HHS Public Access

Author manuscript

Leukemia. Author manuscript; available in PMC 2018 January 11.

Published in final edited form as:

Leukemia. 2018 January ; 32(1): 38–48. doi:10.1038/leu.2017.166.

Deregulation of kinase signaling and lymphoid development in EBF1-PDGFRB ALL leukemogenesis

Seth J. Welsh[1,*], **Michelle L. Churchman**[2,*], **Marco Togni**[2], **Charles G. Mullighan**[2,5], and **James Hagman**[1,3,4,†,5]

[1]Program in Molecular Biology, University of Colorado School of Medicine, Aurora, CO 80045, USA

[2]Department of Pathology, St. Jude Children's Research Hospital, Memphis, TN 38105, USA

[3]Department of Biomedical Research, National Jewish Health, Denver, CO, 80206, USA

[4]University of Colorado Cancer Center, Colorado University Anschutz Medical Campus, Aurora, CO 80045, USA

Abstract

The chimeric fusion oncogene EBF1-PDGFRB is a recurrent lesion observed in Ph-like B-ALL and is associated with particularly poor prognosis. While it is understood that this fusion activates tyrosine kinase signaling, the mechanisms of transformation and importance of perturbation of EBF1 activity remain unknown. EBF1 is a nuclear transcription factor required for normal B-lineage specification, commitment, and development. Conversely, PDGFRB is a receptor tyrosine kinase that is normally repressed in lymphocytes, yet PDGFRB remains a common fusion partner in leukemias. Here, we demonstrate that the *EBF1-PDGFRB* fusion results in loss of EBF1 function, multimerization and autophosphorylation of the fusion protein, activation of STAT5 signaling, and gain of IL-7-independent cell proliferation. Deregulation and loss of EBF1 function is critically dependent on the nuclear export activity of the TM domain of PDGFRB. Deletion of the TM domain partially rescues EBF1 function and restores IL-7 dependence, without requiring kinase inhibition. Moreover, we demonstrate that EBF1-PDGFRB synergizes with loss of IKAROS function in a fully penetrant B-ALL *in vivo*. Thus, we establish that EBF1-PDGFRB is sufficient to drive leukemogenesis through TM-dependent loss of transcription factor function, increased proliferation and synergy with additional genetic insults including loss of IKAROS function.

†**Correspondence:** James Hagman, National Jewish Health, 1400 Jackson Street, K516B, Denver, CO, 80206 USA. Phone: 303-398-1398; Fax: 303-398-1396; hagmanj@njhealth.org.

[5]Co-senior authors

[*]These authors contributed equally to this work

Conflict of interest statement-The authors declare no conflict of interest.

AUTHORSHIP CONTRIBUTIONS

Conceptualization and methodology, S.J.W., M.L.C., M.T., C.G.M. and J.H.; Investigation, S.J.W., M.L.C., M.T.; Writing, S.J.W., M.L.C., C.G.M. and J.H.

Supplementary Information is available at *Leukemia*'s website.

INTRODUCTION

Notwithstanding a 5-year event-free survival rate surpassing 90%, acute lymphoblastic leukemia (ALL) remains a leading cause of cancer-related death for individuals under 40.[1] Hallmarks of leukemic cells include the inability to differentiate into mature lymphocytes and unregulated cellular proliferation.[2] Ph-like (Philadelphia-like; *BCR-ABL1*-like) ALL is a high-risk subtype of B cell precursor ALL defined by a gene expression profile similar to Ph+ ALL.[3] Characteristically, Ph-like ALL has a diverse range of chromosomal rearrangements, mutations, and DNA copy number alterations that deregulate cytokine receptor and tyrosine kinase (TK) signaling.([4] Chromosomal rearrangements in Ph-like ALL commonly result in fusions between lymphoid transcription factor and TK genes. One such fusion, early B cell factor 1–platelet derived growth factor receptor beta (*EBF1-PDGFRB)*, arises from reciprocal translocation or interstitial deletion events between *EBF1* exon 15 to *PDGFRB* exon 11 located at 5q33 (Figure 1a).[3, 5] EBF1 is the most common fusion partner with PDGFRB observed in Ph-like ALL. Like other PDGFRB fusions, it retains both the transmembrane (TM) and tyrosine kinase (TK) domains (Supplementary Figure 1).[6–8] EBF1-PDGFRB occurs in ~8% of Ph-like patients, is enriched in ~30% patients with other B-ALL subtypes who experience induction failure, and is associated with higher relapse rates.[5, 9] Patients harboring the EBF1-PDGFRB fusion frequently have additional genomic lesions resulting in the loss or competitive inhibition of essential B lymphoid transcription factor genes including *IKZF1* (IKAROS), loss of the non-rearranged allele of *EBF1* and/or *PAX5*, and deletion of *CDKN2A/B* (encoding the cell cycle regulators and tumor suppressors ARF and INK4A/B). EBF1-PDGFRB results in cytokine-independent proliferation of non-ALL cell lines and human EBF1-PDGFRB leukemic cells are sensitive to tyrosine kinase inhibitors (TKI).[3]

The transcription factor EBF1 is essential for normal B lymphocyte specification, commitment and differentiation.[10–13] Loss of EBF1 results in developmental arrest at the common lymphoid progenitor (CLP) stage.[13, 14] In mice, *Ebf1* haploinsufficiency results in inappropriate expression of non-B lineage genes and leukemogenesis when paired with constitutively active Stat5.[15–17] Changes in EBF1-dependent transcription have been documented in human ALL blast cells harboring mono- or bi-allelic *EBF1* deletions.[18] Additionally, *EBF1* is mutated or deleted in ~8% of primary B-ALL patients and ~25% of relapsed patients, suggesting it functions as a tumor suppressor.[18, 19]

While it is known that chimeric fusion proteins often drive constitutive kinase signaling in leukemic cells, few data exist revealing how these proteins perturb lymphoid development and contribute to oncogenesis. Mechanistically, it is unclear whether rearrangement of PDGFRB to EBF1 is necessary for PDGFRB activation, or whether the fusion results in loss of EBF1 function. Moreover, it unknown whether EBF1-PDGFRB promotes oncogenesis by other mechanisms in addition to its unregulated TK activity, or how multiple lesions cooperate with EBF1-PDGFRB to promote leukemogenesis in Ph-like B-ALL.[3]

Here, we report that EBF1-PDGFRB drives leukemogenesis through TM-dependent cytoplasmic mislocalization, which prevents EBF1 from activating transcription. Additionally, we use the first genetically faithful Ph-like B-ALL mouse model to quantify

the synergism between EBF1-PDGFRB and dominant negative IKAROS (IK6), which promote leukemogenesis in combination with *Arf* deletion *in vivo*.

MATERIALS AND METHODS

Identification of human PDGFRB fusion genes, cloning, and transduction

PDGFRB fusions were identified from RNA-sequencing and RT-PCR of ALL cohorts with Ph-like ALL, with the exception of TNIP1ex17-PDGFRB, which was identified from RNA-seq of AML cases as previously described.[3, 20] All *PDGFRB* fusions were amplified from leukemic cell cDNA, cloned into Zero Blunt TOPO vector (Thermo Scientific, IL, USA), and then subcloned into the MSCV-IRES-GFP (MIG) or MSCV-IRES-mCFP (expressing mCherry Fluorescent Protein) retroviral vectors. Retroviral supernatants produced using 293T or Phoenix packaging cell lines were used to infect murine $Ebf1^{-/-}$ fetal liver progenitors, Ba/F3 and primary $Arf^{-/-}$ pre-B cells as described.[21] Cell culture conditions and generation of epitope- and GFP-tagged deletion/mutation constructs are detailed in Supplementary Methods.

Quantitative RT-PCR

Isolation of RNA and RT-PCR analysis from retrovirus-infected cells was described previously.[15] Primers are listed in Supplementary Table 1.

Fluorescence microscopy

Detailed methods for the infection and sorting of $Ebf1^{-/-}$ cells, plasmacytomas, and Ba/F3 cells, and analysis using confocal fluorescence microscopy are provided in the Supplementary Methods.

Immunoblotting and co-immunoprecipitation

Preparation of whole cell extracts and co-IP of proteins are available in Supplementary Methods.

Clonogenic assays, FACS/immunophenotyping and phosphoflow

All mice experiments were reviewed and approved by the St. Jude Children's Research Hospital Institutional Animal Care and Use Committee. Culture conditions, staining, and analysis of cells are described in Supplementary Methods.

In vivo leukemogenesis

Generation of retrovirally transduced cells, injection into mice, and subsequent analysis are described in detail in Supplementary Methods.

In vitro drug sensitivity assays

Tyrosine kinase inhibitor (TKI) sensitivity was assessed using the CellTiter-Blue Cell Viability Assay (Promega, WI, USA) as per manufacturer's instructions. IC50 was determined using nonlinear regression (GraphPad Prism, CA, USA). Each experiment was performed three times.

Statistical analyses

Data analyses were performed using GraphPad Prism Version 6.0 (GraphPad, CA, USA). For qRT-PCR, *P* values were obtained using a 2-way ANOVA comparing column means of log transformed values (Y=Log(Y)) with Tukey's correction for multiple comparisons. For *Ebf1*$^{-/-}$ cell counts in proliferation assays, a 2-way repeated measures ANOVA using Tukey's correction for multiple comparisons was used to compare means across continuous time points. All *P* values are described in figures. All data are presented as mean ± SD. For Kaplan Meier curves significance was determined using ANOVA test or Mantle-Cox log rank. *P* values less than 0.05 were considered significant.

