0001; unpaired 2-tailed Student’s t test.

Figure 1. The expression of miR-150 is downregulated in human ALCL cell lines and biopsies. (A) miRNA-specific qPCR analysis of miR-150 in both PBMC and isolated CD4 lymphocytes S or NS with PHA, in 3 NPM-ALK(+) ALCL cell lines (KARPAS-299, SU-DHL-1, and COST) and 2 NPM-ALK(–) ALCL cell lines (FE-PD and Mac-2a). RNU24 was used as an internal control. Relative miR-150 expression was expressed as the 2–ΔΔCt relative to RNU24. **P < 0.001, and ***P < 0.0001; unpaired 2-tailed Student’s t test.

Results

Aberrant miR-150 expression in NPM-ALK(+) ALCL cells. To elucidate the potential role of miRNAs in the pathogenesis of NPM-ALK(+) ALCL, Merkel et al. used a number of model systems to highlight a unique miRNA signature associated with this disease (28). In their study, miR-150 was identified as being deregulated in both human ALCL cell lines and primary tissues, as well as in the CD4/NPM-ALK transgenic mouse model (28). However, the impact of miR-150 deregulation on the oncogenic potency of NPM-ALK(+) cells was not defined. To corroborate the results of Merkel et al., we performed quantitative PCR (qPCR) to examine miR-150 levels in 3 ALCL cell lines carrying the NPM-ALK fusion protein, namely KARPAS-299, SU-DHL-1, and COST, as well as 2 NPM-ALK(–) cell lines, FE-PD and Mac-2a. miR-150 levels in these cells were compared with those in normal peripheral blood mononuclear cells (PBMC) and CD4+ isolated cells stimulated (S) or not (NS) with phytohemagglutinin (PHA) (Figure 1A). These results confirmed the downregulation of miR-150 in ALK(+) cell lines. To validate that these results are relevant to primary patient cases, we measured miR-150 expression in 70 clinical samples of ALCL patients. miR-150 was shown to be significantly reduced in all human primary ALCL samples tested, as was expected due to its recognized roles in the development of hematological malignancies (refs. 35, 39, and Figure 1B). Interestingly, NPM-ALK(+) ALCL samples (n = 56) showed a greater reduction in miR-150 levels than NPM-ALK(–) samples (n = 14), when compared with reactive lymph node (RLN, n = 3) (8.13 ± 0.17 vs. 11.08 ± 0.20 for ALK[+] vs. RLN, P < 0.0001; 8.95 ± 0.27 vs. 11.08 ± 0.20 for ALK[–] vs. RLN, P < 0.001) (Figure 1B). Together, these data suggest that a proportion of the reduction in miR-150 could be an NPM-ALK–dependent phenomenon.

NPM-ALK is responsible for aberrant miR-150 accumulation in lymphoma cells. The miR-150 silencing observed in all of the NPM-ALK(+) cell lines and patient samples tested suggested that the NPM-ALK(+) protein itself might be the driving force behind this phenomenon. To test whether NPM-ALK is involved in miR-150 downregulation, NPM-ALK was silenced in 3 human NPM-ALK(+) ALCL cell lines (KARPAS-299, COST, and SU-DHL-1) using siRNAs directed against ALK mRNA. NPM-ALK knockdown was efficiently achieved, as shown by Western blotting (Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI78488DS1). As a negative control, the same siRNAs were transfected into the FE-PD cell line, which does not express NPM-ALK. In order to check that the knockdown of NPM-ALK expression (si-ALK, Figure 2A) had been performed efficiently, we used Western blotting to detect the accumulation of the activated (phosphorylated) form of NPM-ALK (p-NPM-ALK) and STAT3 (p-STAT3) (Supplemental Figure 1A). As shown in Figure 2A, the inhibition of NPM-ALK corresponded with an increase in the expression of miR-150 in all NPM-ALK(+) cell lines. In addition, and as expected, the level of miR-150 was not modified in FE-PD cells (Figure 2A). In order to determine whether the catalytic activity of ALK can modulate miR-150 expression, we first
treated the KARPAS-299 cell line with either the ALK inhibitor crizotinib or with the drug vehicle alone (PBS). The loss of NPM-ALK autophosphorylation on the tyrosine 1064 residue (Figure 2B) confirmed that the ALK kinase activity was properly inhibited upon crizotinib treatment. Of note, and as expected, a decrease in STAT3 activation (p-STAT3 protein levels) was observed in parallel to ALK kinase activity inhibition (Figure 2B). Next, using qPCR, we observed that miR-150 levels were increased concomitantly to ALK tyrosine kinase inhibition (Figure 2C). In addition, the effect of crizotinib on miR-150 levels was strictly dependent on the presence of NPM-ALK, as no change was observed in FE-PD and Mac-2a cells, the NPM-ALK(–) cell lines (Figure 2C). This result suggests an ALK tyrosine kinase activity–dependent repression of miR-150 expression in NPM-ALK(+) cells.

