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KLF1/EKLF expression in acute leukemia is correlated with chromosomal abnormalities

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Abstract

KLF1 (EKLF) is a master regulator of erythropoiesis and controls expression of a wide array of target genes. We interrogated human tissue microarray samples via immunohistological analysis to address whether levels of KLF1 protein are associated with leukemia. We have made the unexpected findings that higher KLF1 levels are correlated with cells containing abnormal chromosomes, and that high KLF1 expression is not limited to acute myeloid leukemia (AML) associated with erythroid/megakaryoblastic differentiation. Expression of KLF1 is associated with poor survival. Further analyses reveal that KLF1 directly regulates a number of genes that play a role in chromosomal integrity. Together these results suggest that monitoring KLF1 levels may provide a new marker for risk stratification and prognosis in patients with AML.

Keywords

Leukemia; transcription factor; EKLF/KLF1; erythropoiesis; tissue microarrays

1. Introduction

Central to the homeostasis of the hematopoietic system is the correct balance of progenitor cell proliferation versus lineage-committed differentiation [1]. Loss of these controls can lead to unrestricted proliferation and impaired differentiation, both of which are

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characteristic of acute myeloid leukemia (AML) [2, 3]. Although chromosomal translocations leading to oncogene activation are a major contributor to leukemia [4, 5], hypomorphic [6, 7] or mutated [8] transcription factors can also play a role in leukemogenesis.

KLF1 (Erythroid Krüppel-like Factor; EKLF) is a red cell-enriched, zinc finger DNA binding protein whose roles in ß-like globin gene regulation during terminal erythroid differentiation have been well-established with genetic, biochemical, and molecular approaches [9–11]. KLF1 is absolutely critical for the erythroid lineage, of which the most obvious phenotypic effect is a profound ß-thalassemia that leads to lethality in murine embryos at the time of the switch to adult ß-globin expression [12–14]. More recently, KLF1's activation target repertoire has expanded to also include genes in both primitive and definitive erythroid cells [15–17]. In this context, activation of p21, p18, p27, and E2F2 by KLF1 plays a directive role in altering the erythroid cell cycle status from proliferation to differentiation [18–22]. Appropriate expression levels of these KLF1 targets are particularly important for efficient terminal differentiation [22, 23].

KLF1 expression is tissue-restricted throughout early development and in the adult [9, 24]. Although KLF1 is barely detectable in hematopoietic stem cells, it is expressed at low levels in the multipotent progenitor early in hematopoietic differentiation and retains an expression pattern restricted to the common myeloid progenitor (CMP) and megakaryocyte/erythroid progenitor (MEP) [25–27].

Given the regulated targets of KLF1, and particularly their downstream effect on proliferation, it is surprising that KLF1 mutants implicated in malignancy are rare, although KLF1 levels vary considerably within AML subtypes [28]. This is of interest, as links have been established between mutant or haploinsufficient levels of KLF1 and altered human hematology and anemia [15, 29, 30]. In particular, some genes are uniquely sensitive to haploinsufficient levels of KLF1, leading to altered genetic expression patterns and hematologic parameters in humans that can be clinically advantageous [31–35].

We postulated that altered levels of KLF1 may be linked to or even play an active role in a subset of hematopoietic disease. As virtually all previous studies have analyzed variation in KLF1 mRNA expression, we focused on monitoring the presence of or changes in KLF1 protein levels by interrogation of a large cohort of selected tissue microarray AML samples and addressing whether KLF1 levels correlate with leukemic subtypes. Such immunohistochemical analysis of patient biopsies is a powerful approach that can illuminate the relationship between expression and phenotype (e.g., LMO2 [36]).

2. Materials and Methods

2.1. Global data

Hemaexplorer [37] database: http://servers.binf.ku.dk/hemaexplorer(recently updated to http://servers.binf.ku.dk/bloodspot/). Vizome [38] database: http://www.vizome.org/aml/

The human KLF1 and P300 chromatin immunoprecipitation datasets were derived from the data in reference [39]. KLF1 mRNA levels in t(6;9) in DEK/NUP214-expressing AML cells, normal bone marrow, or AML without t(6;9) were derived from the data in reference [40].

