Leukemia (2014) 28, 88–97
© 2014 Macmillan Publishers Limited All rights reserved 0887-6924/14
www.nature.com/leu

ORIGINAL ARTICLE

# BET protein inhibition shows efficacy against JAK2V617F-driven neoplasms

BS Wyspiańska[1,5], AJ Bannister[1,5], I Barbieri[1,5], J Nangalia[2,3], A Godfrey[2,3], FJ Calero-Nieto[2], S Robson[1], I Rioja[4], J Li[2,3], M Wiese[1,2], E Cannizzaro[1,2], MA Dawson[1,2,3], B Huntly[2], RK Prinjha[4], AR Green[2,3], B Gottgens[2] and T Kouzarides[1]

Small molecule inhibition of the BET family of proteins, which bind acetylated lysines within histones, has been shown to have a marked therapeutic benefit in pre-clinical models of mixed lineage leukemia (MLL) fusion protein-driven leukemias. Here, we report that I-BET151, a highly specific BET family bromodomain inhibitor, leads to growth inhibition in a human erythroleukemic (HEL) cell line as well as in erythroid precursors isolated from polycythemia vera patients. One of the genes most highly downregulated by I-BET151 was *LMO2*, an important oncogenic regulator of hematopoietic stem cell development and erythropoiesis. We previously reported that *LMO2* transcription is dependent upon Janus kinase 2 (JAK2) kinase activity in HEL cells. Here, we show that the transcriptional changes induced by a JAK2 inhibitor (TG101209) and I-BET151 in HEL cells are significantly over-lapping, suggesting a common pathway of action. We generated JAK2 inhibitor resistant HEL cells and showed that these retain sensitivity to I-BET151. These data highlight I-BET151 as a potential alternative treatment against myeloproliferative neoplasms driven by constitutively active JAK2 kinase.

*Leukemia* (2014) **28,** 88–97; doi:10.1038/leu.2013.234

**Keywords:** JAK2V617F; BET proteins; LMO2; myeloproliferative neoplasms

## INTRODUCTION

Myeloproliferative neoplasms (MPNs) include a group of diverse and heterogeneous clonal stem cell disorders characterized by overproduction of one or more blood cell types.[1–3] They include polycythemia vera (PV), essential thrombocythemia and primary myelofibrosis. At the molecular level, these pathologies are very often associated with the presence of a gain-of-function point mutation in the Janus kinase 2 (JAK2), JAK2V617F.[4–7] Indeed, the importance of JAK2 mutation in myeloproliferation has been demonstrated both *in vitro* and *in vivo* using a variety of techniques.[5,6,8–10] Furthermore, recent evidence also indicates that the activity of JAK2 can directly control the expression of leukemic transcription factors such as LMO2. It has been shown that JAK2V617F can translocate to the nucleus and phosphorylate tyrosine 41 of histone H3 to prevent binding of HP1$\alpha$ to the promoter of *LMO2*.[11]

Current treatment of MPNs commonly involves phlebotomy and the use of antiproliferative agents such as hydroxycarbamide. Although hydroxycarbamide is safe and effective, resistance or intolerance in patients is not uncommon. In addition, antiproliferative agents do not alter the natural history of the MPN, particularly the risk of transformation to myelofibrosis or acute myeloid leukemia.[12] Therefore, there is a need to develop agents with better clinical efficacy for the treatment of MPNs.

The importance of JAK2 in the development of overactive myeloproliferation promoted efforts to develop small molecule inhibitors of JAK2 activity *in vivo*. Very recently these efforts have seen the approval of a JAK2 inhibitor, ruxolitinib, for the treatment of myelofibrosis.[13,14] However, it is important to stress that JAK2 also has a crucial role in normal hematopoiesis, as well as a host of other physiological processes, and so the levels of JAK inhibitors used in clinical settings are limited by potential widespread toxicity. Indeed, consistent with this perception, treatment of myelofibrosis patients with the licensed dose of ruxolitinib is associated with only a modest decrease in *JAK2V617F* allele burden.[15] Furthermore, chronic exposure to JAK inhibitor therapy induces ‘persistence’ in *in vitro* cultured MPN cells, as well as in ruxolitinib-treated samples isolated from MPN primary patients.[16] There is a clear need, therefore, to develop further drugs in order to achieve molecular remission in MPN patients, to be used either as sole agents or in combination therapy.

Recently, the BET proteins have emerged as a very exciting group of transcriptional co-regulators. They are chromatin readers recognizing acetylated lysines in histones,[17] and comprise four proteins, BRD2, BRD3, BRD4 and BRDT. Each protein possesses two highly conserved bromodomains. Their main function is to recruit members of the pTEF-b complex to promoters to support transcriptional elongation, and their functional importance is underscored by their links to cancer when they become dysregulated.[18,19]

Along with others, we have recently established the therapeutic efficacy of a novel class of epigenetic compounds that selectively target the BET proteins. The BET inhibitors selectively bind to the bromodomains of the BET proteins and inhibit their ability to bind acetylated lysine residue on histones. The therapeutic benefit of BET inhibitors has been demonstrated in pre-clinical models of mixed lineage leukemia (MLL)-fusion protein leukemia,[19,20] in acute myeloid leukemia cell lines lacking MLL rearrangements as well as in multiple myeloma and in Burkitt’s lymphoma.[21,22]

[1]Gurdon Institute and Department of Pathology, University of Cambridge, Cambridge, UK; [2]Department of Haematology, Cambridge Institute for Medical Research and The Wellcome Trust and MRC Stem Cell Institute, University of Cambridge, Cambridge, UK; [3]Addenbrooke’s Hospital, Department of Haematology, University of Cambridge, Cambridge, UK and [4]Epinova DPU, Immuno-Inflammation Centre of Excellence for Drug Discovery, GlaxoSmithKline, Medicines Research Centre, Stevenage, UK. Correspondence: Professor T Kouzarides, Gurdon Institute and Department of Pathology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QN, UK.
E-mail: t.kouzarides@gurdon.cam.ac.uk
[5]These authors contributed equally to this work.
Received 26 July 2013; accepted 6 August 2013; accepted article preview online 9 August 2013; advance online publication, 6 September 2013

In the article, we have investigated the efficacy of a potent BET inhibitor (GSK151A—hereafter referred to as I-BET151) in JAK2-driven neoplastic cells, using human erythroleukemic (HEL) cells as an *in vitro* model system, as well as primary patient samples. We found that I-BET151 efficiently prevented HEL cell proliferation, and induced apoptosis, at least in part, via downregulation of the *LMO2* gene. *LMO2* is also downregulated by JAK2 inhibitors and we found that I-BET151 and a JAK2 inhibitor (TG101209—hereafter referred to as JAK2i) cooperate to downregulate expression of *LMO2* and inhibit HEL cell proliferation. We then investigated whether I-BET151 shows efficacy against a JAK2V617F-driven human MPN, namely PV. We report that I-BET151 efficiently inhibits erythroid colony formation from JAK2 mutant but not wild-type JAK2 containing erythroid colonies cultured from PV patients. Our results highlight the potential for the use of I-BET151 against JAK2-driven neoplasms, especially PV, with the added possibility that this new treatment may reduce disease burden. Furthermore, we provide evidence that the use of I-BET151 in conjunction with JAK2i, may overcome acquired resistance to JAK2 inhibitors.

## MATERIALS AND METHODS

### Cell culture

HEL and human myelogenous leukemia (K562) cells were maintained in RPMI 1640 medium, 10% fetal bovine serum, 1% penicillin/streptomycin/glutamine (GIBCO, Invitrogen, Carlsbad, CA, USA), incubated at 37 °C in 5% $CO_2$ and passaged 1:6 every 2–3 days.

### Compounds

TG101209 JAK2 inhibitor was from TargeGen Inc. (San Diego, CA, USA) and GSK1210151A (I-BET151) was provided by GlaxoSmithKline (Stevenage, UK).

### Immunoblotting

Whole-cell lysates, prepared in 2X Laemmli sample buffer, were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to nitrocellulose (Millipore, Billerica, MA, USA). Signal was detected using ECL (GE Healthcare, Buckinghamshire, UK) and developed on X-ray film (Fuji, Tokyo, Japan). A list of antibodies is provided in the Supplementary Information.

### Cell growth inhibition ($gi_{50}$) assay

HEL and K562 cells ($1 \times 10^5$ cells/ml) were seeded at 200 μl per well. TG101209 and I-BET151 (in dimethylsulphoxide (DMSO)) were serially diluted. Cell growth inhibition was assessed via CellTiter-Glo luminescent viability assay (Promega, Madison, WI, USA), following the manufacturer's instructions.

### Proliferation assays

In all, $1 \times 10^5$ HEL and K562 cells were plated in complete medium containing TG101209, I-BET151 or DMSO. Viable cells were counted twice at each time point using a Countess counter (Invitrogen, Carlsbad, CA, USA).