To further investigate this finding, we took advantage of our previously published conditional NPM-ALK lymphoma transgenic mouse model (Tet-OFF-NPM-ALK murine model). miR-150 expression was assessed in the presence (NPM-ALK(–) OFF) or in the absence (NPM-ALK(+) ON) of doxycycline. In lymph nodes isolated from mice with NPM-ALK(+) lymphoma (NPM-ALK(+) ON, n = 8), we found that miR-150 was one of the most downregulated miRNAs, when compared with lymph nodes isolated from either control normal age–matched WT littermate mice (n = 6; fold change: –7.69; P = 0.01), or healthy transgenic mice who had received doxycycline treatment (NPM-ALK(–) OFF, n = 6; fold change: –3.70; P = 0.04) (F. Meggetto, unpublished observations). We then validated the expression levels of miR-150 in these samples by qPCR. miR-150 expression was significantly decreased in lymph nodes from mice with NPM-ALK(+) lymphoma, and the decrease in miR-150 levels in these cells was statistically significant when compared with cells isolated from either the lymph nodes of WT littermate transgenic

**Figure 2. NPM-ALK expression promotes miR-150 downregulation.** (A) miRNA-specific qPCR analysis of miR-150 in 3 NPM-ALK(+) ALCL cell lines (KARPAS-299, COST, and SU-DHL-1) and one NPM-ALK(–) ALCL cell line (FE-PD) transfected with either negative control siRNA (si-CTL) or si-ALK (siRNA targeting ALK mRNA). RNU24 was used as an internal control. Relative miR-150 expression was expressed as the 2−ΔΔCt relative to the si-CTL experiment. (B and C) Human KARPAS-299 cells were treated with 500 nM crizotinib for 3, 6, 12, 24, or 48 hours or with vehicle (PBS, vehicle control) for 48 hours. (B) Protein levels of total NPM-ALK, p-NPM-ALK (the phosphorylated form of NPM-ALK on tyrosine 1604), p-STAT3 (the phosphorylated form of STAT3 on tyrosine 705), and MYB were assessed by Western blotting using specific antibodies. β-actin served as a loading control. (C) miRNA-specific qPCR analysis of miR-150 in one NPM-ALK(+) ALCL cell line (KARPAS-299) and in 2 NPM-ALK(–) ALCL cell lines (FE-PD and Mac-2a) upon treatment with 500 nM crizotinib for the times shown. RNU24 was used as an internal control. Relative miR-150 expression was expressed as the 2−ΔΔCt relative to no treatment (0 hours). (D and E) Assessment of miR-150 expression by qPCR in WT (n = 6) or NPM-ALK transgenic mice containing a Tet-OFF system. Expression of the NPM-ALK transgene was both induced (NPM-ALK(+) ON, no doxycycline, n = 8) or repressed (NPM-ALK(–) OFF, with doxycycline, n = 6), and miR-150 levels were examined in both. SNORD202 served as an internal control, and relative miR-150 expression was expressed as the 2−ΔΔCt relative to (D) WT or (E) NPM-ALK(–) OFF mice. Data represent mean ± SEM. n = 3; *P < 0.05, **P < 0.001, and ***P < 0.0001; unpaired 2-tailed Student’s t test.
To obtain insights into the relationship between NPM-ALK, miR-150, and MYB, we determined MYB expression levels under ALK knockdown conditions using KARPAS-299 cells. Both crizotinib treatment and si-ALK transfection led to a decrease in MYB at both the mRNA (Figure 3, B and C) and protein (Figure 2B and Figure 3D) levels in parallel to the induction of miR-150 expression (Figure 2A and Figure 3B). In order to check that the knockdown of NPM-ALK expression (si-ALK, Figure 3D) and activity (crizotinib, Figure 2B) had been performed efficiently, we used Western blotting to follow the accumulation of the activated (phosphorylated) form of STAT3 (p-STAT3), known to be the major downstream target of NPM-ALK. Together, these data suggest that the NPM-ALK oncoprotein affects STAT3 activation and the expression of miR-150 and MYB.