2.2. Cells and extracts

Mouse or human KLF1 expression constructs [9, 41] were transfected into fibroblast-like cell lines derived from monkey kidney tissue (COS cells) [42]. Extracts were prepared, and the ensuing western blots were probed with 4B9, 6B3, or 7B2 monoclonal antibodies (all established in-house [43]). As a more stringent test, we used 7B2 to probe an extract from day 10 of the second phase of a culture expanded via a two phase liquid culture from human peripheral blood [44].

2.3. Leukemic tissue samples

Diagnostic bone marrow samples were procured from patients with hematological malignancies from the Tom Baker Cancer Centre, Alberta Canada after IRB approval. Hematoxylin-and-eosin (H&E) sections of bone marrow core biopsies were reviewed for adequacy of tissue, and diagnoses were confirmed according to the WHO classification [45, 46]. Triplicate, representative cores (600-μm) were used to create tissue microarrays (TMA) from FFPE tissue (n=250), as previously described [47]. Sections cut from whole sections or the TMA were baked for 1 hour at 60°C before immunohistochemistry was performed.

Antigen retrieval and immunohistologic detection conditions were optimized for 7B2 using whole bone marrow core biopsy sections. Whole sections as well as the TMA of 250 AML stained with 7B2 were scored as follows: 0, no staining; 1+, faint / weak staining in <10% leukemic blasts 2+, moderate / focal staining in <10 % leukemic blasts; and 3+, strong and diffuse staining in >10% leukemic blasts. In addition, localization (nuclear, cytoplasmic, both) was noted for all positive samples. In the absence of diagnostic tissue due to sampling error or technical difficulties, no score was recorded (n=32; 13%). Using this system, we found the specificity of KLF1 remained high. Fisher exact t test, two tail with >=2 as positive was used as statistical parameter.

All H&E and immunohistochemistry slides were analyzed using a Nikon Eclipse E1000M microscope (Nikon, Burlingame, CA) equipped with a 40x/0.75 NA objective lens and photographed using a SPOT RT color camera (Diagnostic Instruments, Sterling Heights, MI).

2.4. Patient data

Our cohort comprised of consecutive, non-APL (M3) AML patients (n=250) accrued between 2002 and 2007. Inclusion criteria comprised of availability of adequate diagnostic tissue sample; clinical and laboratory data. Therapy related AML and patients with previous diagnosis of myeloproliferative neoplasms were excluded. Patients were characterized based on FAB criteria; however, the diagnosis was also reviewed and revised according to WHO criteria (2008) based on morphology; immunophenotyping and cytogenetic data acquired at accredited clinical laboratories. Cytogenetic data was used for risk stratification (favorable, Intermediate and poor) based on established criteria (Blood. 2010;115(3):453–474). Data

linked with mutational analysis (FLT3/NPM1 and CEBPA) was available in a small subset (18%), hence this data set was not included in risk assessment and further analysis. All patients received induction chemotherapy (Cytarabine and Anthracycline based on 3+7 protocol) with or without bone marrow transplant according to a standardized provincial protocol. Overall survival was calculated from the date of diagnosis to the time of death for any cause or most recent follow-up. The Kaplan Meier method with log rank test (univariate analysis) and cox-regression method (multivariate analysis) was used to estimate the overall survival. All computed results having two-sided P value < 0.05 were considered significant. The statistical analysis was performed using SPSS software v21.0 (IBM, Armonk, NY).

3. Results

3.1 Patients demographic, Clinical and Laboratory Data

All AML patients were adults (≥ 18 years); while more than half (138/250; 55%) were in ≥ 60 yrs. age group. There were 136 men and 114 women (M:F ratio 1.2:1). Inclusion criteria was based on confirmed myeloid lineage by flow cytometry based immunophenotyping; hence patients with ambiguous lineage were excluded. Morphologically, AML with MDS related changes was noted in 51 (23%) while 66 (30%) patients had monocytic differentiation (FAB M4/M5). Erythroid / Megakaryocytic differentiation (FAB M6/M7) was demonstrated in 22 (10%) samples. Cytogenetic data at diagnosis was available in 218 /250 (87%) patients.