RESULTS

The fusion oncoprotein EBF1-PDGFRB lacks EBF1 function

To determine whether EBF1-PDGFRB can activate EBF1 gene targets we generated FLAG-tagged versions of human EBF1, PDGFRB, EBF1-PDGFRB, or kinase-inactive mutant EBF1-PDGFRB(K634R)[22], each with an IRES-driven GFP marker for FACS purification (Figure 1, Supplementary Figure 2 and Supplementary Figure 3). Because it was recently reported that removal of the TM domain from a related fusion (*TEL-PDGFRB*) reduced its ability to impart IL-3 independence to Ba/F3 cells,[23] we also tested EBF1-PDGFRB(ΔTM) lacking the 24-residue TM domain, and EBF1-TM, which fuses the TM domain (plus 22 surrounding juxtamembrane residues) to EBF1 residues 1–583 (Supplementary Figure 2 and 3).

Using retroviruses, we expressed EBF1 and EBF1-PDGFRB proteins in mouse fetal-liver-derived *Ebf1*$^{-/-}$ B-progenitor cells cultured with stem cell factor (SCF), Fms-related tyrosine kinase 3 ligand (FLT3L), and interleukin-7 (IL-7).[24] On day 3 post-infection we purified GFP^{+} cells and quantitated expression of archetypal EBF1 target genes using qRT-PCR. As expected, wild type EBF1 activated transcription of all B cell-specific genes robustly (by as much as 1000-fold for *Igll1* and *Vpreb1*) relative to "empty" MIG (Figure 1b; $P<0.0001$). EBF1-mediated gene activation was unaffected by addition of the TKI imatinib mesylate (Gleevec, STI-571). Unlike EBF1, EBF1-PDGFRB failed to activate five of the six target genes significantly. *Igll1* was activated weakly (2–3-fold), but significantly ($P<0.03$). Similarly weak activation of *Pax5* by EBF1-PDGFRB was observed, but significance was only achieved in the presence of imatinib. Kinase-inactive mutant EBF1-PDGFRB(K634R) produced significant ($P=0.002$, and $P<0.0001$), albeit modest (2–3-fold), activation of *Igll1* and *Cd79b*, respectively, but in considerably smaller amounts relative to the large increases generated by wild type EBF1.

As deletion of the TM domain resulted in re-localization of the fusion protein to nuclei, we examined the consequences of this phenomenon on transcriptional activation of EBF1 targets. Surprisingly, EBF1-PDGFRB(ΔTM) significantly activated five of the six EBF1 target genes examined (Figure 1b; $P<0.0001$, compared with MIG), while also repressing non-B lineage genes, such as *CD244* (Supplementary Figure 4). Moreover, EBF1-PDGFRB(ΔTM) significantly activated EBF1 targets when compared with EBF1-PDGFRB or EBF1-PDGFRB(K634R). Interestingly, *Asb2* was the only EBF1 target not activated

significantly by EBF1-PDGFRB(ΔTM); however, it was activated ~2–3-fold by EBF1-PDGFRB(K634R) relative to EBF1-PDGFRB (P<0.001) or EBF1-PDGFRB(ΔTM). The lack of activation by EBF1-PDGFRB(ΔTM) is likely due to the dependence of *Asb2* transcription on the C-terminal activation domain of EBF1, which may be functionally impaired by its fusion to the TK domain of PDGFRB.[25] Thus, the oncoprotein EBF1-PDGFRB lacks normal EBF1 function, which can be rescued to a far greater degree by TM deletion than by inhibition of kinase activity using imatinib or inactivation of the kinase domain by mutation. Contrary to TM-deletion, fusion of the TM domain to EBF1 (EBF1-TM) reduced the ability of EBF1 ability to activate five of the gene targets examined (*Pax5* is an exception, but overall activation of this gene is weak compared with other genes) when compared to wild type EBF1.

The TM domain drives subcellular mislocalization of EBF1-PDGFRB

To determine whether loss of EBF1 function is due to its subcellular mislocalization, we fused EBF1 and EBF1-PDGFRB to enhanced GFP (Supplementary Figures 2 and 3b), which did not alter function (Supplementary Figure 5a–b, 5d). We then imaged live *Ebf1*$^{-/-}$ cells co-infected to express GFP-tagged proteins as well as untethered mCFP, which served as an internal control. When untethered, GFP and mCFP each localized diffusely throughout both nuclei and cytoplasm (Figure 2a). As expected, EBF1-GFP localized solely within nuclei (Figure 2b). Surprisingly, wild type PDGFRB-GFP localized into cytoplasmic aggregates (Figure 2c) and was not detected on the surface of Ebf1$^{-/-}$ cells by flow cytometry (Supplementary Figure 5c). As a control, retrovirally expressed PDGFRB-GFP was displayed on the surface of plasmacytoma cells (Supplementary Figure 5b,d); therefore, the inability of *Ebf1*$^{-/-}$ progenitors to display this receptor on the cell surface is similar to normal pre-pro-B cells, which do not display surface PDGFRB (S.J.W., data not shown). We conclude that normal pre-pro-B cells and *Ebf1*$^{-/-}$ progenitors may lack a protein(s) necessary for display of surface PDGFRB.

Unlike EBF1 and PDGFRB, EBF1-PDGFRB localized diffusely throughout the cytoplasm and was virtually undetectable in nuclei (Figure 2d). Cytoplasmic localization using direct immunostaining was also observed in Ba/F3 cells expressing EBF1-PDGFRB and other PDGFRB fusions (Supplementary Figure 6a). Ba/F3 subcellular fractionation revealed that PDGFRB fusion proteins were detected only in the total membrane-bound fraction and not in the nuclear or free cytosolic compartments (Supplementary Figure 6b). This cytoplasmic and membrane-associated localization explains the greatly reduced ability of EBF1-PDGFRB to activate EBF1 gene targets. Importantly, inactivation of the kinase domain by imatinib or (K634R)-mutation failed to relocate EBF1-PDGFRB into nuclei (Figure 2e, Supplementary Figures 7d–e).

Examination of the TM domain sequence using the prediction server NetNES1.1 suggested a role as a nuclear export signal peptide (NES).[26] In support of this, we discovered that removing the TM domain completely re-localized EBF1-PDGFRB from the cytoplasm into nuclei (Figure 2f–g; Supplementary Figures 7f–g) where it activated (Figure 1b) or repressed (Supplementary Figure 4) EBF1 targets regardless of TK activity. We confirmed the NES activity of the TM domain by appending it to EBF1 (EBF1-TM) (Figure 2h), and also to

GFP alone (TM:GFP; Supplementary Figures 2, 3b and Supplementary Figure 6c). EBF1-TM localized into cytoplasmic puncti and failed to activate most EBF1 target genes (Figure 1b and Figure 2h, Supplementary Figure 4a).

To determine whether EBF1 is necessary for the cytoplasmic localization of EBF1-PDGFRB, we generated PDGFRB(528–1106) consisting of only PDGFRB-derived fusion protein sequences (Supplementary Figures 2 and 3b). Similar to full-length WT PDGFRB, PDGFRB(528–1106) formed cytoplasmic puncti (Supplementary Figure 8b), which were also observed using imatinib-treated PDGFRB(528–1106) (data not shown) or PDGFRB(528–1106)(K634R) (Supplementary Figure 8c). Unlike EBF1-PDGFRB(ΔTM), PDGFRB(528–1106)(ΔTM) and PDGFRB(528–1106)(ΔTM K634R) fragments did not relocate into nuclei, but localized diffusely (Supplementary Figure 8d–e).

EBF1-PDGFRB homodimerizes, is autophosphorylated, and is stable relative to PDGFRB

Normal PDGFRB signaling requires ligand-induced dimerization at the plasma membrane.[27] To determine whether EBF1-PDGFRB multimerizes we performed co-IP followed by immunoblotting of *Ebf1*$^{-/-}$ progenitors co-transduced with FLAG- and MYC-tagged EBF1-PDGFRB. Pull down of FLAG-tagged EBF1-PDGFRB co-immunoprecipitated MYC-tagged EBF1-PDGFRB, and vice versa, confirming EBF1-PDGFRB multimerization (Figure 3a). Additionally, HA-tagged TNIP1ex14-PDGFRB co-immunoprecipitated His_6-tagged TNIP1ex14-PDGFRB (Supplementary Figure 6d) establishing that cytoplasmic self-association is common among PDGFRB fusion proteins. Importantly, confocal imaging revealed that co-expression of GFP-tagged EBF1-PDGFRB together with mCFP-tagged EBF1 did not alter the cytoplasmic or nuclear localization of either protein, respectively, in B cell progenitors (Figure 3b). This suggests that unlike sequestration of wild type IKAROS to the cytoplasm by IK6, EBF1 and EBF1-PDGFRB are sequestered to different subcellular compartments. This in turn effectively prevents the assembly of heterodimers (EBF1 + EBF1-PDGFRB) in cells. It also explains our inability to detect heterodimers using co-IP (data not shown). Thus, loss of EBF1 function results from its fusion to PDGFRB and not to dominant negative effects of EBF1-PDGFRB on EBF1.

Next, we determined whether EBF1-PDGFRB is capable of autophosphorylation. After transducing *Ebf1*$^{-/-}$ cells with FLAG-tagged versions of EBF1-PDGFRB or EBF1-PDGFRB(K634R) we performed IP followed by immunoblotting with pan-phosphotyrosine (pTyr) antibodies. EBF1-PDGFRB was strongly phosphorylated, which was inhibited by imatinib or the EBF1-PDGFRB(K634R) mutation (Figure 3c).

Typically, PDGFRB is internalized and degraded upon ligand-induced dimerization.[22, 28, 29] Given PDGFRB's lack of surface expression on *Ebf1*$^{-/-}$ cells and punctal localization compared with the diffuse cytoplasmic pattern of EBF1-PDGFRB (Figure 2c–d and Supplementary Figure 5c), we wanted to determine whether PDGFRB was less stable than EBF1-PDBFRB. We incubated *Ebf1*$^{-/-}$ cells expressing these proteins with the translation inhibitor cycloheximide for 0, 4, 8 or 12 hours prior to immunoblotting (Figure 3d). As expected, PDGFRB levels were greatly reduced after only 4 hours, whereas EBF1-PDGFRB, EBF1-PDGFRB(K634R) and EBF1-PDGFRB(ΔTM) levels were unchanged up

to 8 hours and decreased only slightly at 12 hours. Additionally, removal of EBF1 greatly reduced the stability of the PDGFRB(528–1106) fragment.