The NPM-ALK–mediated STAT3 pathway is involved in DNA methylation of the MIR150 gene. In an attempt to identify the mechanisms involved in miR-150 downregulation, we first searched for DNA loss by chromosomal studies: high-resolution array–based comparative genomic hybridization array analysis of tumoral lymph nodes from NPM-ALK conditional transgenic mice and karyotype banding in the 3 NPM-ALK(+) ALCL mice (Figure 2D) or from NPM-ALK(–) OFF healthy mice (Figure 2E). Taken together, these data indicate that the NPM-ALK protein is required for maintaining a low expression of miR-150.

NPM-ALK kinase activity affects miR-150 and MYB expression. In another previous study on the transgenic mice described above, we used transcriptome analysis to compare lymph nodes from conditional NPM-ALK(+) mice (NPM-ALK[+] ON, n = 11) with normal lymph nodes from age-matched WT littermate mice (n = 6) or healthy lymph nodes from doxycycline-treated mice (NPM-ALK[–] OFF, n = 6). The induction of NPM-ALK expression was confirmed by ALK IHC and Western blotting (Supplemental Figure 2, A and B). MYB, a bona fide miR-150 target, was found to be overexpressed in murine NPM-ALK(+) lymphoma cells compared with cells isolated from the lymph nodes of normal littermate transgenic mice (fold change: 6.59; *P < 0.05) or from NPM-ALK(–) OFF healthy mice (fold change: 6.60; P = 0.01) (F. Meggetto, unpublished observations; see Supplemental Methods). We then used qPCR to confirm that MYB was significantly overexpressed in all lymph nodes from mice with NPM-ALK(+) lymphoma compared with lymph nodes from normal littermate transgenic mice (WT) or doxycycline-treated mice (NPM-ALK[–] OFF) (Figure 3A).