3.2. Variation in KLF1 expression and validation of KLF1 antibody for FFPE tissues

Analysis of the Hemaexplorer/Bloodspot compendium data [37] shows that some acute leukemia subtypes exhibit a wide range of KLF1 expression, in many cases achieving levels as high as that seen in the MEP (Figure 1A). Consistent with this, Vizome analysis [38] also shows variation of KLF1 levels across WHO subtypes (Figure 1B). Together, these provide a suitable rationale to address whether differing levels of KLF1 correlate with AML phenotype. We therefore focused on analysis of a large cohort of patients, managed under a standardized and consistent treatment protocol. Two of our anti-mouse KLF1 monoclonal antibodies that cross react with human KLF1 (6B3 and 7B2; Figure 2A,B) were directly tested for their ability to recognize human KLF1 protein in FFPE tissues. 7B2 exhibited a greater specificity (not shown), and was selected for further application. Figure 2C-E shows that the immunohistochemical signal from the antibody is strictly localized to the erythroid cells in the normal bone marrow (Figure 2C and not shown). The FFPE sample from a pure erythroid leukemia contains positive cells (Figure 2D), but the CML sample is negative (Figure 2E). Additional analyses demonstrate that Acute Myelomonocytic Leukemia cells are also negative (not shown). These data demonstrate that the 7B2 anti-KLF1 antibody is capable of monitoring hKLF1 expression in fixed, embedded, and archived FFPE samples, and provides the critical reagent needed for these studies.

3.3. KLF1 expression in leukemic pathologies

Analysis of KLF1 expression and distribution across human AML subtypes enable us to make a number of conclusions. First, in KLF1+ AML cells, a higher level of expression is associated in cells with any chromosomal abnormality in comparison to those with a normal

karyotype ($p<0.0008$) (Figure 3A-C, Table 1). KLF1 expression in AML cells is not linked with any specific recurrent chromosomal translocations, however, higher KLF1 expression is significantly associated with recurrent cytogenetic abnormalities linked with good prognosis, compared to normal cytogenetics ($p<0.002$). When analyzed with respect to karyotype risk, we find positive KLF1 expression is significantly related to intermediate and poor/adverse cytogenetics (Table 2 and Figure 3E).

Second, high level KLF1 expression is seen in AML subtypes that exhibit erythroid as well as megakaryocytic differentiation, with expression seen in 75% of those cases. KLF1 expression (strong, 3+) in samples of Pure Erythroid Leukemia or Acute Megakaryoblastic Leukemia, when compared to expression in samples of AML with MDS-related changes, show a trend towards higher expression ($p=<0.08$). A significant number of various non-APML subtypes of AMLs also show substantial expression of KLF1. Interestingly, comparison of KLF1 expression in various subtypes of AML did not relate to degree of differentiation (monocytic versus erythroid or megakaryocytic) and did not show significant difference between various subtypes of AML ($p= 0.56$).

Third, a slightly higher KLF1 expression is seen in AML samples associated with myelodysplasia in comparison to those without. In the AML with MDS related changes (AML-MRC) category, expression of KLF1 trends more commonly among patients with abnormal cytogenetics (54% vs. 40%; $p<0.09$). Expression of KLF1 in AML-MRC is higher compared to AML with monoblastic/monocytic differentiation (FAB M4/M5) ($p<0.03$); however, expression in AML-MRC vs. AML with maturation (FAB M2) was not significantly different.

3.4 Nuclear/cytoplasmic localization of KLF1

We find that in some cases, KLF1 is strikingly distributed either to the cytoplasm or to the nucleus (Figure 4A-C). Although cytoplasmic distribution of a surprising amount of KLF1 has been seen in murine tissues [48–50], in human AML cases we observe that preferential staining in the cytoplasm is most prevalent in AML blasts, while normal maturing erythroid precursors showed KLF1 staining primarily localized to the nucleus (Figure 4D-F). However, no correlation can be made between cytoplasmic or nuclear distribution and a particular subtype of AML ($p>0.5$).

3.5. Unanticipated KLF1 expression in ALL

Given the highly restricted nature of KLF1 expression to erythroid tissues and its immediate progenitors [9, 24, 25], we were surprised to find lymphoblastic leukemia that express abundant levels of KLF1 (Figure 3D, Table 1). This suggests there is a significant level of genetic dysregulation in these samples, enabling tissue-restricted factors such as KLF1 to be expressed ectopically and possibly confer erythroid characteristics (as seen in some MDS cells [51, 52]) to what would normally be a lymphoid-specific cellular environment.