EBF1-PDGFRB promotes cytokine-independent and clonogenic growth of B cell progenitors, which is targetable by TKI therapy

To determine whether EBF1-PDGFRB is sufficient to transform IL-7-dependent *Ebf1*$^{-/-}$ progenitors, we transduced these cells with various constructs and expanded infected cells over 16 days with SCF and FLT3L, but without IL-7. As expected, only EBF1-PDGFRB-positive cells proliferated (Figure 4a).[3] Removal of SCF and/or FLT3L revealed significant contributions of these cytokines to the growth rates of EBF1-PDGFRB-positive cells (Supplementary Figure 9). Unexpectedly, both EBF1-PDGFRB(ΔTM) and PDGFRB(528–1106)-positive cells failed to proliferate in the absence of IL-7 at any time despite having a functional PDGFRB kinase domain (Figure 4a). Our results establish that, along with a functioning TK domain, fusion of EBF1 to PDGFRB(528–1106) and TM-mediated cytoplasmic localization of EBF1-PDGFRB are also necessary to achieve EBF1-PDGFRB-mediated cytokine independence.

We then confirmed that exogenous cytokines are not required for proliferation of IL3-dependent Ba/F3 pro-B cells or IL7-dependent primary mouse *Arf*$^{-/-}$pre-B cells expressing PDGFRB fusions to EBF1, TNIP1, ATF7IP, or CD74 (Figure 4a–b). In patients harboring rearrangements of *PDGFRB*, these lesions frequently co-occur with *IKZF1* alterations and *CDKN2A* (*Arf*$^{-/-}$) deletions; therefore, we co-expressed the dominant negative IKZF1 isoform (IK6) with each of the fusions in *Arf*$^{-/-}$ pre-B cells, which provide a genetically faithful model of human B-ALL. Co-expression of IK6 did not significantly increase the proliferation rates of ATF7IP-PDGFRB or EBF1-PDGFRB-expressing pre-B cells. However, IK6 co-expression was required for the growth of TNIP1ex14-PDGFRB-positive pre-B cells; *Arf*$^{-/-}$ cells expressing TNIP1ex14-PDGFRB without IK6 do not survive in the absence of IL-7, and therefore, were not included in the growth assay (Figure 4b).

Mechanistically, PDGFRB fusions to EBF1, TNIP1, or ATF7IP activate the STAT5 pathway to bypass the cytokine dependence in both Ba/F3 and *Arf*$^{-/-}$cells (Figure 4c).[3, 8] This activation can be reversed by treatment with the TKI dasatinib. Likewise, in *Ebf1*$^{-/-}$ cells lacking IL-7, EBF1-PDGFRB expression activated STAT5 signaling, which was blocked by imatinib or mutant EBF1-PDGFRB(K634R) kinase inhibition (Supplementary Figure 10). Surprisingly, cells expressing EBF1-PDGFRB(ΔTM), which failed to expand in the absence of IL-7, still activated STAT5 but to a lesser extent than full-length EBF1-PDGFRB (Figure 4a, Supplementary Figure 10). Next, we performed cytotoxicity assays *in vitro* to assess the relative sensitivities of fusion proteins to the commonly used TKI dasatinib, but also to the class III TK inhibitors crenolanib and dovitinib.[30–32] Crenolanib binds the active confirmation of PDGFRA and has been utilized for treatment of imatinib-resistant gastrointestinal stromal tumors, as has the multi-kinase-inhibitor dovitinib. (Figure 4d). Each of the three TKI's potently inhibited proliferation of cells expressing the PDGFRB fusion proteins.

We then expressed EBF1-PDGFRB, TNIP1ex14-PDGFRB, and empty vector in C57Bl/6 WT and *Arf*$^{-/-}$ lineage-negative bone marrow hematopoietic progenitors and assessed

colony-forming potential over serial re-platings *in vitro* as a surrogate measure of self-renewal. All fusions failed at serial re-plating under myeloid conditions (IL3, IL6, SCF, GM-CSF; data not shown), but induced serial re-plating under lymphoid conditions (IL7, SCF, FLT3L; Figure 5a). Morphological (Figure 5b) and flow-cytometric (Figure 5c) analyses of colony-forming cells harvested after rounds 3–6 of re-plating revealed a lymphoid phenotype. TNIP1ex14-PDGFRB expression required concomitant loss of *Arf* in order to promote serial re-plating for B-progenitor lymphoid colonies. In contrast, EBF1-PDGFRB potently supported serial B-progenitor colony re-plating in a WT background with enhanced re-plating in Arf-null cells, supporting the notion that concomitant activation of kinase signaling and perturbation of lymphoid maturation (by inhibition of EBF1 by EBF1-PDGFRB, or bypassing kinase induced senescence by inactivation of ARF in the case of TNIP1ex14-PDGFRB) is required for lymphoid transformation, proliferation and self-renewal.

EBF1-PDGFRB is leukemogenic, synergizes with IK6, and is antagonized by EBF1

Because both *IKZF1* alterations and *CDKN2A* (*Arf*) deletions are frequently observed in Ph-like cases harboring PDGFRB fusions, we co-modeled EBF1-PDGFRB with either empty vector or the dominant negative isoform of IKAROS (IK6) in primary $Arf^{-/-}$pre-B cells to determine oncogenicity *in vivo*. In the absence of an oncogenic driver $Arf^{-/-}$ pre-B cells, without or with IK6-expression, are not leukemogenic.[33] We then transplanted 1×10^6 $Arf^{-/-}$ pre-B cells expressing EBF1-PDGFRB together with empty vector or with IK6 by tail vein injection into sublethally irradiated WT recipients. EBF1-PDGFRB induced a fully penetrant leukemia with a median survival of 44 days (Figure 6a). Disease onset and spleen weight were significantly increased with co-expression of IK6, resulting in a median survival of 37 days (Figure 6a–b). Flow cytometric analysis of bone marrow and spleen from moribund mice revealed outgrowth of B-progenitor leukemia ($CD43^+$ $CD19^+BP1^+IgM^-$), with reduced B220 expression in the IK6-co-expressing pre-B cells compared with their EBF1-PDGFRB-only expressing counterparts (Figure 6c). Histological examination revealed that both EBF1-PDGFRB and EBF1-PDGFRB+IK6 leukemias were highly infiltrative across multiple tissues including liver, lung and the central nervous system (Figure 6d).

In leukemic patients, the high frequency of co-occurring lesions resulting in rearrangement or loss or *EBF1* suggests that intact EBF1 may antagonize leukemogenesis. To test this, we infected $Ebf1^{-/-}$ cells with EBF1-PDGFRB alone, or together with the 4-hydroxytamoxifen (4-OHT)-dependent EBF1:estrogen receptor fusion, which allows for intracellular EBF1 titration *in vitro*.[24] We then sorted positive cells and expanded them in the absence of IL-7 across increasing dosages of 4-OHT (increasing EBF1 levels) while maintaining constant EBF1-PDGFRB levels (Supplementary Figures 11a–b). Increasing the dosage of active EBF1 significantly reduced the ability of EBF1-PDGFRB to confer IL-7 independence to $Ebf1^{-/-}$ progenitors in a dose-dependent manner. These results further support a model in which loss of EBF1 activity contributes to leukemogenesis.

DISCUSSION

In this study, we defined novel mechanisms of EBF1-PDGFRB-dependent leukemogenesis beyond dysregulated tyrosine kinase activity. Furthermore, we describe the first genetically faithful mouse model of Ph-like B-ALL, which confirms that EBF1-PDGFRB is sufficient to drive leukemogenesis *in vivo*. Additionally, EBF1-PDGFRB synergizes with the dominant negative form of IKAROS, IK6.

Mechanistically, we observed dual contributions of cytoplasmic mislocalization of EBF1-PDGFRB, which not only promotes constitutive TK signaling via STAT5 activation but also prevents EBF1 from localizing within nuclei, activating B cell specific genes, and promoting B lymphoid development (Figure 7). Surprisingly, removal of the TM domain resulted in nuclear relocalization of EBF1-PDGFRB and partial restoration of EBF1 function. Removal of the TM domain also restored IL-7 dependence to B cell progenitors expressing EBF1-PDGFRB, despite the presence of a functional TK domain. Interestingly, the EBF1 portion of EBF1-PDGFRB was not only required for IL-7 independence, but also appeared to protect the oncoprotein from TM-dependent cytoplasmic degradation as EBF1-PDGFRB was the only TM-containing construct that did not form cytoplasmic puncti (Supplementary Figure 12).

We conclude that the PDGFRB TM domain facilitates both nuclear export and interactions with other cytosolic proteins, which in turn promote TK activity and transformation by EBF1-PDGFRB. Mislocalization by the TM/NES is likely a shared property of other PDGFRB-containing fusion proteins (e.g. ETV6-PDGFRB), which generally include this motif.[34] Subcellular mislocalization and enhanced protein stability also contribute to leukemogenesis by other TK fusion proteins. For example, transforming activities of the NUP214-ABL1 fusion is dependent on its association with nuclear pore complexes.[35] The mechanism that enhances stability of EBF1-PDGFRB is unknown, but it may be similar to the attenuation of proteosomal degradation reported for ETV6-PDGFRB, FIP1L1-PDGFRA, and ZMYM2(ZNF198)-FGFR1.[36, 37]

The loss of EBF1 function in EBF1-PDGFRB is likely a key determinant of perturbed lymphoid maturation in B-ALL. This may, in part, account for the notably poor outcome of human EBF-PDGFRB+ B-ALL.[38] Similar to the loss of IKZF1 due to deletion or dominant negative mutations, deletions of *EBF1* genes or inhibition of EBF1 function in EBF1-PDGFRB impair B cell maturation and are associated with poor outcomes.[39] In this regard, it is notable that restoration of EBF1 function in EBF1-PDGFRB, or enforced expression of EBF1, activated EBF1 target genes and blocked EBF1-PDGFRB-driven cell proliferation. These observations indicate that re-establishment of EBF1 function in EBF1-PDGFRB+ B-ALL, *i.e.* using inhibitors of the TM/NES of PDGFRB,[40] may provide an additional strategy for treating a subset of TKI-refractory leukemias.