### Clonogenic assays in methylcellulose

Clonogenic potential of HEL and K562 cells was assessed via colony growth in the presence of DMSO or 1 μM I-BET151. Cells were plated in methylcellulose supplemented with human cytokines (Methocult H4435, Stemcell Technologies, Vancouver, BC, Canada) in duplicate at $1 \times 10^2$ and $1 \times 10^3$ per plate. Colonies were analyzed 7–10 days after seeding.

### Human samples and assays

All patients gave written informed consent; the study was approved by the Cambridge and Eastern Region Ethics Committee, and research was carried out in accordance with the Declaration of Helsinki. The selection of patients and colonies analysis were performed as previously described.[23] Following isolation of peripheral blood mononuclear cells from PV5, lineage-negative cells enriched for hematopoietic progenitors were obtained by magnetic separation as per manufacturer's instructions (Human Progenitor Enrichment Kit, Easysep, Stemcell Technologies). Cells were incubated in DMEM, 15% fetal bovine serum, 1 unit/ml EPO and either DMSO, 200 nM or 1 uM I-BET151. Cells were lysed in Trizol and RNA extraction was performed using phenol/chloroform.

### Flow cytometry

Apoptosis was assessed using Annexin V-FITC kit (Miltenyi Biotec Ltd, Bergisch Gladbach, Germany) supplemented with 7-AAD (BD Bioscience, Franklin Lakes, NJ, USA), according to the manufacturer's instructions. Flow cytometry experiments were performed on a CyAn ADP flow cytometer (Dako, Glostrup, Denmark), and data were analyzed with FlowJo software (Tree Star, Inc., Ashland, OR, USA).

### Chromatin immunoprecipitation (ChIP) assay

HEL cells were treated with 1 μM I-BET151 or DMSO for 4 h. ChIP was carried out as previously described.[11]

### Quantitative real-time reverse transcriptase (RT)-PCR

Cells were treated for 4 h with TG101209, I-BET151 or DMSO. mRNA was extracted from equal numbers of cells using the RNAeasy kit (Qiagen, Velno Limburg, Netherland), following the manufacturer's protocol. First-strand complementary DNA was synthesized using the SuperScript III Reverse Transcriptase kit (Invitrogen). Analysis of ChIP and complementary DNA samples was carried out on an ABI 7900 real-time PCR machine, using fast SYBR green PCR Master Mix (ABI, Foster City, CA, USA) according to the manufacturer's instructions. Primers used in this study are listed in the Supplementary Information.

### Lentiviral production and transduction

Short hairpin RNA (shRNA) against LMO2 (in pLL3.7 vector) was kindly provided by Dr Jiro Kikuchi (Jichi Medical University, Japan[24]). Lentivirus production was carried out as previously described.[25]

### Flow cytometry and competitive proliferation assay

Green fluorescent protein fluorescence analysis was performed using a Cyan ADP analyser (Beckman Coulter, High Wycombe, UK). Competitive proliferation assays in liquid culture were performed by monitoring the green fluorescent protein-positive cell-fraction over a 13-day time course.

### Gene expression and bioinformatic analysis

HEL cell RNA (treated with 1 μM I-BET151 or TG101209) was extracted after 4 h and processed as described before after hybridization to Illumina Human HT12 v4 BeadChips. Gene expression data were analyzed as previously described.[19]

## RESULTS

Using an end point viability assay, we have recently shown that HEL cells are sensitive to I-BET151 treatment with an $IC_{50}$ of approximately 1 μM.[19] To further investigate the effect of I-BET151 on HEL cells, we performed a cell proliferation assay over a range of I-BET151 concentrations (Figure 1a). These data clearly show that HEL cells are acutely sensitive to I-BET151 exposure, exhibiting an $IC_{50}$ of 300 nM in this assay. In contrast, K562 cells were less effected by I-BET, exhibiting an $IC_{50}$ of 6 μM; 20-fold greater than the $IC_{50}$ in HEL cells (Figure 1a). Consistent with the strong effects in liquid culture, the colony-forming potential of HEL cells was significantly reduced by I-BET151, whereas K562 cells were unaffected (Figure 1b).

We next asked whether I-BET151 treatment induces apoptosis in HEL cells, as it does in MLL fusion-driven cell lines. Figure 1c shows that two markers of apoptotic cells, cleaved poly ADP-ribose polymerase and active CASPASE 3, are both detectable after 24-h exposure to I-BET151 and expression of both is significantly upregulated after 48-h exposure. A high level of I-BET151-induced apoptosis was also detected via fluorescence-activated cell sorting analysis, where 30% of cells were positive for ANNEXIN V staining after a 48-h treatment with 1 μM I-BET151 (Figure 1d) compared with just 3% positive cells in DMSO-treated cells. These data suggest that I-BET151 alters transcriptional programs in HEL cells that ultimately regulate apoptosis.