3.6. Regulation of relevant targets by KLF1

Although our data is correlative between KLF1 levels and abnormal karyotype, many genes that are directly or indirectly linked to chromosomal abnormalities in MDS and AML [53–

57] are direct targets as judged by ChIP analysis (*NPM1, SF3B1, KDM6A,* and *CREBBP* examples are shown in Figure 5A), raising the possibility that high levels of KLF1 may be causally related to chromosomal aberrancies via a direct gain-of-function effect. Such a change in KLF1 level may result from a change in protein stability or from altered transcription. For example, the DEK oncogene, which aids in the optimal expression of KLF1 [58], forms a DEK/NUP214 chimeric protein in cells with the t(6;9) rearrangement [59, 60], As a result of DEK disruption, KLF1 mRNA levels are lower in t(6;9) DEK/NUP214-expressing AML cells compared to normal bone marrow or AML without t(6;9) (Figure 5B) [40].

4. Discussion

We have made the unexpected discovery that leukemic cells containing abnormal chromosomes correlate with higher levels of KLF1 protein. Many genes whose mutation is associated with chromosomally abnormal MDS and AML are directly bound by KLF1 in vivo. This suggests that loss of KLF1 expression control may modify normal levels of these targets, ultimately altering chromosomal properties. Such a postulate is supported by the observation that cellular levels of KLF1 are known to directly influence downstream gene expression, such that some targets are adversely affected by a 50% drop in KLF1 expression [15, 29–32]. On the other hand, KLF1 gain-of-function quickly leads to an increase in cell cycle inhibitors and cessation of cell division [18–21], yielding precocious terminal erythroid differentiation and inhibition of megakaryopoiesis [25, 61–63].

Changes in KLF1 expression control can arise in a number ways. First, we have shown that alteration of DEK function via its fusion with NUP214 in t(6;9) [40] decreases KLF1 levels. Second, the converse occurs in AML cells with altered RUNX1 function (e.g., fusion with ETO in t(8;21)), which is an upstream repressor of KLF1 [64]. Third, KLF1 levels increase in mice containing deficits in both Dnmt3a and Tet2, an effect also observed in the AML subset with these dual mutations [65]. Fourth, AML cells with amplified 8q24, a region that harbors MYC and other linked genes, express increased levels of KLF1 and downstream erythroid signature genes [66]. Finally, a minimal common segment on chromosome 19 targeted by chromothripsis in acute erythroid leukemia harbors KLF1 [67]. Although these studies are suggestive of possible mechanisms for our present observations, they still remain correlative.

The high level of cytoplasmic KLF1 in some cells is quite intriguing, but its function there remains unexplained [17]. Given that the present studies used bone marrow core biopsies, coupled to the extent of hypoxic conditions within the marrow [68, 69], it is possible that oxygen tension is playing a role in KLF1 nuclear/cytoplasmic distribution. This has not been directly tested and inspection of additional samples will be needed to see whether a subtle correlation exists between subcellular KLF1 distribution and a particular AML subtype.

In contrast to our observations on event-free survival, one study has suggested that AML patients with a higher level of KLF1 have a longer survival [70]. There are a number of issues with that study. First, the cohort was small (about 50 samples) with numerous divisions with no documentation of statistical power. Second, that study utilized mRNA

from a whole sample (normal plus leukemic cells), likely explaining the high levels of KLF1 seen in patients with low blast count. Third, almost half of patients were treated with autologous bone marrow transplant, leaving unclear how the treatment was equalized for event-free survival between various groups. Fourth, no multivariate analysis was provided. Our analysis of KLF1 in a normal cytogenetics cohort with over 100 standardized patients addresses the event-free survival issue more powerfully.

Ultimately, our study suggests that monitoring levels of KLF1 may be useful in ascertaining treatment strategies and/or stratification of patients with AML. Correlating KLF1 levels with chromosomal aberrance is of clinical relevance, as chromosomal abnormalities are central to the pathogenesis of AML [3, 53, 71]. In addition, given that the classification of myeloid neoplasms including AML has evolved [72] and mutation profiling is more easily attainable, the identification of new expression markers such as KLF1 that can be used individually and in combination to simplify the extensive heterogeneity of the disease [73] and enable prognostic classification remains highly desired.