To obtain insights into the relationship between NPM-ALK, miR-150, and MYB, we determined MYB expression levels under ALK knockdown conditions using KARPAS-299 cells. Both crizotinib treatment and si-ALK transfection led to a decrease in MYB at both the mRNA (Figure 3, B and C) and protein (Figure 2B and Figure 3D) levels in parallel to the induction of miR-150 expression (Figure 2A and Figure 3B). In order to check that the knockdown of NPM-ALK expression (si-ALK, Figure 3D) and activity (crizotinib, Figure 2B) had been performed efficiently, we used Western blotting to follow the accumulation of the activated (phosphorylated) form of STAT3 (p-STAT3, form of STAT3 phosphorylated on tyrosine 705), MYB, and DNMT1 were assessed by Western blotting after knockdown of NPM-ALK. GAPDH served as an internal control to ensure equal loading.
cell lines: KARPAS-299, COST, and SU-DHL-1. DNA karyotype changes did not seem to be the cause of miR-150 downregulation in the NPM-ALK(+) mouse (chromosome 7) model and human (19q13.33 chromosomal region) cell lines (F. Meggetto, unpublished observations; see Supplemental Methods). As observed in these 2 NPM-ALK(+) lymphoma models, genomic hybridization array analysis shows that miR-150 downregulation in 50 ALCL patients (ALK[+] [n = 27] and ALK[–] [n = 23]) was not related to DNA deletion on the 19q13.33 region containing the independent transcription unit coding for miR-150 (L. Lamant, unpublished observations; see Supplemental Methods). In order to determine whether the observed miR-150 downregulation in NPM-ALK(+)
cell lymphomas was achieved by DNA methylation, we measured the levels of miR-150 after decitabine treatment (Figure 4A). A significant increase in miR-150 expression was observed in decitabine-treated cells compared with the drug vehicle alone in the 2 NPM-ALK(+) cell lines KARPAS-299 and SU-DHL-1. In contrast, miR-150 expression was not affected in the FE-PD and Mac-2a (ALK(-)) cell lines treated with decitabine (Figure 4A). We next analyzed the methylation status of miR-150 in a panel of 20 tumor specimens from ALCL ALK(+) and ALK(-) patients and in 4 ALCL cell lines: 3 ALK(+) (KARPAS-299, SU-DHL-1, and COST) and 1 ALK(-) (FE-PD). Using methylated DNA IP assays (MeDIP assays), with human peripheral blood T lymphocytes (PBTL) as a control, we identified a differentially methylated region of about 1 kb surrounding the miR-150 coding sequence in the NPM-ALK(+) ALCL biopsies compared with the NPM-ALK(-) biopsies and PBTL. In this region, we detected what we termed DMR (differentially methylated region), a significantly elevated level of methylation in the NPM-ALK(+) ALCL biopsies (Figure 4B and Supplemental Figure 3A). In contrast, limited methylation was detected in NPM-ALK(-) ALCL biopsies, indicating that methylation of the MIR150 gene is an NPM-ALK-predominant phenomenon (Figure 4B and Supplemental Figure 3A). Of note, in alike biopsies, we observed a limited MIR150 gene methylation in the NPM-ALK(-) FE-PD cell line when compared with the 3 ALCL cell lines carrying the NPM-ALK fusion, KARPAS-299, SU-DHL-1, and COST. In addition, using bisulfite sequencing, we evaluated the level of DNA methylation of the region surrounding the MIR150 gene in KARPAS-299 and COST cells (Supplemental Figure 4A). This showed a significant level of methylation of some of the CpG dinucleotides in this region (Supplemental Figure 4B). As expected, this DNA methylation pattern was lost in cells treated with decitabine (Supplemental Figure 4B). Most importantly, we observed that the inhibition of NPM-ALK, using either crizotinib (Supplemental Figure 4C) or an siRNA targeting NPM-ALK (Supplemental Figure 4D), also induced a clear reduction in MIR150 methylation. Finally, we confirmed that the level of miR-150, measured by qPCR, was significantly lower in NPM-ALK(+) ALCL biopsies compared with healthy RLNs (Supplemental Figure 3B).