© 2014 Macmillan Publishers Limited

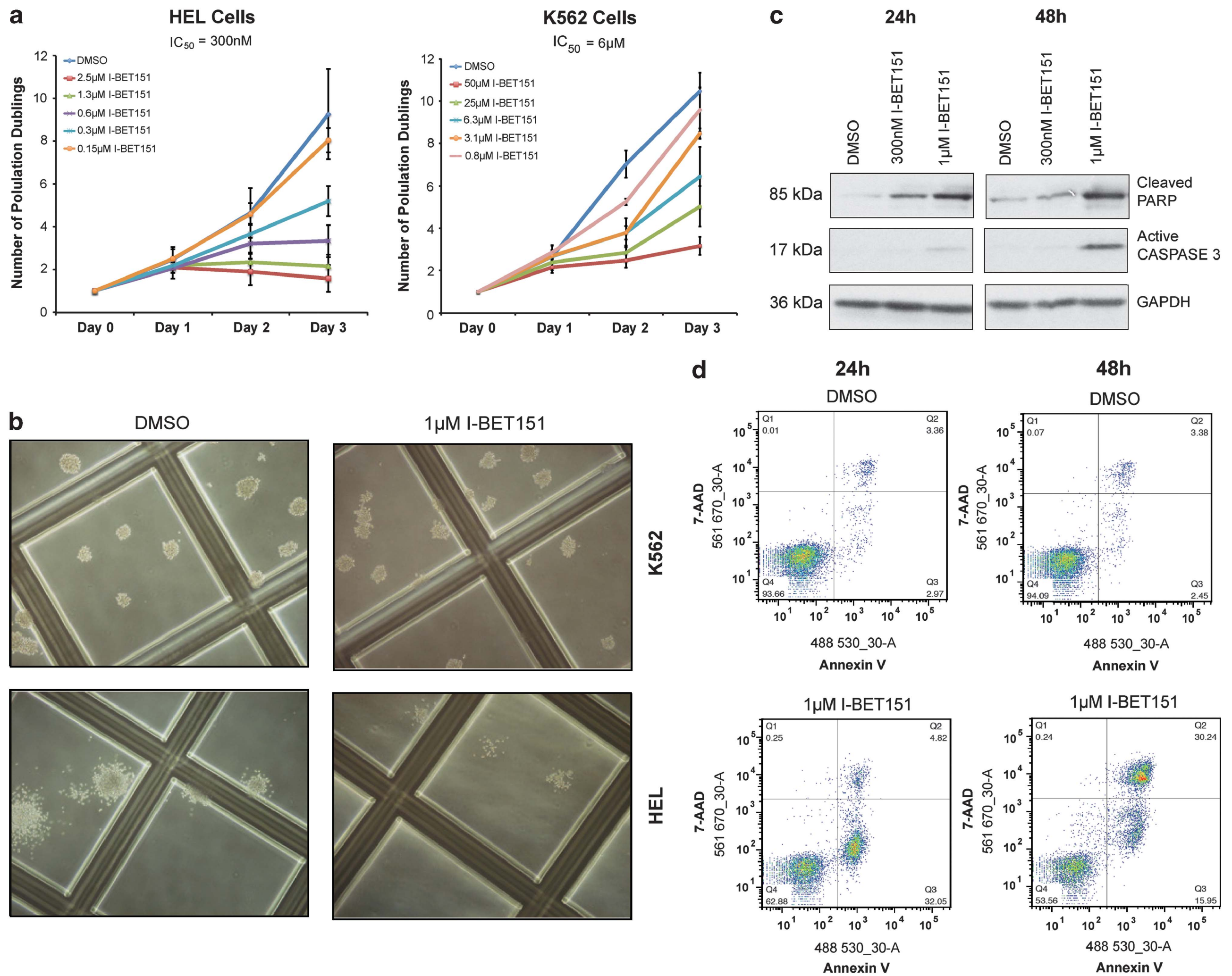


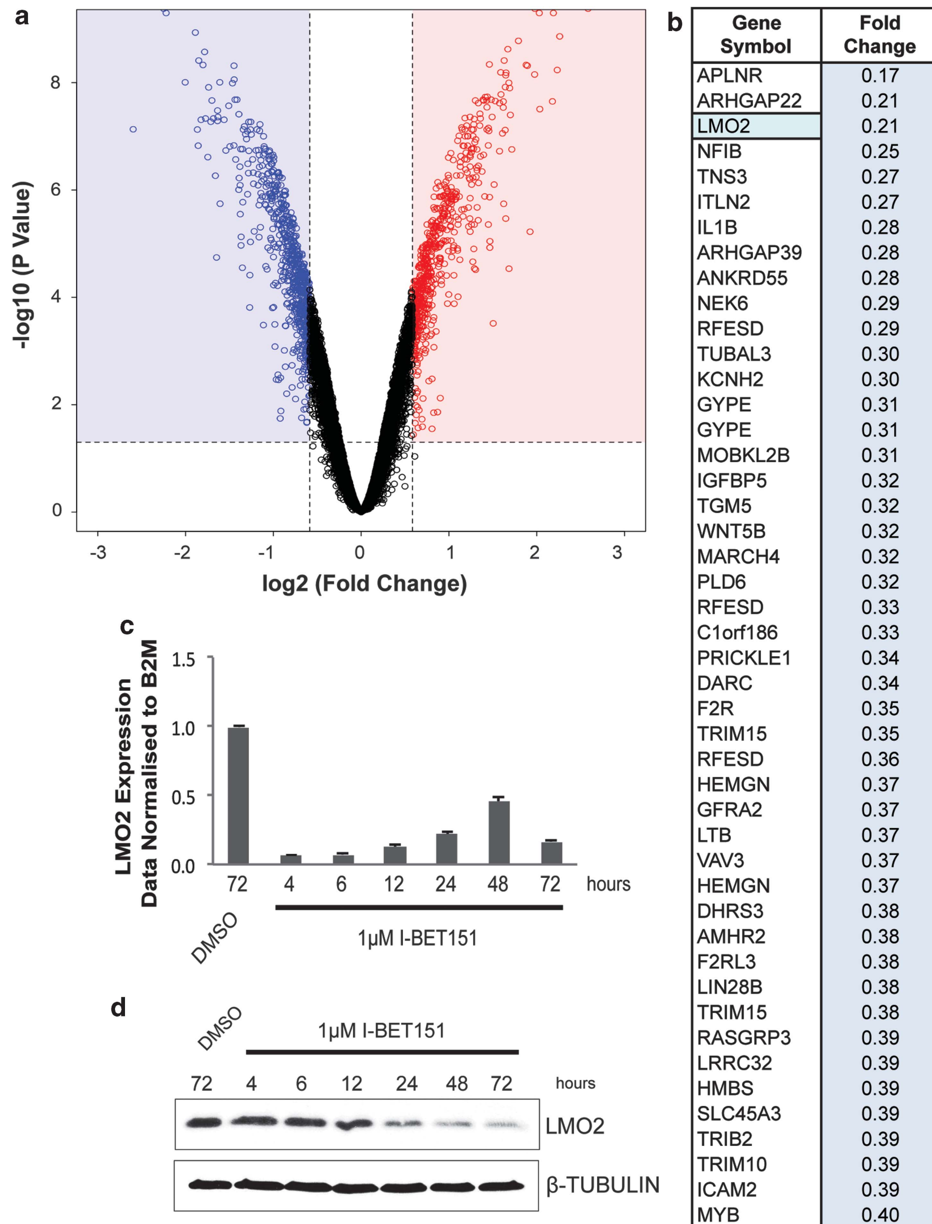

**Figure 1.** I-BET151 inhibits growth and induces apoptosis in erythroleukemia cells. (**a**) HEL and myelogenous leukemia (K562) cells were treated with a range of I-BET151 concentrations and the population doubling was monitored over 3 days. (**b**) Clonogenic assay performed in the presence of DMSO or 1 μM I-BET151. (**c**) Immunoblotting demonstrating an increase in the cleaved poly ADP-ribose polymerase (PARP) and active CASPASE 3 after 24 h and 48 h of I-BET151 treatment. (**d**) Apoptosis was assessed by fluorescence-activated cell sorting analysis after 24- and 48-h incubation with DMSO or 1 μM I-BET151 by monitoring ANNEXIN V.

To identify the precise transcriptional pathways controlled by I-BET151, global gene expression analyses were performed in HEL cells after treatment with 1 μM I-BET151 or DMSO for 4 h. This strategy allowed us to identify early, and most likely directly regulated, I-BET151-responsive genes. As demonstrated previously in MLL-driven cells, we observed in HEL cells differential expression of only a selective subset of genes rather than global transcriptional dysregulation; using a cutoff threshold of twofold, 125 genes were downregulated and 154 genes were upregulated by I-BET151 treatment (Figure 2a and Supplementary Figure 1) Strikingly, the third most downregulated gene (from over 19 000 probes on the array) was *LMO2* (Figure 2b), an important regulator of hematopoietic stem cell development and erythropoiesis. I-BET151 induced changes in *LMO2* mRNA levels were confirmed by RT-PCR, and changes in LMO2 protein levels were detected by western blotting (Figures 2c and d). Furthermore, changes in expression of three of the most downregulated genes (*LMO2*, *MYB* and *NEK6*), and a control gene whose expression did not change (*CISH*), were validated directly by RT-PCR in a least two separate experiments (Supplementary Figure 2).

Given the importance of *LMO2* to hematopoietic stem cell development, leukemogenesis and erythropoiesis, we decided to focus on this gene in order to better understand how the BET inhibitors exerted their effects in HEL cells. The *LMO2* gene contains three transcriptional start sites[26] (TSSs; sites 1, 2 and 3 in Figure 3a). Using ChIP analysis, we found the active gene marker H3K4me3 at the three TSSs, especially at the proximal (site 1) and intermediate (site 2) TSSs but not at an upstream region (site 4; Figure 3a). Importantly, we also found significant levels of BRD3 and 4 at the TSSs, which were markedly reduced after treatment with I-BET151. ChIP of the elongation competent form of RNA polymerase II, phosphorylated at serine 2 within its carboxy terminal domain by the BRD3/4 recruited cdk9 kinase, indicated that the active polymerase is present primarily at the proximal and intermediate TSSs and that exposure to I-BET151 significantly reduces the amount of transcriptionally competent polymerase (Figure 3b). Finally, the BET proteins associate with the RNA polymerase II associated factor complex to promote transcription. ChIP with antibodies against LEO1, an integral polymerase II associated factor complex member shows that LEO1 is indeed present within the *LMO2* locus, especially at the proximal and intermediate TSSs, and that it is displaced by I-BET151 treatment (Figure 3b). These observations provide mechanistic insight into how the *LMO2* gene is downregulated upon I-BET151 treatment.

We next addressed whether I-BET151-mediated repression of *LMO2* is sufficient to inhibit HEL cell proliferation. To this end, we


© 2014 Macmillan Publishers Limited




| Gene Symbol | Fold Change |
|---|---|
| APLNR | 0.17 |
| ARHGAP22 | 0.21 |
| LMO2 | 0.21 |
| NFIB | 0.25 |
| TNS3 | 0.27 |
| ITLN2 | 0.27 |
| IL1B | 0.28 |
| ARHGAP39 | 0.28 |
| ANKRD55 | 0.28 |
| NEK6 | 0.29 |
| RFESD | 0.29 |
| TUBAL3 | 0.30 |
| KCNH2 | 0.30 |
| GYPE | 0.31 |
| GYPE | 0.31 |
| MOBKL2B | 0.31 |
| IGFBP5 | 0.32 |
| TGM5 | 0.32 |
| WNT5B | 0.32 |
| MARCH4 | 0.32 |
| PLD6 | 0.32 |
| RFESD | 0.33 |
| C1orf186 | 0.33 |
| PRICKLE1 | 0.34 |
| DARC | 0.34 |
| F2R | 0.35 |
| TRIM15 | 0.35 |
| RFESD | 0.36 |
| HEMGN | 0.37 |
| GFRA2 | 0.37 |
| LTB | 0.37 |
| VAV3 | 0.37 |
| HEMGN | 0.37 |
| DHRS3 | 0.38 |
| AMHR2 | 0.38 |
| F2RL3 | 0.38 |
| LIN28B | 0.38 |
| TRIM15 | 0.38 |
| RASGRP3 | 0.39 |
| LRRC32 | 0.39 |
| HMBS | 0.39 |
| SLC45A3 | 0.39 |
| TRIB2 | 0.39 |
| TRIM10 | 0.39 |
| ICAM2 | 0.39 |
| MYB | 0.40 |

**Figure 2.** I-BET151 potently inhibits the expression of an important hematopoietic regulator, *LMO2*. Messenger RNA was isolated from HEL cells treated for 4 h with 1 μM I-BET151 or DMSO. Four biological replicates were analyzed by microarray to generate a global gene expression profile. (**a**) Volcano plot for I-BET151 against DMSO-treated samples, showing the adjusted significance *P*-value (−log10) versus fold change (log2). (**b**) The 46 most downregulated genes are illustrated; shading highlights the position of *LMO2* gene. (**c**) *LMO2* gene expression after treatment with 1 μM I-BET151 was validated with qRT-PCR. Data are normalized to *B2M* expression; the expression level of *LMO2* in DMSO was assigned a value of 1 and the error bars reflect s.d. (**d**) Immunoblotting demonstrating reduced levels of LMO2 protein after treatment with 1 μM I-BET151.

transduced HEL cells with previously validated retroviral constructs expressing shRNAs against *LMO2* together with a *GFP* reporter gene[24] to allow identification of transduced cells. We then monitored the ability of the transduced cells to proliferate relative to non-transduced cells. The results clearly indicate that cells transduced with shRNAs against *LMO2* are rapidly outgrown by non-transduced cells (Figure 4a). In contrast, cells transduced with control shRNAs proliferate at the same rate as non-transduced cells. Importantly, shRNAs targeting *LMO2* mRNA caused a dramatic reduction in LMO2 protein levels (Figure 4b). In contrast, K562 cells transduced with shRNAs against *LMO2* did not show any growth disadvantage compared with the cells treated with control shRNAs (Supplementary Figure 3). Together, these data indicate that HEL cells require LMO2 for efficient cellular proliferation and they strongly implicate the *LMO2* gene as a major target for the efficacy of I-BET151 in HEL cells. Consistent with this notion, targeted inhibition of *LMO2* in HEL cells induced apoptosis (Figure 4c) similarly to treatment of the cells with I-BET-151 (Figures 1c and d).