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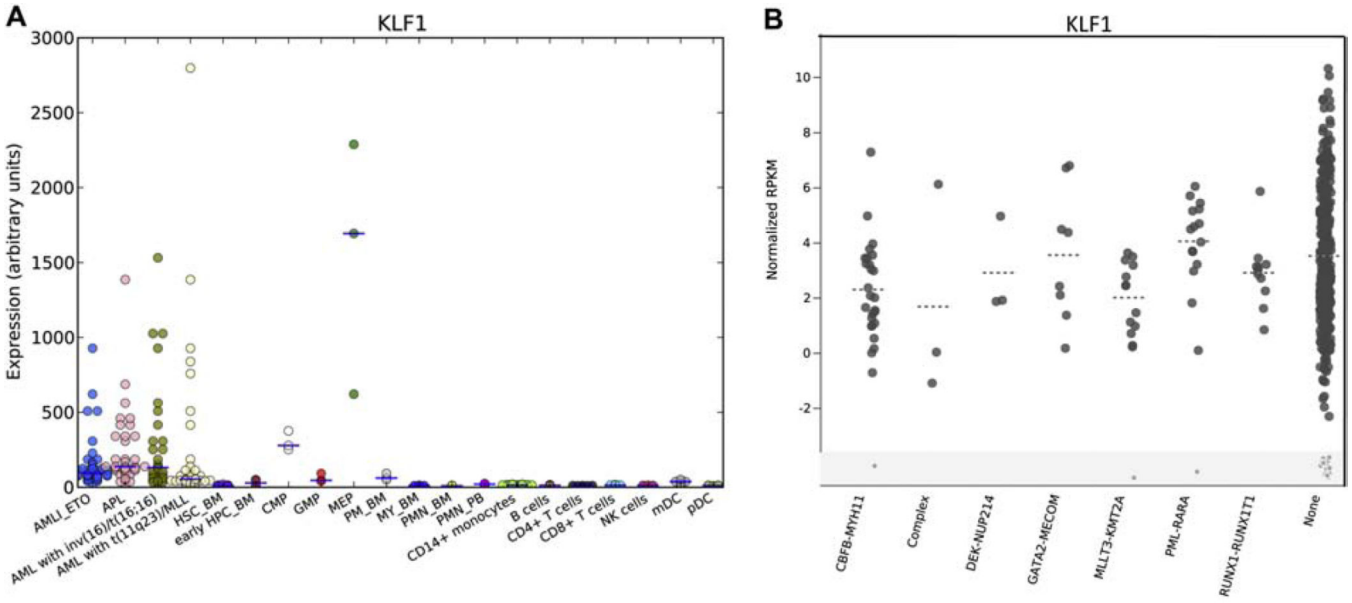

Figure 1.

Analysis of KLF1 levels. **(A)** Hemaexplorer analysis of KLF1 RNA levels in various leukemic (**left**) and normal cells (**right**) [37], shown on the same scale. The restricted pattern of human KLF1 expression in normal cells (highest in the CMP and MEP while not detectable in other cells) mirrors that seen in prospectively isolated normal mouse hematopoietic cells [25]. **(B)** Vizome analysis [38] of KLF1 expression stratified by WHO fusion categories.