In NPM-ALK(+) cells, 2 members of the DNMT family, DNMT1 and DNMT3b, are known to be directly responsible for the DNA methylation of microRNA genes. Therefore, we investigated whether these proteins associate with the MIR150 gene (at locations [-1],000 to [+1],000 relative to the first nucleotide of pre-miR-150). Employing the KARPAS-299 cell line and a combination of ChIP and qPCR analysis (q-CHIP) with primers amplifying 4 chromatin fragments evenly distributed within the DMR region of MIR150, we determined that DNMT1, but not DNMT3A and DNMT3B, is markedly associated with the MIR150 gene (Figure 4C and data not shown). As in NPM-ALK(+) cells, STAT3 is known to enhance the binding of DNMT1 to some promoters; we used q-CHIP experiments to analyze the association of STAT3 with the MIR150 gene. As shown in Figure 4C, no STAT3 binding was observed. Finally, in NPM-ALK(+) cells, the STAT3 protein has also been shown to induce methylation of some host genes/microRNAs through transcriptional activation of DNMT1; therefore, we sought to determine the level of DNMT1 under NPM-ALK and STAT3 knockdown conditions. In si-ALK conditions, we observed, as expected, a decrease in STAT3 activation (reduced p-STAT3 protein levels) and, in parallel, a decrease in the level of DNMT1 protein (Figure 3D and Supplemental Figure 1B). In conditions of strong inhibition of STAT3 in KARPAS-299 (compared with its inhibition via si-ALK, Figure 3D), DNMT1 protein levels were decreased (Figure 4D), with a concomitant increase in miR-150 expression (Figure 4E). The levels of the DNMT1 protein were also evaluated in the conditional NPM-ALK lymphoma transgenic mouse model. As shown in Supplemental Figure 2B, DNMT1 is markedly overexpressed in NPM-ALK(+) ON mice compared with WT or NPM-ALK(-) OFF mice. Thus, the downregulation of miR-150 in NPM-ALK(+) cell lymphomas is likely to be regulated, at least in part, by epigenetic silencing via STAT3 and DNMT1 activation.

**Ectopic expression of miR-150 inhibits proliferation and blocks S-phase entry of NPM-ALK(+) cells in vitro.** To investigate the effect of miR-150 on cell proliferation, we transfected the NPM-ALK(+) cell line KARPAS-299 with miR-150 microRNA mimics. The successful overexpression of miR-150 in the cells was confirmed by qPCR (Supplemental Figure 5). Using Western blotting, we checked that the expression of MYB, a bona fide miR-150 target, was reduced in NPM-ALK(+) cells transfected with miR-150 (Figure 5A). MTS and colony-formation assays showed that ectopic expression of miR-150 could markedly inhibit cell viability (Figure 5B) and block clonogenicity (Figure 5C and D) of NPM-ALK(+) cells compared with the mimic microRNA control (miR-CTL). This antiproliferation effect could be partially due to the disruption of cell growth regulation, such as cell cycle arrest. Thus, we next explored the effect of miR-150 on cell cycle regulation. Flow cytometric cell cycle analysis showed that miR-150 transfection in KARPAS-299 NPM-ALK(+) ALCL cells increased the number of cells in GO/G1 phase and decreased the number of cells in S and G2/M phases of the cell cycle compared with miR-CTL transfection (Figure 5E). There was no detectable increase in the sub-G0/G1 fraction. Accordingly, we noted the lack of an increase in apoptosis (7AAD/Annexin-V staining, data not shown), which was further supported by the absence of a detectable increase in the cleaved caspase-3 level shown on Western blots (data not shown). In addition, we observed that the mitotic index was markedly different among KARPAS-299 cells with ectopic miR-150 expression (1.8% of cells) and those expressing miR-CTL (7%) (Figure 5F). In addition, we could show that the ectopic expression of miR-150 induces a decrease of the MYB protein level (Supplemental Figure 6A) and affects the in vitro growth of the COST cells, another NPM-ALK(+) ALCL cell line (Supplemental Figure 6, B and C). All these results suggest that miR-150 has a key role in the growth of NPM-ALK(+) cell lines, it attenuates MYB expression, and it works as a tumor suppressor.