We previously reported transcriptional regulation of the *LMO2* locus by JAK2V617F in HEL cells. We also evaluated the global gene expression effect of JAK2 inhibition in HEL cells using the JAK2-specific inhibitor TG101209 (JAK2i).[11] *LMO2* appeared within the first 20 most downregulated genes after JAK2 inhibition in HEL cells. To better evaluate the common features of JAK2 and BET inhibition, the global gene expression effects of the two inhibitors

© 2014 Macmillan Publishers Limited

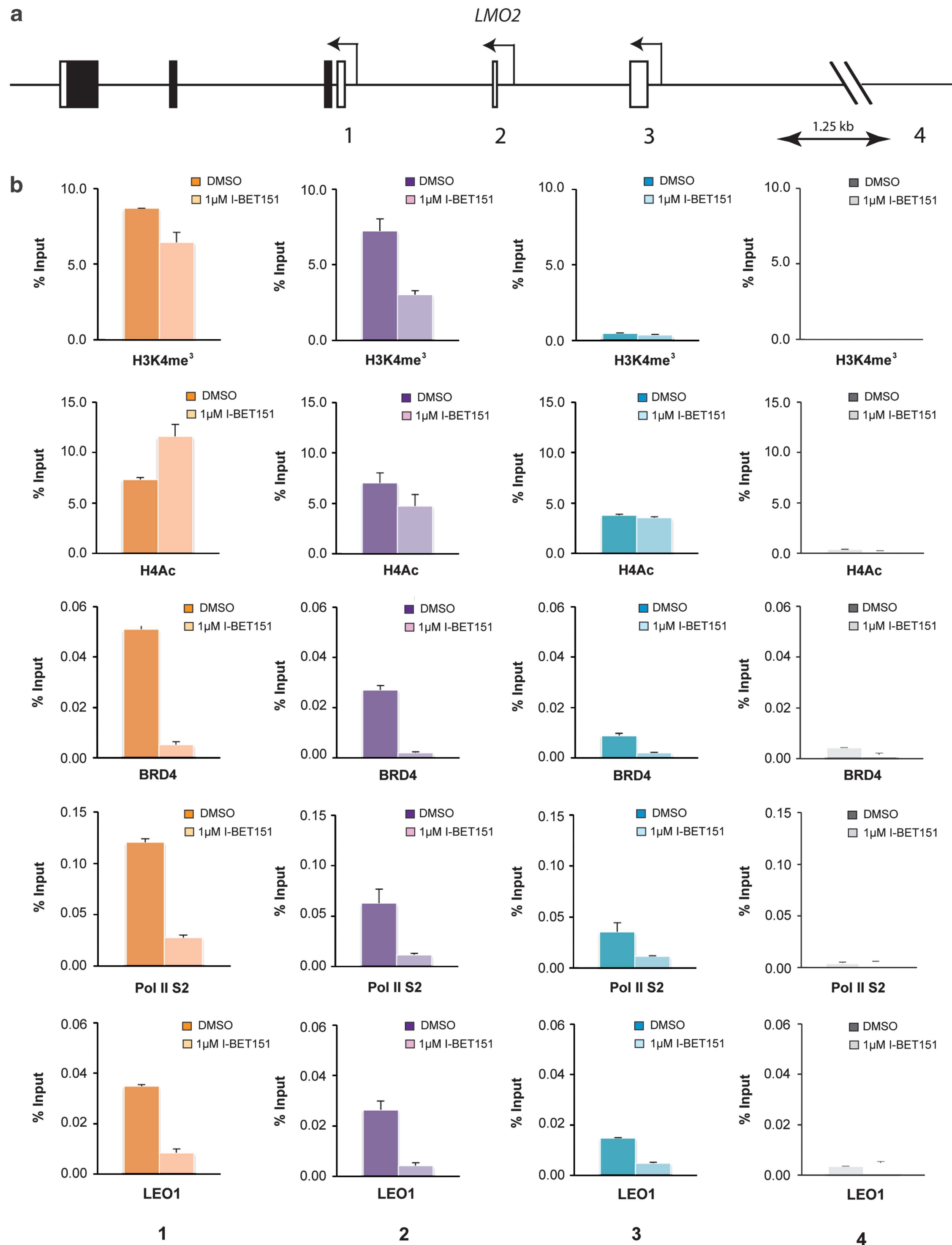



**Figure 3.** ChIP analysis at the *LMO2* locus. (**a**) Schematic representation of *LMO2* locus. Black boxes indicate coding, and white boxes non-coding exons. Arrows mark transcription start sites. (**b**) Four regions within the *LMO2* locus were investigated (amplicons 1–4; see schematic representation of *LMO2* locus) by ChIP analyses with antibodies against H3K4me3, panH4ac, BRD4, Pol II S2, and an integral member of the PAF complex, LEO1. Bar graphs are represented as the mean enrichment relative to input and error bars reflect s.d.

were compared. The gene expression changes induced by the inhibitors are highly correlated showing a Pearson correlation coefficient of 0.683 (Figure 5a). We also identified a consistent overlap between the two data sets comparing the most differentially expressed genes. We found that 38% (48 out of 125) of I-BET151 downregulated genes are also downregulated by JAK2i (Figure 5c). Similarly, 31% (48 out of 154) of I-BET151 upregulated genes are also upregulated by JAK2i (Figure 5d). Together, these data indicate a striking overlap between the genes regulated by I-BET151 and those regulated by JAK2i, suggesting that these compounds function in the same or very similar pathways. Indeed, this notion is reinforced by the observation that the two


© 2014 Macmillan Publishers Limited

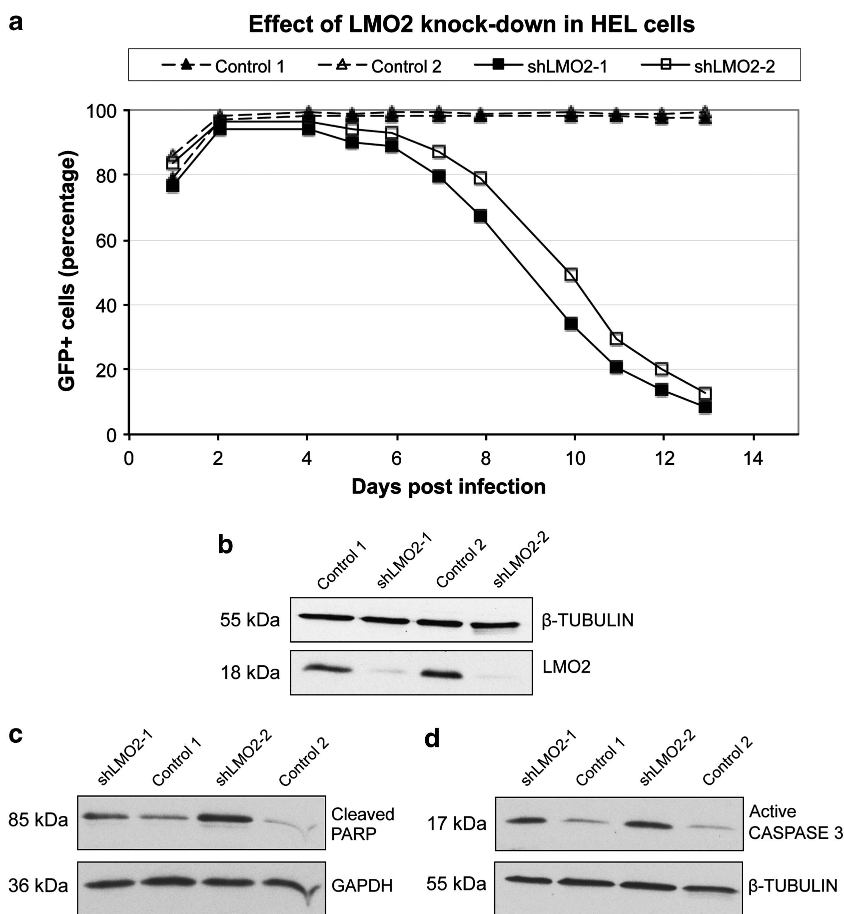



**Figure 4.** LMO2 is required for the continuous proliferation of erythroleukemia cells. (**a**) Knockdown of *LMO2* in HEL cells results in a competitive growth disadvantage. HEL cells were transduced with constructs containing shRNA against *LMO2* or empty vector as a control. Green fluorescent protein (GFP) was included as an indicator of transduced cells and its presence was monitored for 13 days following infection. Percentages of GFP-positive cells are indicated. Shown are the results from a representative experiment performed in duplicate. (**b**) shRNA knockdown validation by immunoblotting showing LMO2 protein disappearing at day 5 post-infection with shLMO2 retrovirus. (**c**) Knockdown of *LMO2* induces apoptosis in HEL cells. Immunoblotting showing the induction of poly ADP-ribose polymerase (PARP) cleavage and (**d**) the activation of CASPASE 3 in cells transduced with shLMO2-1 and shLMO2-2.

compounds have similar effects on HEL cells, inducing arrest of growth and apoptosis with comparable $IC_{50}$s (Supplementary Figure 4).