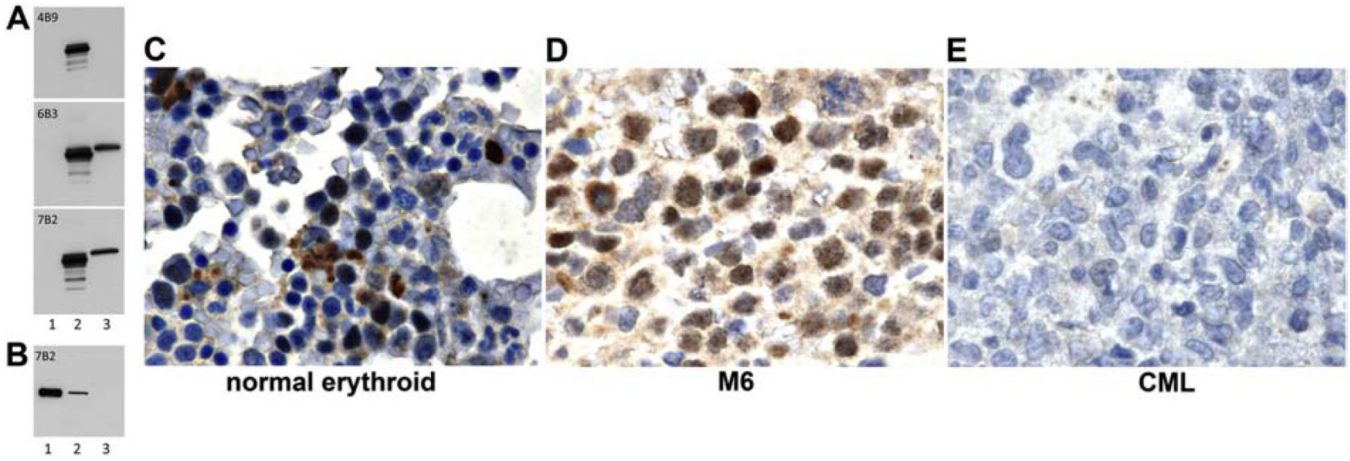

Figure 2.

Tests of anti-mouse KLF1 monoclonal antibodies' ability to recognize human KLF1 protein. **(A)** Extracts from mock (1), mKLF1- (2), or hKLF1- (3) transfected COS cells were probed via western blot with each of three anti-mKLF1 monoclonals as indicated. hKLF1 protein migrates at a slightly larger apparent molecular weight than mKLF1 protein. **(B)** Extracts from hKLF1-transfected COS cells (1), human erythroid culture (2), or mock transfected COS cells (3) were probed with 7B2 anti-mKLF1 monoclonal via western blot analysis. hKLF1 protein expression in tissues were examined by Immunohistochemistry with 7B2 antibody: normal erythroid bone marrow **(C),** AML-M6 **(D),** or CML **(E)** sample photographs were taken at 100x.

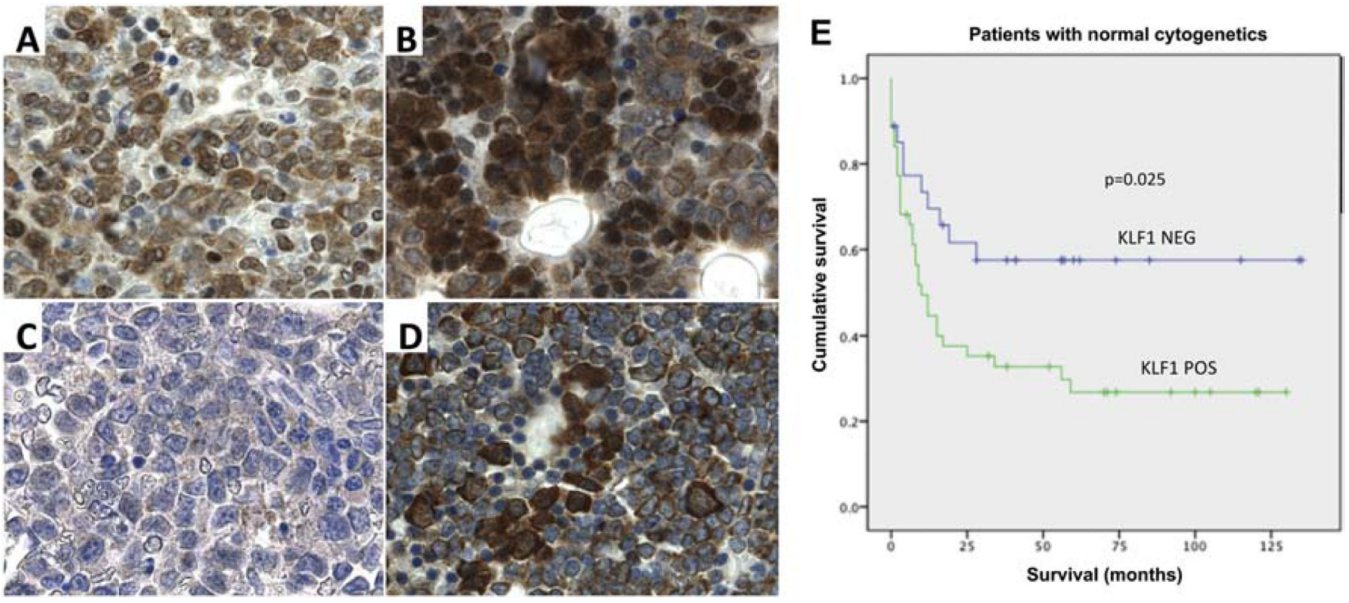

Figure 3.