**The overexpression of miR-150 hampers the growth of NPM-ALK(+) ALCL in vivo.** We next investigated the efficiency of miR-150 transfection on tumor growth of 2 NPM-ALK(+) ALCL cell lines in vivo (KARPAS-299 and COST). miR-CTL- or miR-150–transfected KARPAS-299 or COST cells were inoculated s.c. into the left or right flank of each mouse, respectively (n = 5 for KARPAS-299 and n = 6 for COST). Transfection of miR-150
The effect of crizotinib treatment or a combination of the 2 agents (crizotinib + decitabine) on miR-150 expression. A significant increase in miR-150 expression was observed in crizotinib-treated CR06 cells upon decitabine treatment compared with untreated cells or cells only treated with crizotinib (Supplemental Figure 7C). Subsequently, we analyzed cell growth. As expected, crizotinib had no inhibitory effect on viability and on the ability of KARPAS-299-CR06 cells to form colonies (Figure 7, A and B, and Supplemental Figure 8A), in accordance with the more aggressive phenotype of these cells compared with the more sensitive parental KARPAS-299 cells. In contrast, decitabine markedly reduced cell viability and blocked clonogenicity in soft agar assays (Figure 7, A and B, and Supplemental Figure 8A). The same results were obtained after the addition of decitabine and crizotinib (Figure 7, A and B, and Supplemental Figure 8A). Thus, resistance to crizotinib due to NPM-ALK substitution mutation does not confer cross-resistance to decitabine and does not affect the antiproliferative activity of the demethylating drug in KARPAS-299-CR6 cells treated with the drug alone or in combination with crizotinib. Since the hypomethylating agent caused miR-150 overexpression in these crizotinib-resistant NPM-ALK(+) cells (Supplemental Figure 7C). We examined the effect of crizotinib treatment or a combination of the 2 agents (crizotinib + decitabine) on miR-150 expression. A significant increase in miR-150 expression was observed in crizotinib-treated CR06 cells upon decitabine treatment compared with untreated cells or cells only treated with crizotinib (Supplemental Figure 7C). Subsequently, we analyzed cell growth. 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into KARPAS-299 and COST cells resulted in decreased growth and tumor weight of s.c. xenograft tumors in NOD/SCID mice when compared with those transfected with miR-CTL (Figure 6, A–C). Morphological analyses showed that forced expression of miR-150 caused phenotypic hallmarks of cellular degeneration (uncommon chromatin condensation, nuclear piknosis, cellular volume decrease, and nuclear envelope disruption) (Figure 6D). These results show that miR-150 overexpression drastically disadvantages the growth of NPM-ALK(+) cells in vivo.

Demethylating treatment or ectopic miR-150 expression correlates with growth inhibition of crizotinib-resistant NPM-ALK(+) ALCL cells. KARPAS-299-CR06 cells express a form of NPM-ALK presenting the substitution mutation L1196Q within the KD. This NPM-ALK mutant is insensitive to the crizotinib inhibitor and, therefore, no change in STAT3 activation is observed upon crizotinib treatment (Supplemental Figure 7A). We examined the expression level of miR-150 in crizotinib-resistant KARPAS-299-CR06 cells. miR-150 was found to be significantly downregulated in KARPAS-299-CR06 cells compared with parental KARPAS-299 cells (Supplemental Figure 7B). In order to examine the possible role of miR-150 in crizotinib-resistance, we first induced an increase in miR-150 expression with decitabine...
Combination with decitabine (Supplemental Figure 9). All of these results suggest that the restoration of miR-150 could inhibit the growth of crizotinib-resistant NPM-ALK(+) ALCL cells.

Discussion

Epigenetic gene silencing plays an important role in carcinogenesis. By inhibiting the expression of many tumor-suppressor genes, DNA methylation of gene promoter regions is a key component of this process (45). DNA methylation is also an important epigenetic mechanism for silencing genes transcribed by RNA polymerase II, including miRNA genes (15, 18, 19). Recent reports have shown that miRNAs undergo the same epigenetic regulatory laws as any other protein-coding genes (20, 46, 47). Methylation is mediated by members of the DNMT family that can be inactivated by small-molecule inhibitors such as decitabine (22). High DNMT1 expression is found in human ALCL cell lines and primary tumors (41). In NPM-ALK(+) ALCL cell lines, transcription of the
cell cycle arrest in the G2/M cell cycle phase and significantly enhanced the antiproliferative effect of decitabine (57). They also showed that the efficacy of decitabine was more significant in resistant cells than in sensitive cells. These data suggest that effective delivery of decitabine and prolonged DNMT1 depletion are critical to overcome drug resistance (57).