Our studies highlight the importance of lineage maturation in preventing leukemogenesis. In B-ALL, the B cell-specific transcriptional network is perturbed, and genomic profiling studies have revealed that factors involved in B lymphoid specification, including *IKZF1*, *PAX5* and *EBF1* are commonly lost via mutation or deletion in >60% of patients, with a

higher percentage in Ph+ and Ph-like cases.[41–42] These alterations are associated with transcriptional dedifferentiation and poor outcomes. Loss of normal EBF1 function is important for leukemogenesis by EBF1-PDGFRB, because intact EBF1 antagonizes functions of the fusion protein. We propose that EBF1-PDGFRB drives significant features of Ph-like B-ALL by itself, but is more potent in cells that have impaired homeostatic functions due to the loss of additional genes including the second allele of *EBF1* itself, *IKZF1*, or by *FLT3* gene duplications.[3, 39] Synergy between loss of these alterations and EBF1-PDGFRB has not been characterized at the molecular level; however, the loss of EBF1 and IKZF1 (e.g. IK6) together likely perturbs regulation of common genes and pathways.[43] For example, alterations of *IKZF1* results in arrested differentiation, acquisition of a hematopoietic stem cell-like phenotype, and confers resistance to TKI therapy in models of BCR-ABL1 positive ALL.[44] Lesions in *IKZF1* also activate expression of integrins and integrin signaling pathways.[43, 45] Together, effects of the loss of IKZF1 compound the loss of EBF1. In summary, our data confirm that loss of the tumor suppressor functions of EBF1, together with proliferative advantages provided by the TK function of PDGFRB, constitute a potent driver of leukemogenesis in B-ALL.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors would like to thank Jay Hesselberth, John Cambier, Mark Johnston, Laurent Gapin, Aaron M. Johnson, Hua Huang, and Mikael Sigvardsson for helpful suggestions and reagents. We also thank the National Jewish Health Flow Cytometry Core and Josh Loomis for support and technical assistance, the St. Jude Children's Research Hospital Flow Cytometry and Cell Sorting Shared Resource, and Cell and Tissue Imaging Center. This work was funded by grants to J.H. from the National Institutes of Health R01 AI081878 and AI098417, The Wendy Siegel Fund for Leukemia and Cancer Research, and the Cancer League of Colorado. The Victor W. Bolie and Earleen D. Bolie Graduate Scholarship Fund supported S.J.W. This work was supported by the American Lebanese Syrian Associated Charities of St Jude Children's Research Hospital. C.G.M. was supported by a St. Baldrick's Foundation Scholar Award, A St. Baldrick's Foundation Robert J. Arceci Innovation Award, A Specialized Center of Research Award from the Leukemia and Lymphoma Society, and an Outstanding Investigator Award (R35 CA197695) from the National Cancer Institute.

References

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2016. CA Cancer J Clin. 2016; 66:7–30. [PubMed: 26742998]
2. Kelly LM, Gilliland DG. Genetics of myeloid leukemias. Annu Rev Genomics Hum Genet. 2002; 3:179–198. [PubMed: 12194988]
3. Roberts KG, Morin RD, Zhang J, Hirst M, Zhao Y, Su X, et al. Genetic alterations activating kinase and cytokine receptor signaling in high-risk acute lymphoblastic leukemia. Cancer Cell. 2012; 22:153–166. [PubMed: 22897847]
4. Jain N, Roberts KG, Jabbour E, Patel K, Eterovic AK, Chen K, et al. Ph-like acute lymphoblastic leukemia: a high-risk subtype in adults. Blood. 2017; 129:572–581. [PubMed: 27919910]
5. Schwab C, Ryan SL, Chilton L, Elliott A, Murray J, Richardson S, et al. EBF1-PDGFRB fusion in paediatric B-cell precursor acute lymphoblastic leukaemia (BCP-ALL): genetic profile and clinical implications. Blood. 2016; 127:2214–2218. [PubMed: 26872634]
6. Demoulin JB, Montano-Almendras CP. Platelet-derived growth factors and their receptors in normal and malignant hematopoiesis. Amer J Blood Res. 2012; 2:44–56. [PubMed: 22432087]

7. Demoulin JB, Essaghir A. PDGF receptor signaling networks in normal and cancer cells. Cytokine Growth Factor Rev. 2014; 25:273–283. [PubMed: 24703957]
8. Ishibashi T, Yaguchi A, Terada K, Ueno-Yokohata H, Tomita O, Iijima K, et al. Ph-like ALL-related novel fusion kinase ATF7IP-PDGFRB exhibits high sensitivity to tyrosine kinase inhibitors in murine cells. Exp Hematol. 2016; 44:177–188. e5. [PubMed: 26703895]
9. O'Connor D, Moorman AV, Wade R, Hancock J, Tan RM, Bartram J, et al. Use of Minimal Residual Disease Assessment to Redefine Induction Failure in Pediatric Acute Lymphoblastic Leukemia. J Clin Oncol. 2017; 35:660–667. [PubMed: 28045622]
10. Vilagos B, Hoffmann M, Souabni A, Sun Q, Werner B, Medvedovic J, et al. Essential role of EBF1 in the generation and function of distinct mature B cell types. J Exp Med. 2012; 209:775–792. [PubMed: 22473956]
11. Pongubala JM, Northrup DL, Lancki DW, Medina KL, Treiber T, Bertolino E, et al. Transcription factor EBF restricts alternative lineage options and promotes B cell fate commitment independently of Pax5. Nat Immunol. 2008; 9:203–215. [PubMed: 18176567]
12. Treiber T, Mandel EM, Pott S, Györy I, Firner S, Liu ET, et al. Early B cell factor 1 regulates B cell gene networks by activation, repression, and transcription- independent poising of chromatin. Immunity. 2010; 32:714–25. [PubMed: 20451411]
13. Lin H, Grosschedl R. Failure of B-cell differentiation in mice lacking the transcription factor EBF. Nature. 1995; 376:263–267. [PubMed: 7542362]
14. Györy I, Boller S, Nechanitzky R, Mandel E, Pott S, Liu E, et al. Transcription factor Ebf1 regulates differentiation stage-specific signaling, proliferation, and survival of B cells. Genes Dev. 2012; 26:668–682. [PubMed: 22431510]
15. Lukin K, Fields S, Lopez D, Cherrier M, Ternyak K, Ramirez J, et al. Compound haploinsufficiencies of Ebf1 and Runx1 genes impede B cell lineage progression. Proc Natl Acad Sci USA. 2010; 107:7869–7874. [PubMed: 20385820]
16. Heltemes-Harris LM, Willette MJ, Ramsey LB, Qiu YH, Neeley ES, Zhang N, et al. Ebf1 or Pax5 haploinsufficiency synergizes with STAT5 activation to initiate acute lymphoblastic leukemia. J Exp Med. 2011; 208:1135–1149. [PubMed: 21606506]
17. Heckl D, Schwarzer A, Haemmerle R, Steinemann D, Rudolph C, Skawran B, et al. Lentiviral vector induced insertional haploinsufficiency of Ebf1 causes murine leukemia. Mol Ther. 2012; 20:1187–95. [PubMed: 22472950]
18. Mullighan CG, Goorha S, Radtke I, Miller CB, Coustan-Smith E, Dalton JD, et al. Genome-wide analysis of genetic alterations in acute lymphoblastic leukaemia. Nature. 2007; 446:758–64. [PubMed: 17344859]
19. Yang JJ, Bhojwani D, Yang W, Cai X, Stocco G, Crews K, et al. Genome-wide copy number profiling reveals molecular evolution from diagnosis to relapse in childhood acute lymphoblastic leukemia. Blood. 2008; 112:4178–4183. [PubMed: 18768390]
20. Roberts KG, Li Y, Payne-Turner D, Harvey RC, Yang Y-L, Pei D, et al. Targetable Kinase-Activating Lesions in Ph-like Acute Lymphoblastic Leukemia. New Eng J Med. 2014; 371:1005–1015. [PubMed: 25207766]
21. Williams RT, Roussel MF, Sherr CJ. Arf gene loss enhances oncogenicity and limits imatinib response in mouse models of Bcr-Abl-induced acute lymphoblastic leukemia. Proc Natl Acad Sci USA. 2006; 103:6688–6693. [PubMed: 16618932]
22. Sorkin A, Westermark B, Heldin CH, Claesson-Welsh L. Effect of receptor kinase inactivation on the rate of internalization and degradation of PDGF and the PDGF beta-receptor. J Cell Biol. 1991; 112:469–478. [PubMed: 1846866]
23. Toffalini F, Hellberg C, Demoulin JB. Critical role of the platelet-derived growth factor receptor (PDGFR) beta transmembrane domain in the TEL-PDGFRbeta cytosolic oncoprotein. J Biol Chem. 2010; 285:12268–12278. [PubMed: 20164181]
24. Gao H, Lukin K, Ramirez J, Fields S, Lopez D, Hagman J. Opposing effects of SWI/SNF and Mi-2/NuRD chromatin remodeling complexes on epigenetic reprogramming by EBF and Pax5. Proc Natl Acad Sci USA. 2009; 106:11258–11263. [PubMed: 19549820]