To further investigate the possibility that I-BET151 and JAK2i function in a common pathway we tested the two inhibitors together, in a combinatorial manner. To this end, we analyzed a wide range of concentrations of both inhibitors, both separately and combined, in 72-h proliferation assays. I-BET151 and JAK2i showed an additive effect for all concentrations tested (Figure 5d and Supplementary Figure 5). We also tested the combined effect of the two inhibitors on the expression of *LMO2*. Once again we find that the two compounds act in an additive manner (Figure 5e). Furthermore, each inhibitor has a maximal effective dose with respect to *LMO2* inhibition. Once this level of *LMO2* inhibition has been reached, the gene becomes insensitive to further additions of either inhibitor (Supplementary Figure 6). This strongly suggests that the inhibitors are functioning in a very similar manner. As with the global gene expression analysis and cell proliferation analysis described above, these data are fully consistent with I-BET151 and JAK2i sharing key downstream targets.

The *LMO2* promoter is under the control of JAK2 and constitutive activation of the pathway, by JAK2V617F, leads to dysregulated expression of *LMO2*.[11] Furthermore, *LMO2* expression is very sensitive to JAK2 inhibition.[27] The *JAK2V617F* mutation is also tightly linked to multiple MPNs especially PV. Indeed, JAK2 inhibitors are now being used in clinical trials in the treatment of PV, but the mechanism(s) of their action downstream of JAK2 remain uncertain. As enforced expression of *LMO2* in progenitor cells increases erythroid differentiation enhancing transcription of erythroid genes, we reasoned that the dysregulated drive to produce red blood cells in PV might be because of constitutively active JAK2 (JAK2V617F) inducing production of aberrantly high levels of LMO2 protein in red blood cell progenitors. If so, I-BET151 might provide an alternative treatment for PV patients by reducing LMO2 levels and consequently diminishing the drive to erythropoiesis. To investigate this possibility, circulating hematopoietic progenitors purified from a *JAK2V617F*-positive PV patient were incubated with either DMSO vehicle or I-BET151 (200 nM or 1 μM) for 4 h and the levels of *LMO2* mRNA were determined via RT-PCR. Figure 6a clearly shows that treatment of the progenitor cells with I-BET151 induces a dose-dependent decrease in *LMO2* mRNA levels irrespective of which housekeeping gene the data are normalized to (*ACTIN* and *GAPDH*, Figure 6a).

We next asked whether exposure to I-BET151 decreases the erythroid colony potential of hematopoietic cells from a single PV patient in cytokine-supplemented methylcellulose containing just DMSO vehicle or increasing concentrations of I-BET151 (Figure 6b). We genotyped a total of 187 burst forming units-erythroid colonies in order to identify those that were wild type (WT) for

© 2014 Macmillan Publishers Limited

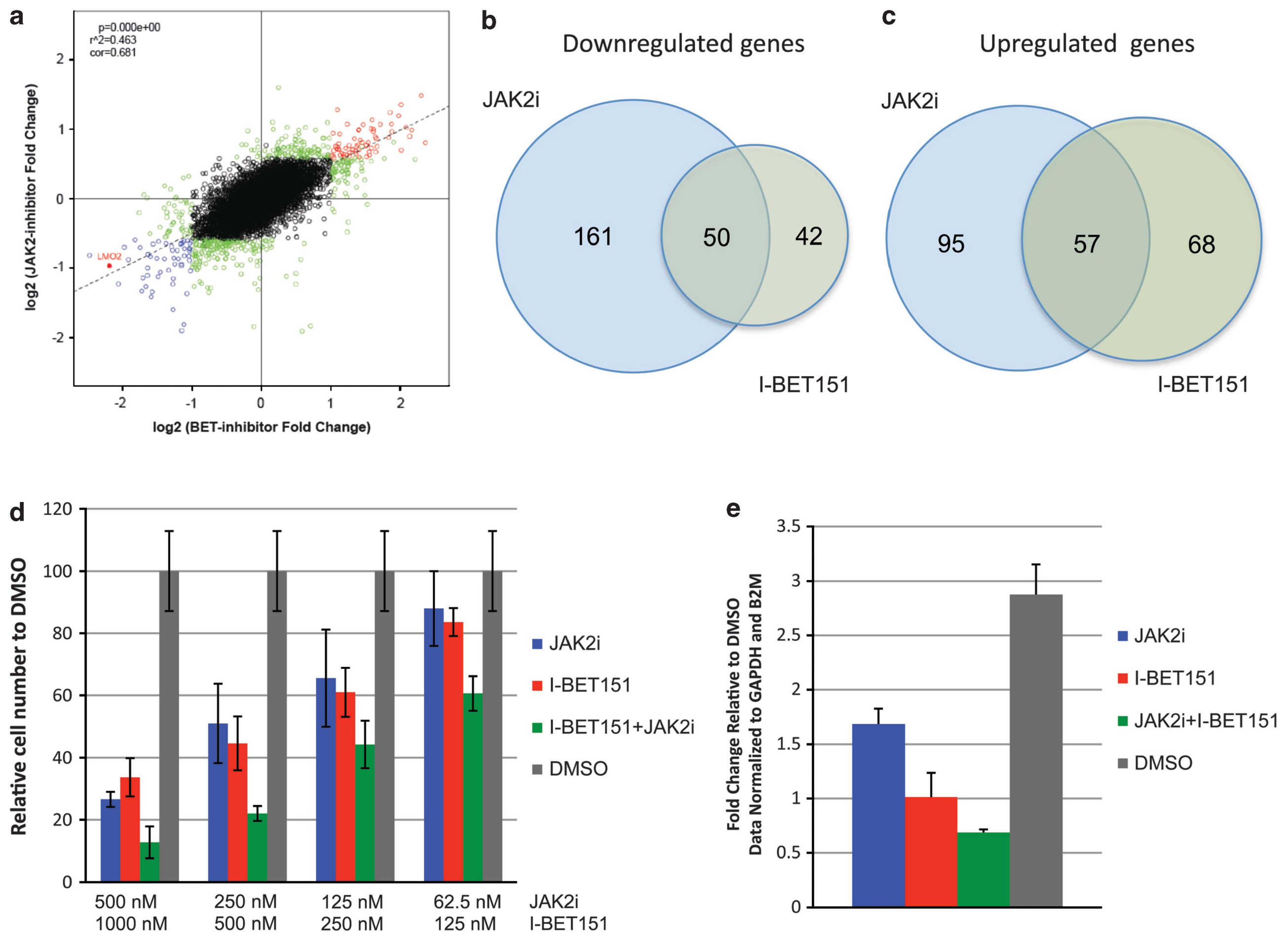



**Figure 5.** JAK2i and I-BET151 induce similar transcriptional changes and cooperate in HEL cells. (**a**) Correlation of gene expression profiles induced by JAK2i and I-BET151 in HEL cells. Log2 fold change values were scaled to have equal mean and variance and a threshold was selected which corresponded to an absolute fold change of 2.0 in the I-BET151 experiment. Genes significantly upregulated by both inhibitors are represented as red hollow dots, whereas genes significantly downregulated by both inhibitors are represented as blue hollow dots. Genes significantly regulated by only one of the two inhibitors are represented as green hollow dots. (**b**) Overlapping of gene sets significantly down-regulated by JAK2i and I-BET151. (**c**) Overlapping of gene sets significantly upregulated by JAK2i and I-BET151. (**d**) HEL cell 72-h proliferation assay using JAK2i and I-BET151 at the indicated concentrations individually or in combination. (**e**) *LMO2* mRNA levels were analyzed by RT-qPCR in HEL cells after treatment with DMSO, 125 nM I-BET151, 500 nM JAK2i or a combination of the two inhibitors.