Only one study has demonstrated that decitabine exhibits an antineoplastic activity against NPM-ALK(+) ALCL cells and xenografted tumors both in vitro and in vivo (41). It is thus tempting to propose that decitabine could be an effective treatment against NPM-ALK(+) ALCL, possibly through causing miR-150 reexpression. In NPM-ALK(+) KARPAS-299 ALCL cells, we observed that miR-150 expression was increased upon decitabine treatment. In vivo, upregulation of miR-150 expression exhibited an antineoplastic activity against xenografted NPM-ALK(+) KARPAS-299 tumors. The overexpression of miR-150 induced a significant decrease in the number of viable cells, and cell cycle analysis showed a decrease in the proportion of cells in S phase as well as G2/M phase. This cell cycle blockage was not associated with an increase in apoptosis, similar to that reported by Han and coworkers, who also treated KARPAS-299 cells with decitabine (16). Moreover, in NPM-ALK(+) KARPAS-299 ALCL cells, we observed that upregulation of miR-150
for ALCL, new treatments are needed for resistant or relapsing patients (3, 7, 68, 69). The use of crizotinib, an ALK TKI, has revolutionized the treatment of ALK(+) patients with advanced-stage or relapses (70). However, acquired resistance, defined as progression after initial benefit, inevitably occurs upon such targeted treatment (10, 71). Although drug resistance can be overcome using epigenetic therapies in experimental models, clinical studies have highlighted the challenges of current epigenetic therapies for solid tumors and the need to identify more targeted approaches (72). From this has arisen the potential of hypomethylating agents, such as decitabine, which have been assessed to be able to cooperate with TKIs (22). Ceccon and coworkers generated a stable subpopulation of KARPAS-299 cells that are able to live and proliferate in the presence of crizotinib, naming them CR06 (for crizotinib resistant growing with 600 nM of crizotinib) (73). KARPAS-299-CR06 cells express a form of NPM-ALK presenting the substitution mutation L1196Q within the KD. A leucine substitution in residue position 1196 is found at relatively high frequency in NSCLC patients. This mutation was shown to confer resistance to the drug in vitro. Interestingly, Leu1196 corresponds to a key residue that has been observed to confer resistance to many TKIs (10). The same position is exploited by BCR-ABL (the most infamous T315I mutant, ref. 74) to enable it to survive TKI therapy, and the corresponding EGFR T790M mutation gives complete resistance to EGFR (75).

We have shown that this NPM-ALK–gatekeeper mutation, at the Leu1196 position, does not confer cross-resistance to decitabine and does not affect the antiproliferative activity of decitabine on KARPAS-299-CR06 cells. Using crizotinib-resistant KARPAS-299-CR06 cells, we identified that restoration of miR-150 by
ALK fusions that harbor either different ALK fusions or overexpressed/neoplastic ALK(+) cells. As for crizotinib-naive or -sensitive NPM-ALK(+) cells, the response to decitabine appears to be induced by modifications at both the mir-150 and MYB levels, and cell cycle arrest was induced with no significant increase in apoptosis. To the best of our knowledge, our study reveals for the first time that hypomethylating/antileukemic drugs show great potential for the treatment of crizotinib-resistant ALK(+) cells (Figure 8). In accordance with our findings, La Rosée and coworkers used resistant cell lines caused by the expression of mutated Bcr-Abl to demonstrate in vitro that combined treatment with decitabine and imatinib, a selective inhibitor of the BCR-ABL tyrosine kinase, is beneficial for imatinib-resistant cells (76). Clinical trials are now underway to determine whether these results are valid in a physiological environment.