25. Boller S, Ramamoorthy S, Akbas D, Nechanitzky R, Burger L, Murr R, et al. Pioneering Activity of the C-Terminal Domain of EBF1 Shapes the Chromatin Landscape for B Cell Programming. Immunity. 2016; 44:527–541. [PubMed: 26982363]
26. la Cour T, Gupta R, Rapacki K, Skriver K, Poulsen FM, Brunak S. NESbase version 1.0: a database of nuclear export signals. Nucl Acids Res. 2003; 31:393–396. [PubMed: 12520031]
27. Claesson-Welsh L. Signal transduction by the PDGF receptors. Prog Growth Factor Res. 1994; 5:37–54. [PubMed: 8199353]
28. Pahara J, Shi H, Chen X, Wang Z. Dimerization drives PDGF receptor endocytosis through a C-terminal hydrophobic motif shared by EGF receptor. Exp Cell Res. 2010; 316:2237–2250. [PubMed: 20580638]
29. Mori S, Tanaka K, Omura S, Saito Y. Degradation process of ligand-stimulated platelet-derived growth factor beta-receptor involves ubiquitin-proteasome proteolytic pathway. J Biol Chem. 1995; 270:29447–29452. [PubMed: 7493983]
30. Stein EM. Molecularly targeted therapies for acute myeloid leukemia. Hematology Am Soc Hematol Educ Prog. 2015; 2015:579–583.
31. Katoh M. FGFR inhibitors: Effects on cancer cells, tumor microenvironment and whole-body homeostasis. Int J Mol Med. 2016; 38:3–15. [PubMed: 27245147]
32. Zimmerman EI, Turner DC, Buaboonnam J, Hu S, Orwick S, Roberts MS, et al. Crenolanib is active against models of drug-resistant FLT3-ITD-positive acute myeloid leukemia. Blood. 2013; 122:3607–3615. [PubMed: 24046014]
33. Churchman ML, Low J, Qu C, Paietta EM, Kasper LH, Chang Y, et al. Efficacy of Retinoids in IKZF1-Mutated BCR-ABL1 Acute Lymphoblastic Leukemia. Cancer Cell. 2015; 28:343–56. [PubMed: 26321221]
34. Toffalini F, Hellberg C, Demoulin JB. Critical role of the platelet-derived growth factor receptor (PDGFR) beta transmembrane domain in the TEL-PDGFRBbeta cytosolic oncoprotein. J Biol Chem. 2010; 285:12268–12278. [PubMed: 20164181]
35. De Keersmaecker K, Rocnik JL, Bernad R, Lee BH, Leeman D, Gielen O, et al. Kinase activation and transformation by NUP214-ABL1 is dependent on the context of the nuclear pore. Mol Cell. 2008; 31:134–42. [PubMed: 18614052]
36. Toffalini F, Kallin A, Vandenberghe P, Pierre P, Michaux L, Cools J, et al. The fusion proteins TEL-PDGFRbeta and FIP1L1-PDGFRalpha escape ubiquitination and degradation. Haematologica. 2009; 94:1085–1093. [PubMed: 19644140]
37. Kasyapa CS, Kunapuli P, Cowell JK. HSPA1A is an important regulator of the stability and function of ZNF198 and its oncogenic derivative, ZNF198-FGFR1. J Cell Biochem. 2007; 102:1308–1317. [PubMed: 17471537]
38. Schwab C, Ryan SL, Chilton L, Elliott A, Murray J, Richardson S, et al. EBF1-PDGFRB fusion in pediatric B-Cell precursor acute lymphoblastic leukemia (BCP-ALL): genetic profile and clinical implications. Blood. 2016; 127:2214–2218. [PubMed: 26872634]
39. Mullighan CG, Su X, Zhang J, Radtke I, Phillips LA, Miller CB, et al. Deletion of IKZF1 and prognosis in acute lymphoblastic leukemia. N Eng J Med. 2009; 360:470–480.
40. Petti LM, Talbert-Slagle K, Hochstrasser ML, DiMaio D. A single amino acid substitution converts a transmembrane protein activator of the platelet-derived growth factor beta receptor into an inhibitor. J Biol Chem. 2013; 288:27273–27286. [PubMed: 23908351]
41. Harvey RC, Mullighan CG, Wang X, Dobbin KK, Davidson GS, Bedrick EJ, et al. Identification of novel cluster groups in pediatric high-risk B-precursor acute lymphoblastic leukemia with gene expression profiling: correlation with genome-wide DNA copy number alterations, clinical characteristics, and outcome. Blood. 2010; 116:4874–4884. [PubMed: 20699438]
42. Hunger SP, Mullighan CG. Redefining ALL classification: toward detecting high-risk ALL and implementing precision medicine. Blood. 2015; 125:3977–87. [PubMed: 25999453]
43. Schwickert TA, Tagoh H, Gültekin S, Dakic A, Axelsson E, Minnich M, et al. Stage-specific control of early B cell development by the transcription factor Ikaros. Nat Immunol. 2014; 15:283–293. [PubMed: 24509509]
44. Churchman ML, Mullighan CG. Ikaros: Exploiting and targeting the hematopoietic stem cell niche in B-progenitor acute lymphoblastic leukemia. Exp Hematol. 2017; 46:1–8. [PubMed: 27865806]

45. Joshi I, Yoshida T, Jena N, Qi X, Zhang J, Van Etten RA, et al. Loss of Ikaros DNA-binding function confers integrin-dependent survival on pre-B cells and progression to acute lymphoblastic leukemia. Nat Immunol. 2014; 15:294–304. [PubMed: 24509510]

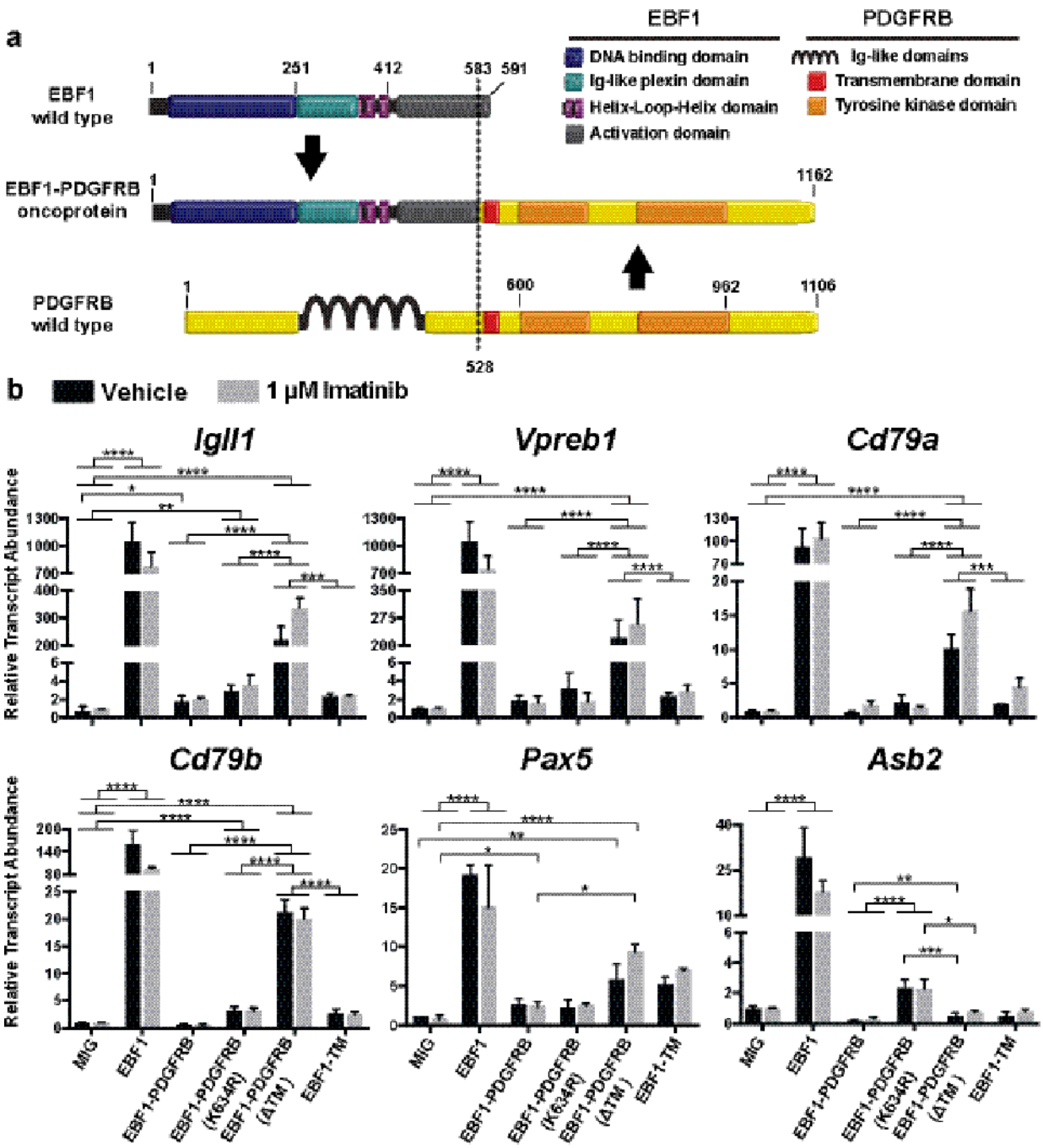

Figure 1.