JAK2 and those that were heterozygous or homozygous for the *JAK2V617F* mutation (Figure 6b). We found that I-BET151 treatment inhibits erythroid colony formation by cells containing mutated *JAK2V617F*—both heterozygous and homozygous cells in the case of this patient (Figure 6b). At the highest concentration of I-BET151 (1 μM), the treatment significantly reduced total colony number (only 47 burst forming unit-erythroid grew) suggesting cytotoxicity effects at this top concentration. In contrast, however, 200 nM I-BET151 treatment reduced colonies from *JAK2V617F* containing cells, especially homozygous cells, while having little or no effect on WT *JAK2* colonies. This was a striking result and it prompted us to investigate the effect in erythroid progenitors from additional patients. Consequently, we repeated the burst forming unit-erythroid colony-forming assay in three additional patients (Figure 6c). We performed these assays at 200 nM I-BET151 since this concentration showed negligible effect on total burst forming unit-erythroid colony numbers (Figure 6b). The data indicated that in all the primary cultures 200 nM I-BET151 had a significant effect on *JAK2V617F*-mutant colonies, especially those homozygous for the mutation, with no negative effect on WT *JAK2* colonies. To confirm this, Poisson regression analyses were performed for either count of total mutant colonies, or of homozygous mutant colonies, with total colonies as an offset, also controlling for the effect of differences between individuals. These analyses indicated that the proportion of total mutant or homozygous mutant colonies is reduced with addition of 200 nM iBET ($P = 0.003$ and $P < 0.001$, respectively). In two of the patient samples (PV2 and PV4), there was a clear reduction in total colonies formed—however, this is not a drug toxicity effect because the total number of WT JAK2 colonies in each case were actually slightly increased by I-BET151 treatment. Thus, the growth inhibitory effect is limited to the progenitor cells carrying *JAK2V617F*.

As discussed above, the clinical efficacy of JAK2 inhibitors is far from optimal. Despite a beneficial effect on the symptoms associated with myelofibrosis, JAK2i treatment does not lead to a significant reduction in the burden of *JAK2V617F*-positive cells. Unfortunately, a significant number of patients develop adverse effects that lead to suspension or reduction of the drug dosage.[15,28] Furthermore, a recent study showed that cell lines carrying the *JAK2V617F* mutation (including HEL cells) could become reversibly persistent to JAK2i without new mutations appearing or clonal selection occurring, highlighting this as a potential problem for patients being treated with JAK2i. Our data suggest that the two inhibitors have similar effects on HEL cell transcription programs and that they may function in the same or highly overlapping pathways. Therefore, we decided to test the


© 2014 Macmillan Publishers Limited

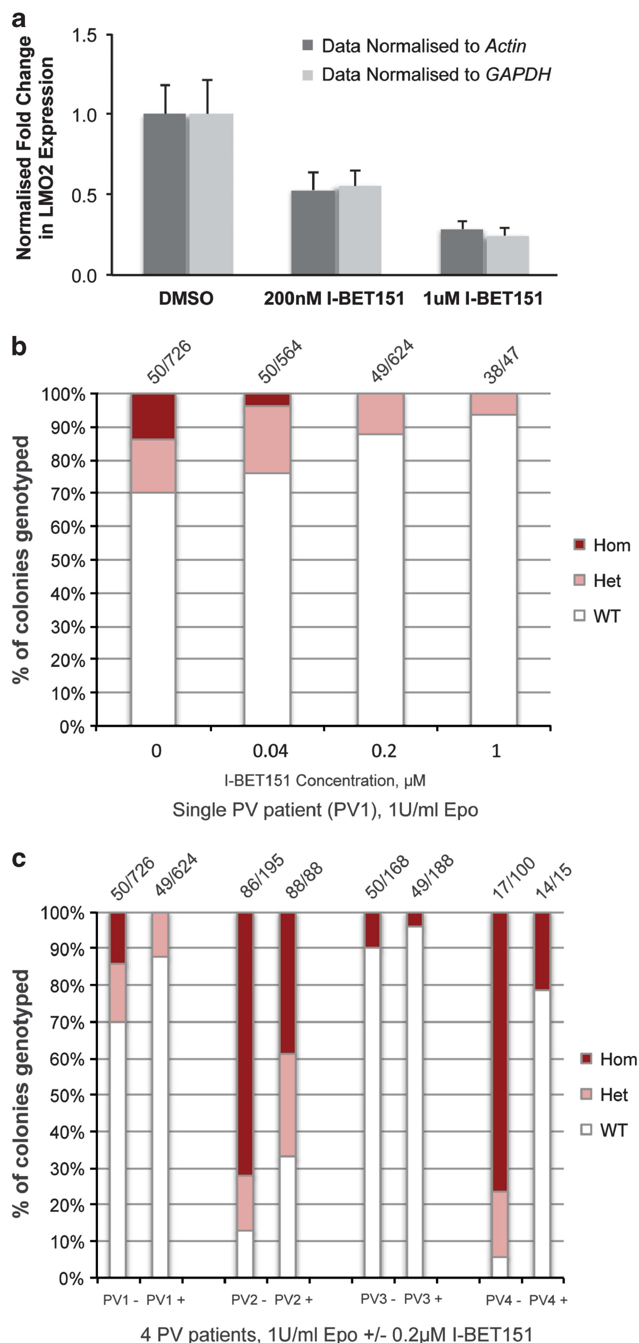



**Figure 6.** I-BET151 shows efficacy in samples isolated from patients with PV. (**a**) Hematopoietic progenitor cells were isolated from whole blood obtained from a PV patient and treated with DMSO, 200 nM I-BET151 or 1 μM I-BET151. Expression of *LMO2* was examined 4 h after treatment. Data are normalized individually to *Actin* or *GAPDH*. The expression level of *LMO2* in DMSO was assigned a value of 1 and error bars reflect s.d. of three biological replicates. (**b**) Peripheral blood mononuclear cells were isolated from a single PV patient and cultured in methylcellulose media supplemented with 1 unit/ml Epo and with either DMSO or I-BET151 at the concentrations indicated burst forming unit-erythroid colonies were genotyped after 14 days in culture. The numbers at the top of the graph show the number of colonies genotyped for *JAK2V617F* mutation over total colonies derived from plating $1.6 \times 10^6$ cells. The Y axis shows the relative percentages of homozygous *JAK2V617F* colonies (Hom; red), heterozygous *JAK2V617F* colonies (Het; pink) and wild type JAK2 colonies (WT; white). (**c**) The burst forming unit-erythroid assay was repeated in a further three PV patients in the presence of DMSO or 200 nM I-BET151 and the results are presented as described for panel (**c**).

ability of HEL cells to develop resistance to I-BET151. To do this, HEL cells were treated constantly with the $IC_{20}$ of each inhibitor (I-BET151 or JAK2i as positive control) or DMSO as determined in Supplementary Figure 7. Cells were counted and cultures split at the same rate every 3 days. The concentration of inhibitor was progressively increased according to the flowchart in Figure 7a. As expected, HEL cells rapidly developed resistance to JAK2i and they were able to grow in 500 nM JAK2i after 30 days of continuous treatment (Figure 7b). The resistant cells were unable to persist at concentrations of JAK2i $>500$ nM, as previously reported.[16] In contrast, HEL cells treated constantly with I-BET151 developed only a very moderate resistance and only after 30 days of treatment with no subsequent rise (Figure 7c). Finally, we tested the cross sensitivity of the persistent cells to the two inhibitors. Naive HEL cells, DMSO chronically treated cells, JAK2i persistent cells and I-BET151 persistent cells were treated with 250 nM JAK2i (Figure 7d) or 125 nM I-BET151 (Figure 7e). As expected, the JAK2i persistent cells showed no sensitivity to JAK2i while the cells chronically treated with DMSO showed growth inhibition comparable to naive HEL cells. On the other hand, the I-BET151 persistent cells showed a slightly decreased sensitivity to 125 nM I-BET151 compared with naive HEL or cells chronically treated with DMSO. Importantly though, the JAK2i highly persistent cells remained completely sensitive, if not hypersensitive, to I-BET151. Also, the I-BET151 persistent cells showed an unchanged sensitivity to JAK2i. Both JAK2i persistent and I-BET151 persistent cells maintain their sensitivity to high concentrations of the two inhibitors (Supplementary Figure 8). These results indicate that the moderate resistance achieved by JAK2V617F-expressing HEL cells to I-BET151 occurs over a much longer time than the significant acquired resistance to JAK2i. Most importantly though, persistent cells retain sensitivity to the other inhibitor.

## DISCUSSION

In this paper, we have shown that a BET protein inhibitor decreases the proliferation and survival of HEL cells. We report that this sensitivity is due, at least in part, to I-BET151 inhibiting expression of the *LMO2* gene. *LMO2* was originally identified as a T-ALL oncogene, and has long been known as a positive regulator of erythroid differentiation.[29] More recently, sustained expression of *LMO2* was also shown to be required for growth of a subset of preB-ALL leukemias[24] although *LMO2* activation, in contrast to T-ALL, does not represent a recognized initiating event in this type of leukemia. Importantly, normal B-cell development does not require *LMO2* as shown elegantly using conditional knockout mice,[30] thus establishing *LMO2* as a potentially significant therapeutic target in preB-ALL leukemias. Moreover, elevated *LMO2* expression has also recently been shown to be required for proliferation of an acute myeloid leukemia cell line carrying an MLL translocation, as well as primary mouse bone marrow progenitors transduced with an MLL-eleven-nineteen-leukemia protein retrovirus.[25] This study again observed exquisite sensitivity of the MLL-eleven-nineteen-leukemia protein transduced progenitor cells to lowering *LMO2* levels, whereas the growth of non-leukemic multipotential progenitor cells was unaffected.