Case examples 7B2 staining in **(A)** acute myeloid leukemia with normal karyotype; **(B)** acute myeloid leukemia with abnormal karyotype; **(C)** acute myelomonocytic leukemia; (**D)** acute lymphoblastic leukemia. **(E)** Survival curves for patients without cytogenetic abnormalities, dependent on KLF1 levels. A score <3 was taken as negative, ≥3 as positive.

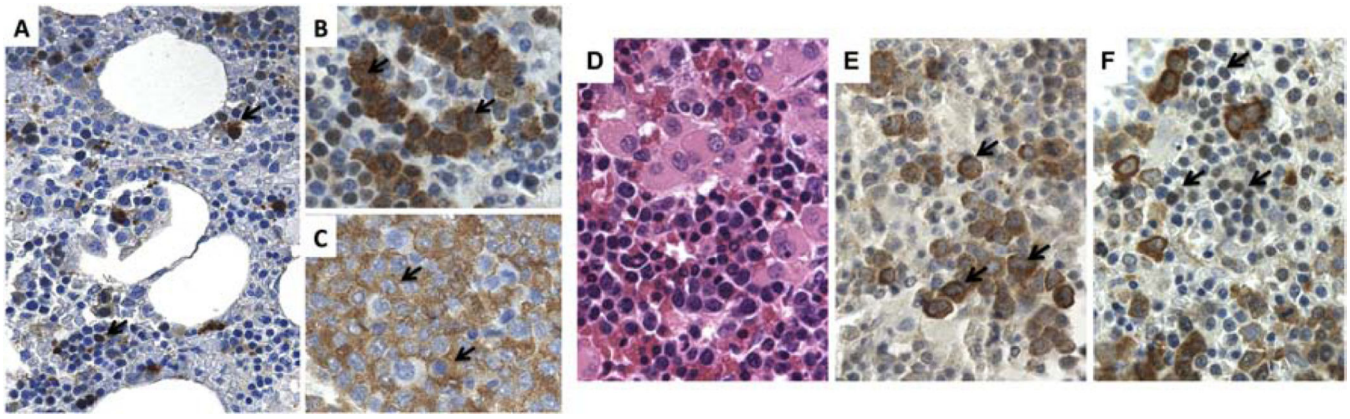

Figure 4.

Nuclear/cytoplasmic partitioning of KLF1. **(A)** 7B2 staining is localized to the nuclei of erythroid precursor cells, but not found in myeloid precursors or megakaryocytes in normal human bone marrow; **(B)** Case of erythroleukemia with nuclear and cytoplasmic staining for 7B2; **(C)** Case of erythroleukemia with predominantly cytoplasmic staining for 7B2. H & E staining shows **(D)** a case of erythroleukemia with immature erythroid blasts and maturing erythroid precursors; **(E)** blasts show 7B2 staining primarily localized to the cytoplasm; **(F)** maturing erythroid precursors show 7B2 staining primarily localized to the nucleus.