EBF1-PDGFRB fails to activate EBF1 gene targets in B cell progenitors. (**a**) Schematic diagrams of WT EBF1, EBF1-PDGFRB, and WT PDGFRB proteins. Functional domains are listed along with relevant amino acid positions. Ig: Immunoglobulin-like (**b**) Quantitative RT-PCR analysis of endogenous gene activation by EBF1, EBF1-PDGFRB, or modified versions of these proteins in retrovirally transduced *Ebf1*$^{-/-}$ cells, without or with 1μM imatinib 72 hours post infection. All conditions were normalized to *Hprt1* transcripts.

MSCV-IRES-GFP (MIG) was used as a negative control. Error bars represent the mean ± SD of three independent replicates. **** $P<0.0001$, *** $P<0.0002$, ** $P<0.002$, * $P<0.03$.

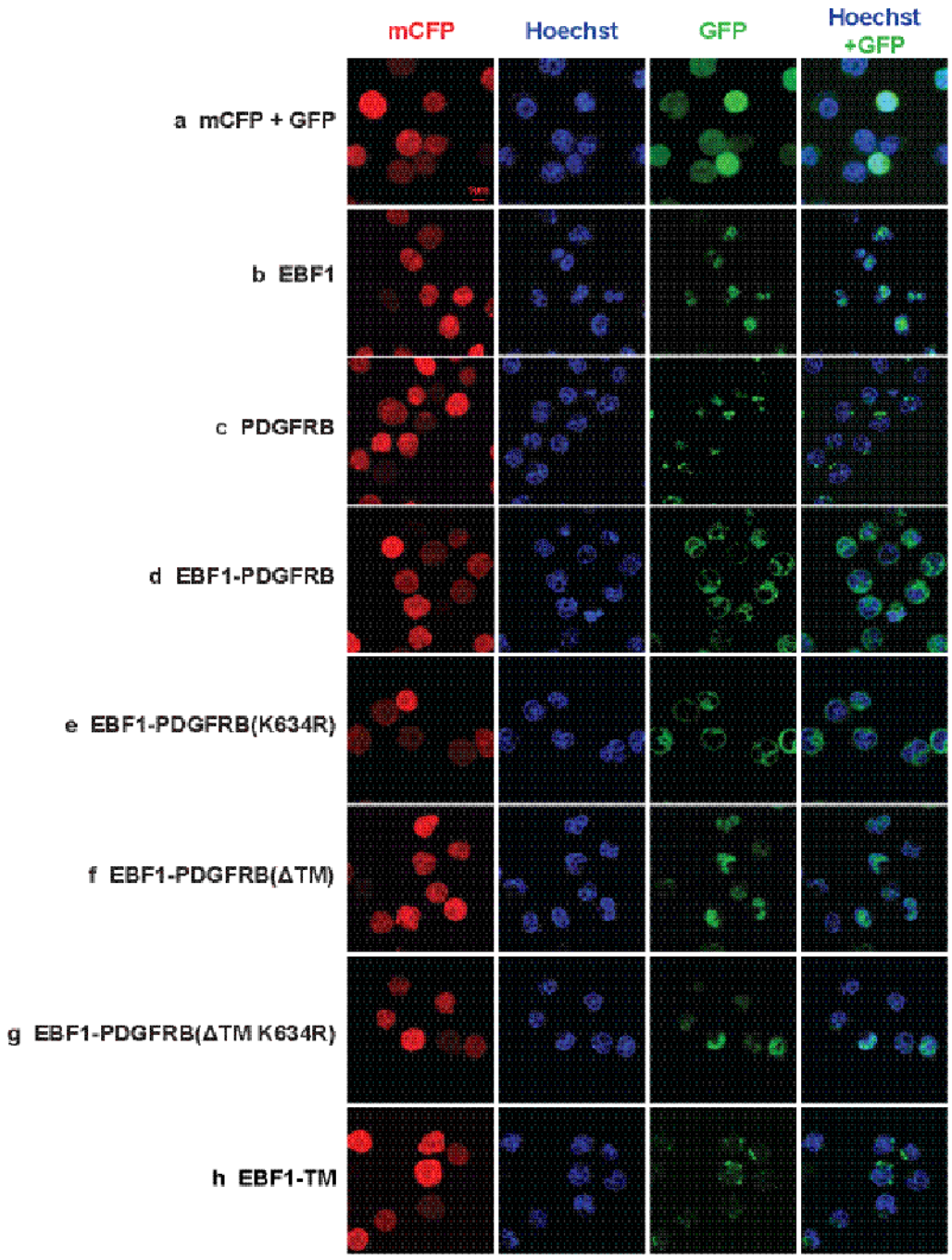

Figure 2.

Mislocalization of EBF1-PDGFRB to the cytoplasm requires the TM domain of PDGFRB. (**a-h**) Confocal images of live, unfixed *Ebf1*$^{-/-}$progenitors (100X magnification) expressing various constructs tagged with GFP (Supplementary Figures 2 and 3b). Untethered mCFP was included in all experiments as an internal control to visualize whole cells. In overlays (column 4), Hoechst and GFP images are merged. (**a**) Untagged GFP and mCFP diffusely localizes to both nuclei and cytoplasm. (**b-c**) Nuclear vs. puncti localization patterns of EBF1-GFP compared with PDGFRB-GFP, respectively. (**d**) EBF1-PDGFRB-GFP localizes

diffusely throughout the cytoplasm, which is not affected by kinase inactivation (**e**), but is highly dependent upon the presence of the intact TM domain (**f-g**). (**h**) EBF1-TM localizes in cytoplasmic puncti.

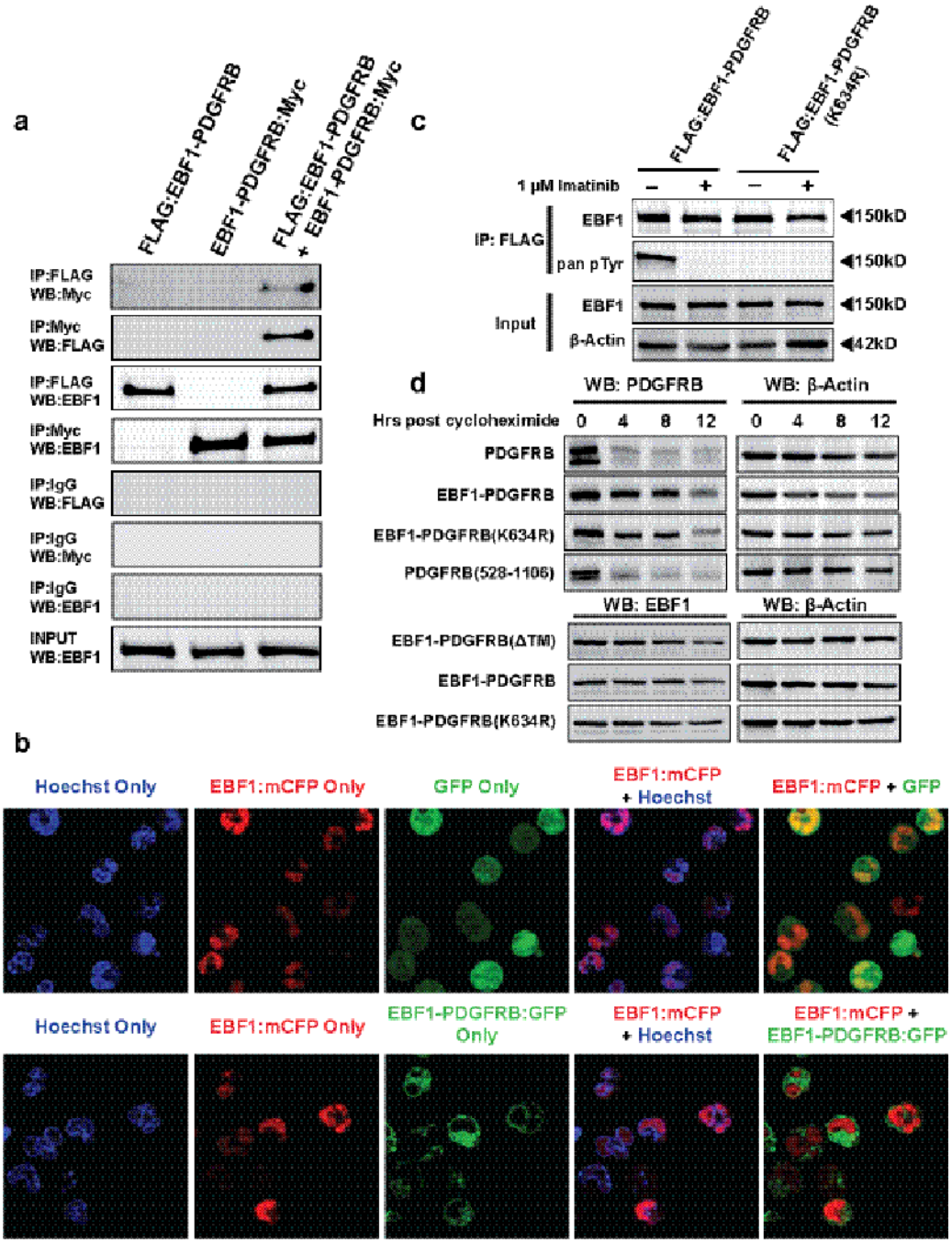

Figure 3.

EBF1-PDGFRB multimerizes, is phosphorylated on tyrosine residues, and is stabilized in *Ebf1*$^{-/-}$ progenitors. (**a**) Co-IP demonstrating multimerization of Myc- and FLAG-tagged EBF1-PDGFRB in *Ebf1*$^{-/-}$cells. (**b**) EBF1-PDGFRB and EBF1 localize to non-overlapping compartments in live cells. Confocal microscopy detecting co-expression of EBF1-PDGFRB-GFP and EBF1-mCFP in *Ebf1*$^{-/-}$ B progenitor cells. EBF1-PDGFRB is restricted to cytoplasm, while EBF1 is detected only in nuclei of the same cells (**c**) IP followed by immunoblots demonstrate that EBF1-PDGFRB is autophosphorylated. Phosphorylation is

blocked by imatinib and the K634R mutation. (**d**) Stability of EBF1-PDGFRB fusion, PDGFRB, and PDGFRB(528–1106) in the presence of cycloheximide. Antibodies used for IP and blotting are indicated.