LMO2 is therefore rapidly emerging as a major therapeutic target for a range of hematopoietic malignancies, yet given its nature as a small nuclear protein, it has been difficult to think of efficient therapeutic strategies by which its inhibit its action. By showing efficient downregulation of the *LMO2* gene in both cell lines and primary patient samples through the use of specific small molecule inhibitors, this article for the first time provides a strategy for *LMO2* inhibition that should be readily transferable to clinical trial settings. Further rationale for such approaches is provided by the recurring theme that neoplastic cells appear to be more sensitive to *LMO2* inhibition than their non-malignant counterparts.

© 2014 Macmillan Publishers Limited

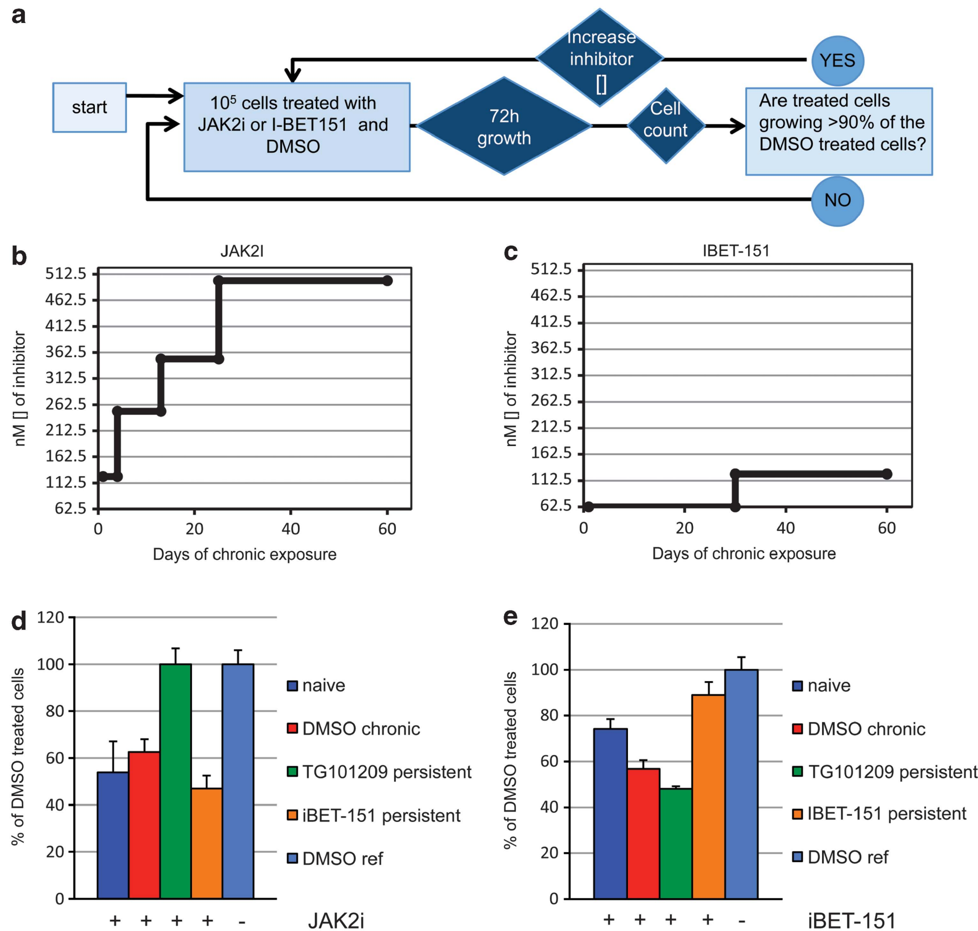



**Figure 7.** I-BET is effective against JAK2i-resistant HEL cells. (**a**) Schematic representation of the strategy used to establish JAK2i and I-BET151 persistent cells. (**b**) Concentration increase over time during the establishment of JAK2i persistent cells. (**c**) Concentration increase over time during the establishment of I-BET151 persistent cells. (**d**) HEL cells 72-h proliferation assay. The indicated cells were treated with 250 nM JAK2i and counted after 72-h proliferation. The indicated values are expressed as % of the DMSO-treated control cells. (**e**) HEL cells 72-h proliferation assay. The indicated cells were treated with 125 nM I-BET151 and counted after 72-h proliferation. The indicated values are expressed as % of the DMSO-treated control cells.

How the reduction in LMO2 levels leads to loss of cellular proliferation is not clear but presumably it involves reduced expression of downstream LMO2 target genes, a number of which have been shown to promote cell proliferation and self-renewal.[31] Furthermore, specific reduction of *LMO2* in HEL cells via shRNA targeting not only reduced cellular proliferation but it also induced apoptosis, indicating LMO2 regulates genes controlling cell death.

Although our global transcriptome analysis indicates that *LMO2* was the third most repressed gene, there were other notable significantly repressed genes highlighted in this analysis. For example, a number of anti-apoptotic genes, such as *BCL2L1*, were identified. The combined action of this class of genes, together with the effects of LMO2 depletion, presumably explains the I-BET151-induced apoptosis. Indeed, BET inhibition-induced apoptosis, via inhibition of anti-apoptotic genes, seems to be a common mechanism operating across different cell types.[19–21]

The transcriptional effects of JAK2i and I-BET151 display a high degree of correlation indicating that the BET proteins and JAK2 signaling can converge on the same transcriptional regulatory processes. It seems likely that one role of JAK2V617F is to maintain a high level of transcription at the *LMO2* locus. We believe this study extends our understanding of the transcriptional control of *LMO2* by placing the BET proteins in the same regulatory pathway as JAK2. Together, these data indicate that the BET proteins and JAK2 are involved in a common transcriptional regulation system controlling a specific subset of genes, including the sustained expression of *LMO2*.

Our findings suggest that inappropriate *LMO2* expression is a molecular feature of PV. *LMO2* mRNA is expressed in progenitor cells from PV patients and its expression is rapidly and robustly reduced when the cells are exposed to I-BET151. This correlates tightly with the ability of I-BET151 to block colony formation from these progenitor cells. In particular, I-BET151 is especially potent in preventing growth of colonies derived from homozygous *JAK2V617F* progenitor cells. In PV, expansion of a dominant homozygous *JAK2V617F* subclone appears to be fundamental to erythrocytosis and disease progression in many PV patients.[23] Thus, our findings indicate that I-BET151 treatment against PV may be highly effective as the compound specifically targets *JAK2* mutant cells, especially homozygous *JAK2V617F* cells. This finding may have important implications for the propensity of MPN to progress. Using the analogy of imatinib where *BCR-ABL*+ hematopoiesis is specifically targeted and normal hematopoiesis is restored, significant clonal response, as our results suggest for I-BET treatment, translates into a decreased progression to advanced disease.

Continuous treatment of *JAK2V617F*-positive cells with JAK inhibitors leads to resistance to the drug.[16] Interestingly, this resistance seems to be reversible and not because of the generation of new mutations in the JAK-STAT pathway.[16] Our data suggest that the induction of resistance to I-BET151 occurs at a much slower rate compared with the acquired JAKi resistance.


© 2014 Macmillan Publishers Limited

We suggest that the use of the two inhibitors in combination, perhaps as an alternating dosage strategy, may prevent the establishment of JAK2i persistent cells.

Recently, JAK2 inhibitors have been approved for the treatment of myelofibrosis as they show an improvement of the clinical symptoms of the pathology. However, the JAK2 inhibitors do not induce a strong and consistent reduction in the burden of *JAK2V617F*-positive progenitor cells.[14,32] The observed clinical effect of the JAK2 inhibitors may be mediated by nonspecific targeting of JAK1 and JAK2 in both *JAK2V617F*-negative and -positive cells. The clinically approved JAK2 inhibitors have also been found to cause a range of adverse side effects. Our findings suggest that I-BET151 represents a promising alternative treatment for JAK2V617F-driven diseases, where combinatorial use of the both JAK and BET inhibitors may enhance our ability to target the neoplastic cells, while simultaneously reducing the risk of adverse side effects.

## CONFLICT OF INTEREST

T Kouzarides is a founder of Abcam Ltd. RK Prinja and I Rioja are employees and shareholders of GlaxoSmithKline. The remaining authors declare no conflict of interest.

## ACKNOWLEDGEMENTS

We thank members of the Kouzarides laboratory for the thoughtful discussion. The Kouzarides laboratory was supported by Cancer Research UK, Leukaemia and Lymphoma Research, GlaxoSmithKline and BBSRC. Work in the Green laboratory is supported by Leukemia and Lymphoma Research, Cancer Research UK, the Kay Kendall Leukaemia Fund, the NIHR Cambridge Biomedical Research Centre, the Cambridge Experimental Cancer Medicine Centre, and the Leukemia and Lymphoma Society of America. The Gottgens laboratory was supported by Cancer Research UK and Leukaemia and Lymphoma Research, UK. The Huntly laboratory was supported by Cancer Research UK and Leukaemia and Lymphoma Research, UK. MA Dawson is a Wellcome Trust Beit Fellow and E Cannizzaro and M Wiese are funded by this Fellowship.