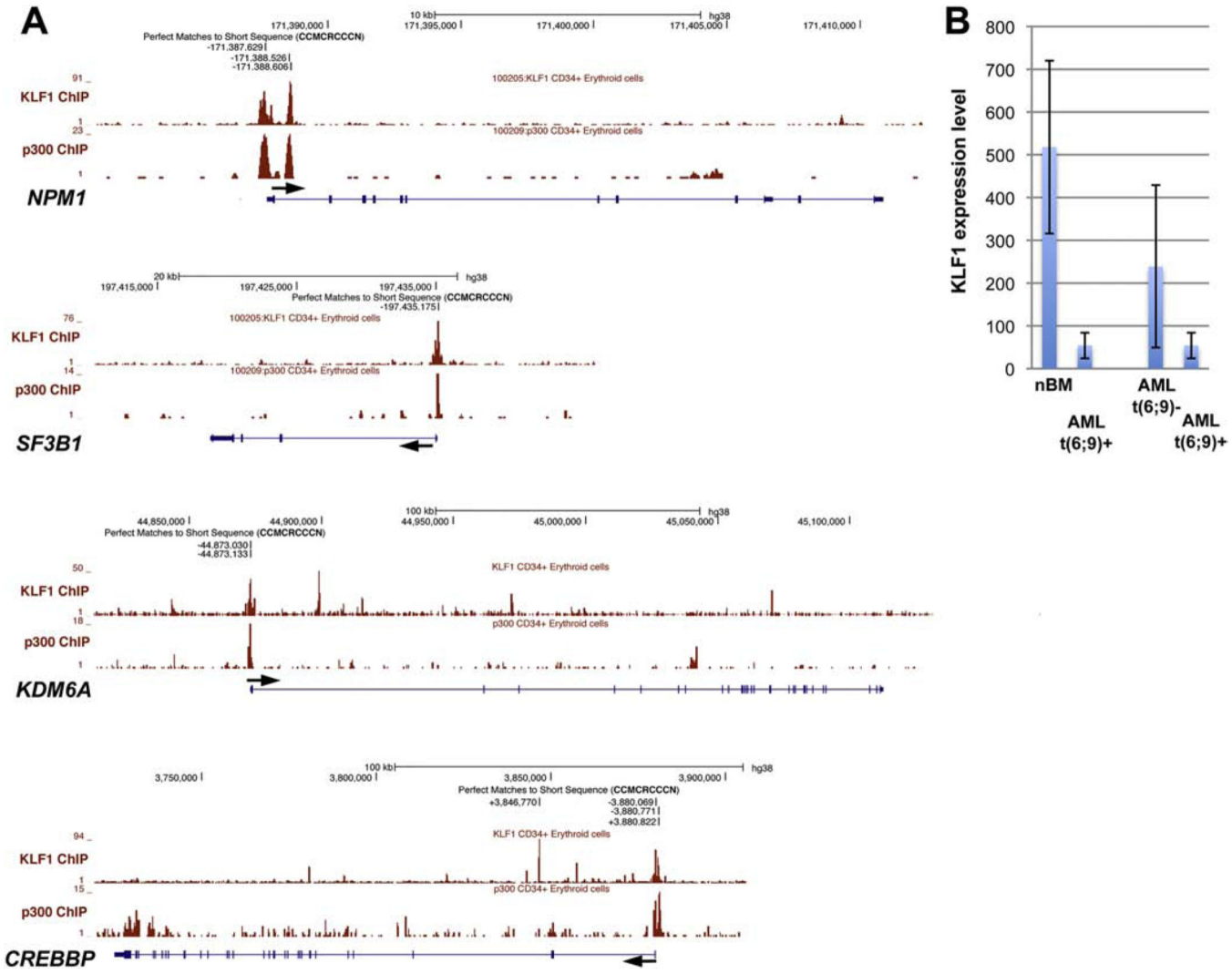

Figure 5.

Regulation by and of KLF1. **(A)** KLF1 ChIP data [39] identifies potential KLF1-regulated targets (*NPM1, SF3B1, KDM6A, CREBBP*) that are mutated or exhibit altered levels in MDS or AML cells with abnormal chromosomes [53, 54, 56]. p300 ChIP is shown for comparison, as this coactivator is known to interact with KLF1 [42] and is present at many KLF1-regulated targets. Also shown is the overlap of KLF1/p300 peaks with the known KLF1 consensus sequence (5'-CCMCRCCCN) [74]. **(B)** Expression levels of KLF1 is shown in cells that express a normal DEK protein (BM and AML with no t(6;9) translocation) or in AML cells that express the DEK/NUP214 fusion that follows from the t(6;9) translocation [40].

Table 1.

Summary of KLF1 protein staining in human acute leukemia subtypes

	Total	Negative	Positive - Strong Cytoplasmic	Positive - Strong Nuclear & Cytoplasmic	Positive - Weak Nuclear or Cytoplasmic
AML - normal cytogenetics	79	20 (25%)	20 (35%)	23 (29%)	3 (10%)
AML - abnormal cytogenetics	157	59 (38%)	59 (38%)	30(19%)	9 [6%)
ALL	74	44 (60%)	24 (32%)	6 (8%)	0

Table 2.

Comparison of KLF1 expression across various cytogenetic risk categories

Risk group	KLF1 positive	KLF1 negative	Total	p-value
Favorable cytogenetics	12(11%)	25 (23%)	37	
Intermediate cytogenetics	59 (53%)	40 (45%)	107	0.022 *
Adverse cytogenetics	40 (36%)	34 (32%)	74	0.043 *
TOTAL	111 (100%}	107 (100%)	218	

* = compared against good prognosis karyotype