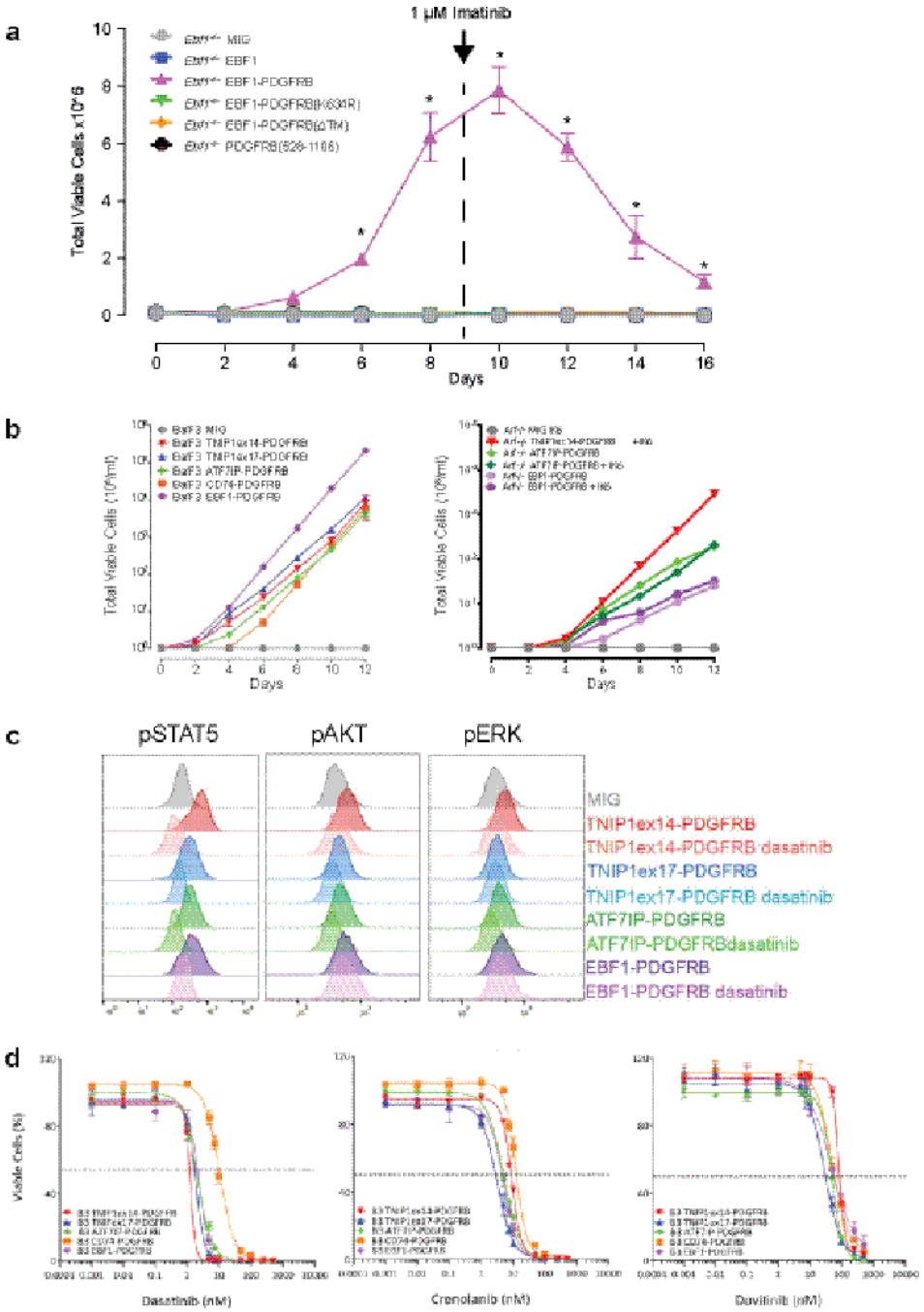

Figure 4.

EBF1-PDGFRB and other PDGFRB fusions promote cytokine-independent and clonogenic growth of B cell progenitors through STAT5, ERK and AKT phosphorylation. EBF1-PDGFRB-mediated transformation requires a TM domain. (**a**) Growth curve of *Ebf1*$^{-/-}$ cells infected (in triplicate) to express proteins as shown. GFP^{+} cells were sorted and grown over 16 days in the absence of IL-7, and counted every 48 hours. 1μM imatinib was added at day 9 to all cultures. Asterisks represent P<0.0001 for EBF1-PDGFRB compared with negative control. (**b**) Ba/F3 and *Arf*$^{-/-}$ pre-B cells were transduced with TNIP1ex14-PDGFRB,

TNIP1ex17-PDGFRB, ATF7IP-PDGFRB, CD74-PDGFRB, or EBF1-PDGFRB and grown in the absence of IL-3 or IL-7, respectively, and counted every two days. (**c**) Phosflow analysis of pSTAT5, pAKT, and pERK in transduced Ba/F3 cells with or without 100nM dasatinib treatment for one hour. (**d**) Cytotoxicity assays of Ba/F3 cells transduced to express fusion proteins as in (b), followed by incubation with dasatinib, crenolanib, or dovitinib. Cultures were sampled at Error bars represent means ± SD.

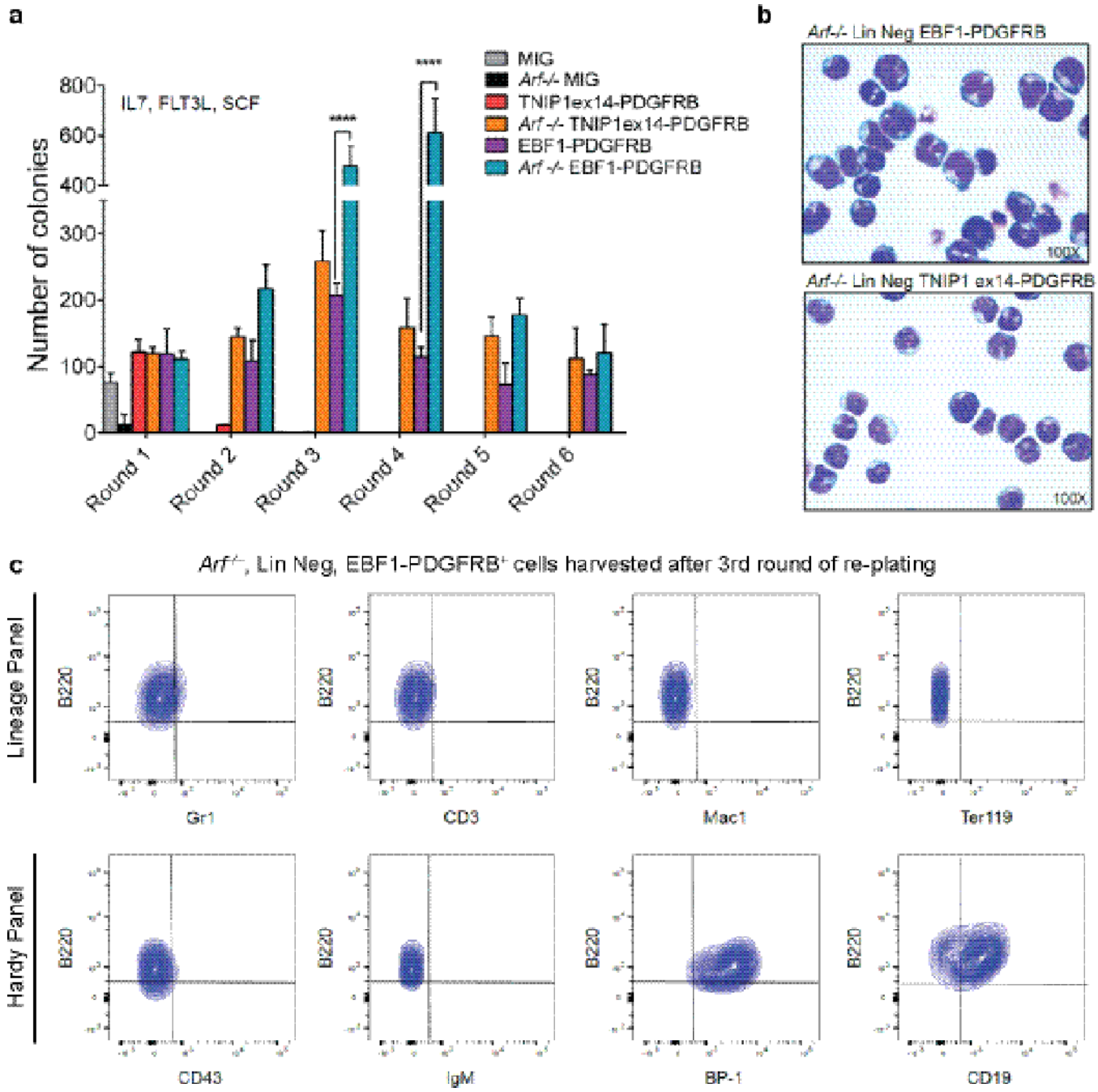

Figure 5.

Re-plating activity of progenitors expressing PDGFRB fusions. (**a**) EBF1-PDGFRB or TNIP1ex14-PDGFRB transduction of lineage-negative WT or $Arf^{-/-}$ cells in semi-solid methylcellulose containing IL7, FLT3L, and SCF resulted in sustained re-plating of B lymphoid colonies. All cells collected from rounds three to six of re-plating were analyzed for (**b**) cell morphology and (**c**) flow cytometric detection of Lineage Panel vs. Hardy Panel staining. Data in (**c**) is representative of $Arf^{-/-}$, EBF1-PDGFRB-positive cells collected from

methylcellulose after three rounds of re-plating. Error bars represent the mean ± SD of three biological replicates.