The discoveries of the EML4-ALK fusion in 2007 as the oncogenic driver in NSCLC and then the identification of activating mutations of the ALK gene in the development of neuroblastoma have made ALK one of the most extensively studied targets in the field of TKI drug development (77–80). Our findings suggest that an alternative option in these cases would be to target deregulated epigenetic mechanisms and apply decitabine in combination with already-established drugs, such as crizotinib or other ALK inhibitors, to TKI-resistant ALK(+) tumors or in combination with other agents to improve the overall response rates without exposing patients to long-term toxicities. Consequently, our study should promote further investigation into the effect of epigenetic modulation in other ALK-related malignancies that harbor either different ALK fusions or overexpressed/overactivated full-length ALK oncogenes.

**Methods**

**Human cell lines, and tumoral and normal samples.** KARPAS-299, COST, and SU-DHL-1 ALK(+) ALCL cell lines bearing the t(2;5) (p23;q35) translocation were obtained from DSMZ (German Collection of Microorganisms and Cell Culture) or established in our laboratory. Both the ALK(-) ALCL cell lines, Mac2a and FE-PD, were provided by O. Merkel (Medical University, Vienna, Austria) and K. Pulford (Oxford University, Oxford, United Kingdom) respectively, as gifts. Cells were cultured in Iscove Modified Dulbecco’s medium (IMDM, Invitrogen) supplemented with 15% FCS and were maintained in exponential growth phase. The KARPAS-299-CR06 cell line was provided by C. Gambacorti-Passerini and M. Ceccon (Department of Health Sciences, University of Milano-Bicocca, Milan, Italy) (73) and was cultured in RPMI-1640 supplemented with 10% FCS. Medium was replaced with fresh RPMI-1640 supplemented with 600 nM crizotinib every 48 or 72 hours. PBMCs from 3 healthy donors were used as normal lymphoid cells and were provided by A. Quillet-Mary (UMR1037, CRCT). PBMCs and CD4+ isolated cells were stimulated using 7 × 10^6 of KARPAS-299 cells and 1,000 gel units of micrococal nuclease for each IP performed (Supplemental Table 2). The ChIP and Input (2% of the starting material) samples were analyzed by qPCR (Supplemental Table 1). Results are expressed as percentage of input ([100×^2]([C_bas]-[log2 of 50])- C_tn60)).

**Bisulfite sequencing.** Genomic DNA (gDNA) extraction, bisulfite conversion, and Illumina sequencing were performed by Active Motif (http://www.activemotif.com). gDNA was extracted from 3 million KARPAS-299 cells treated for 5 days with 5 μM decitabine or 500 nM crizotinib, and 3 million COST cells transfected with si-RNA targeting NPM-ALK. gDNA samples were then subjected to bisulfite conversion. PCR amplicons were generated using forward and reverse bisulfite primers (Supplemental Table 1) designed to amplify a 541-bp region of the MIR150 gene containing 22 CpG dinucleotides. PCR products were sequenced on Illumina NextSeq 500. As reference sequence, the human chr19 (hg19 assembly) was used.

**Statistics.** Differences between 2 groups were examined using 2-tailed Student’s t test. All analyses were performed using GraphPad Prism version 6.00 for Windows. P < 0.05 was considered significant.

**Study approval.** Frozen tumor samples were retrieved from CHU de Toulouse tumor tissue bank. The study was carried out in accordance with the institutional review board–approved protocols, and the procedures followed were in accordance with the Helsinki Declaration of 1975, as revised in 2000. Mice were housed under specific pathogen-free conditions, in a constant temperature (20°C–22°C) animal room, with a 12-hour light/12-hour dark cycle and free access to food and water. All animal procedures were performed following the principle guidelines of INSERM, and our protocol was approved by the Midi-Pyrénées Ethics Committee on Animal Experimentation.

**Accession number.** Bisulfite sequencing data were deposited to the Gene Expression Omnibus (GEO) public database according to MINSEQE standards (accession no. GSE69836).

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48. Maia BM, Rocha RM, Califin GA. Clinical significance of