## REFERENCES

1 Adamson JW, Fialkow PJ, Murphy S, Prchal JF, Steinmann L. Polycythemia vera: stem-cell and probable clonal origin of the disease. *N Engl J Med* 1976; **295**: 913–916.
2 Fialkow PJ, Faguet GB, Jacobson RJ, Vaidya K, Murphy S. Evidence that essential thrombocythemia is a clonal disorder with origin in a multipotent stem cell. *Blood* 1981; **58**: 916–919.
3 Campbell PJ, Baxter EJ, Beer PA, Scott LM, Bench AJ, Huntly BJ *et al.* Mutation of JAK2 in the myeloproliferative disorders: timing, clonality studies, cytogenetic associations, and role in leukemic transformation. *Blood* 2006; **108**: 3548–3555.
4 Baxter EJ, Scott LM, Campbell PJ, East C, Fourouclas N, Swanton S *et al.* Acquired mutation of the tyrosine kinase JAK2 in human myeloproliferative disorders. *Lancet* 2005; **365**: 1054–1061.
5 Kralovics R, Passamonti F, Buser AS, Teo SS, Tiedt R, Passweg JR *et al.* A gain-of-function mutation of JAK2 in myeloproliferative disorders. *N Engl J Med* 2005; **352**: 1779–1790.
6 Levine RL, Wadleigh M, Cools J, Ebert BL, Wernig G, Huntly BJ *et al.* Activating mutation in the tyrosine kinase JAK2 in polycythemia vera, essential thrombocythemia, and myeloid metaplasia with myelofibrosis. *Cancer Cell* 2005; **7**: 387–397.
7 Zhao R, Xing S, Li Z, Fu X, Li Q, Krantz SB *et al.* Identification of an acquired JAK2 mutation in polycythemia vera. *J Biol Chem* 2005; **280**: 22788–22792.
8 James C, Ugo V, Le Couedic JP, Staerk J, Delhommeau F, Lacout C *et al.* A unique clonal JAK2 mutation leading to constitutive signalling causes polycythaemia vera. *Nature* 2005; **434**: 1144–1148.
9 Mullally A, Lane SW, Ball B, Megerdichian C, Okabe R, Al-Shahrour F *et al.* Physiological Jak2V617F expression causes a lethal myeloproliferative neoplasm with differential effects on hematopoietic stem and progenitor cells. *Cancer Cell* 2010; **17**: 584–596.
10 Li J, Spensberger D, Ahn JS, Anand S, Beer PA, Ghevaert C *et al.* JAK2 V617F impairs hematopoietic stem cell function in a conditional knock-in mouse model of JAK2 V617F-positive essential thrombocythemia. *Blood* 2010; **116**: 1528–1538.
11 Dawson MA, Bannister AJ, Gottgens B, Foster SD, Bartke T, Green AR *et al.* JAK2 phosphorylates histone H3Y41 and excludes HP1alpha from chromatin. *Nature* 2009; **461**: 819–822.
12 Hernandez-Boluda JC, Alvarez-Larran A, Gomez M, Angona A, Amat P, Bellosillo B *et al.* Clinical evaluation of the European LeukaemiaNet criteria for clinicohaematological response and resistance/intolerance to hydroxycarbamide in essential thrombocythaemia. *Br J Haematol* 2011; **152**: 81–88.
13 Harrison C, Kiladjian JJ, Al-Ali HK, Gisslinger H, Waltzman R, Stalbovskaya V *et al.* JAK inhibition with ruxolitinib versus best available therapy for myelofibrosis. *N Engl J Med* 2012; **366**: 787–798.
14 Verstovsek S, Mesa RA, Gotlib J, Levy RS, Gupta V, DiPersio JF *et al.* A double-blind, placebo-controlled trial of ruxolitinib for myelofibrosis. *N Engl J Med* 2012; **366**: 799–807.
15 Verstovsek S, Kantarjian H, Mesa RA, Pardanani AD, Cortes-Franco J, Thomas DA *et al.* Safety and efficacy of INCB018424, a JAK1 and JAK2 inhibitor, in myelofibrosis. *N Engl J Med* 2010; **363**: 1117–1127.
16 Koppikar P, Bhagwat N, Kilpivaara O, Manshouri T, Adli M, Hricik T *et al.* Heterodimeric JAK-STAT activation as a mechanism of persistence to JAK2 inhibitor therapy. *Nature* 2012; **489**: 155–159.
17 Barbieri I, Cannizzaro E, Dawson MA. Bromodomains as therapeutic targets in cancer. *Brief Funct Genomics* 2013; **12**: 219–230.
18 Yang Z, Yik JH, Chen R, He N, Jang MK, Ozato K *et al.* Recruitment of P-TEFb for stimulation of transcriptional elongation by the bromodomain protein Brd4. *Mol Cell* 2005; **19**: 535–545.
19 Dawson MA, Prinjha RK, Dittmann A, Giotopoulos G, Bantscheff M, Chan WI *et al.* Inhibition of BET recruitment to chromatin as an effective treatment for MLL-fusion leukaemia. *Nature* 2011; **478**: 529–533.
20 Zuber J, Shi J, Wang E, Rappaport AR, Herrmann H, Sison EA *et al.* RNAi screen identifies Brd4 as a therapeutic target in acute myeloid leukaemia. *Nature* 2011; **478**: 524–528.
21 Delmore JE, Issa GC, Lemieux ME, Rahl PB, Shi J, Jacobs HM *et al.* BET bromodomain inhibition as a therapeutic strategy to target c-Myc. *Cell* 2011; **146**: 904–917.
22 Mertz JA, Conery AR, Bryant BM, Sandy P, Balasubramanian S, Mele DA *et al.* Targeting MYC dependence in cancer by inhibiting BET bromodomains. *Proc Natl Acad Sci USA* 2011; **108**: 16669–16674.
23 Godfrey AL, Chen E, Pagano F, Ortmann CA, Silber Y, Bellosillo B *et al.* JAK2V617F homozygosity arises commonly and recurrently in PV and ET, but PV is characterized by expansion of a dominant homozygous subclone. *Blood* 2012; **120**: 2704–2707.
24 Hirose K, Inukai T, Kikuchi J, Furukawa Y, Ikawa T, Kawamoto H *et al.* Aberrant induction of LMO2 by the E2A-HLF chimeric transcription factor and its implication in leukemogenesis of B-precursor ALL with t(17;19). *Blood* 2010; **116**: 962–970.
25 Calero-Nieto FJ, Joshi A, Bonadies N, Kinston S, Chan WI, Gudgin E *et al.* HOX-mediated LMO2 expression in embryonic mesoderm is recapitulated in acute leukaemias. *Oncogene* 2013; e-pub ahead of print 27 May 2013; doi:10.1038/onc.2013.175.
26 Oram SH, Thoms JA, Pridans C, Janes ME, Kinston SJ, Anand S *et al.* A previously unrecognized promoter of LMO2 forms part of a transcriptional regulatory circuit mediating LMO2 expression in a subset of T-acute lymphoblastic leukaemia patients. *Oncogene* 2010; **29**: 5796–5808.
27 Ma AC, Ward AC, Liang R, Leung AY. The role of jak2a in zebrafish hematopoiesis. *Blood* 2007; **110**: 1824–1830.
28 Pardanani A, Gotlib JR, Jamieson C, Cortes JE, Talpaz M, Stone RM *et al.* Safety and efficacy of TG101348, a selective JAK2 inhibitor, in myelofibrosis. *J Clin Oncol* 2011; **29**: 789–796.
29 Nam CH, Rabbitts TH. The role of LMO2 in development and in T cell leukemia after chromosomal translocation or retroviral insertion. *Mol Ther* 2006; **13**: 15–25.
30 McCormack MP, Forster A, Drynan L, Pannell R, Rabbitts TH. The LMO2 T-cell oncogene is activated via chromosomal translocations or retroviral insertion during gene therapy but has no mandatory role in normal T-cell development. *Mol Cell Biol* 2003; **23**: 9003–9013.
31 McCormack MP, Young LF, Vasudevan S, de Graaf CA, Codrington R, Rabbitts TH *et al.* The Lmo2 oncogene initiates leukemia in mice by inducing thymocyte self-renewal. *Science* 2010; **327**: 879–883.
32 Dawson MA, Curry JE, Barber K, Beer PA, Graham B, Lyons JF *et al.* AT9283, a potent inhibitor of the Aurora kinases and Jak2, has therapeutic potential in myeloproliferative disorders. *Br J Haematol* 2010; **150**: 46–57.

Supplementary Information accompanies this paper on the Leukemia website (http://www.nature.com/leu)

© 2014 Macmillan Publishers Limited