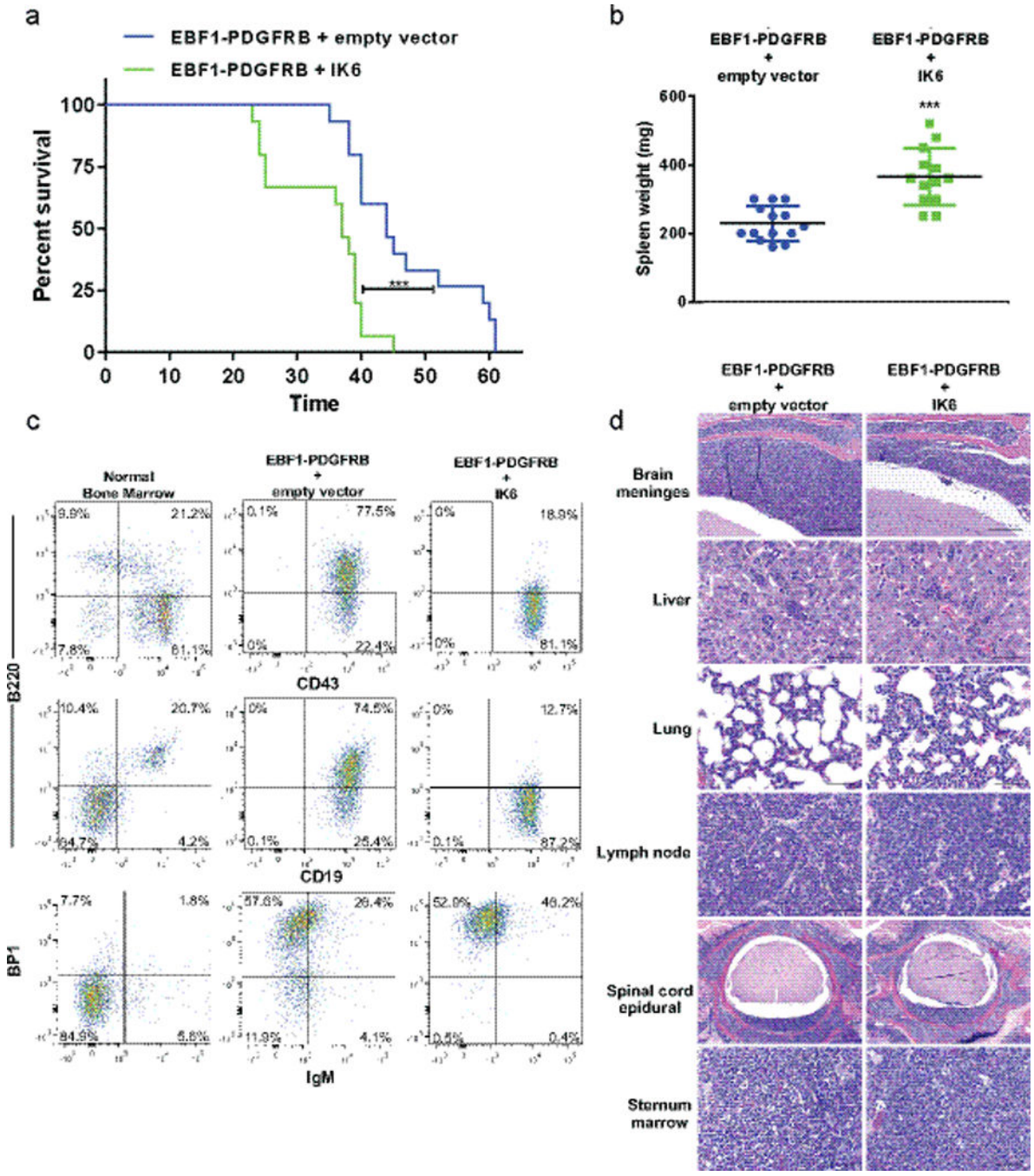

Figure 6.

EBF1-PDGFRB is leukemogenic, cooperates with IK6, and produces a fully penetrant disease. (**a**) Transplantation of *in vitro*-derived EBF1-PDGRFB transformed $Arf^{-/-}$ pre-B cells with co-transduction of empty vector or IKAROS dominant negative IK6, which lacks the N-terminal DNA-binding zinc fingers due to deletion of exons 4–7. Statistical significance was assessed by log rank Mantel-Cox (***$P < 0.0005$) and n = 15 mice per group (5 mice each from three independent pre-B cell transductions). (**b**) Mice inoculated with pre-B cells co-expressing EBF1-PDGFRB and IK6 had increased splenic infiltration, as

determined by spleen weight. The data points ± SD are plotted, ***$P < 0.0005$. (**c**) Representative flow cytometric analysis of bone marrow from moribund mice detecting hematopoietic lineage markers CD43, B220, CD19, BP1, and IgM. (**d**) Representative histology from EBF1-PDGFRB or EBF1-PDGFRB + IK6 leukemia infiltrated tissues.

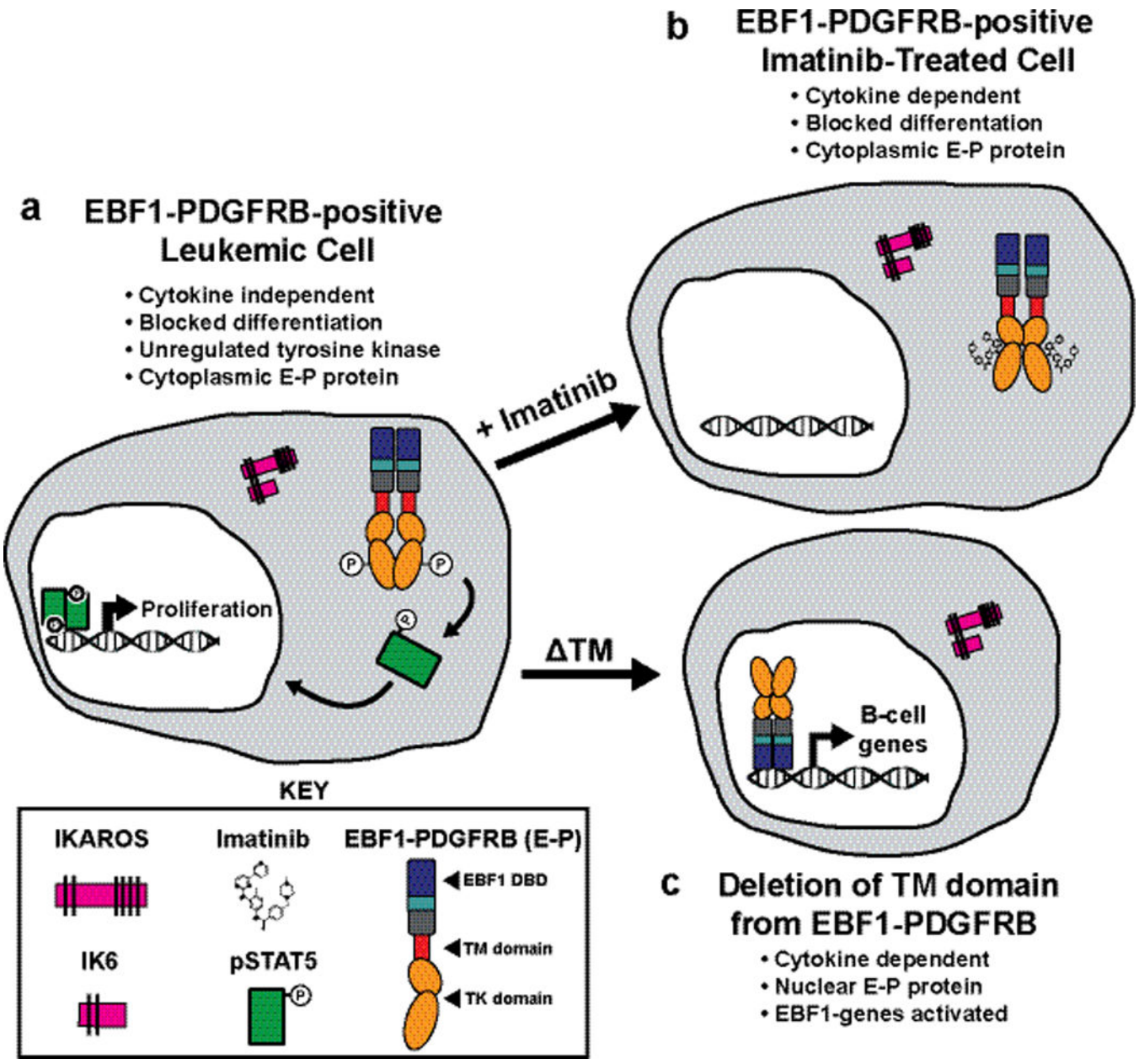

Figure 7.

Proposed model of EBF1-PDGFRB (E-P) leukemogenesis. (**a**) Leukemic cells harbor the EBF1-PDGFRB (E-P) fusion protein, which homodimerizes, autophosphorylates, and activates STAT5 signaling promoting aberrant proliferation. Leukemia cells fail to differentiate due to sequestration of EBF1 (in the form of E-P) outside of nuclei. E-P can synergize with IKAROS loss-of-function, which is imposed by the dominant-negative isoform IK6. (**b**) Treatment with imatinib blocks E-P TK activity and downstream STAT5 signaling, but fails to restore differentiation. (**c**) Deletion of the TM motif results in relocalization of E-P proteins into nuclei, resulting in partial restoration of the B cell program.