BET protein inhibition shows efficacy against *JAK2V617F* driven neoplasms

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Abstract

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 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 down regulated 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 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.

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 (ET), and primary myelofibrosis (PMF). At the molecular level these pathologies are very often associated with the presence of a gain of function point mutation in the Janus kinase 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, 9, 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 HP1α to the promoter of *LMO2* (ref. 11).

Current treatment of MPNs commonly involves phlebotomy and the use of antiproliferative agents such as hydroxycarbamide. Although hydroxy-carbamide 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 plays 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 recognising acetylated lysines in histones (17), and comprise 4 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).

We, and others, 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 MLL-FP leukemia (19, 20), in AML cell lines lacking MLL rearrangements as well as in multiple myeloma and in Burkitt's lymphoma (21, 22).

In this manuscript we have investigated the efficacy of a potent BET inhibitor (GSK151A - hereafter referred to as I-BET151) in JAK2 driven neoplastic cells, employing 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 down-regulation of the *LMO2* gene. *LMO2* is also down-regulated by JAK2 inhibitors and we found that I-BET151 and a JAK2 inhibitor (TG101209 - hereafter referred to as JAK2i) cooperate to down-regulate 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

Human erythroleukemia (HEL) and human myelogenous leukemia (K562) cells were maintained in RPMI 1640 medium, 10% FBS, 1% penicillin/streptomycin/glutamine (GIBCO, Invitrogen), incubated at 37°C in 5% CO_2 and passaged 1:6 every 2-3 days.

Compounds

TG101209 JAK2 inhibitor was from TargeGen Inc. and GSK1210151A (I-BET151) was provided by GlaxoSmithKline.

Immunoblotting

Whole cell lysates, prepared in 2X Laemmli sample buffer, were separated by SDS-PAGE and transferred to nitrocellulose (Millipore). Signal was detected using ECL® (GE Healthcare) and developed on X-ray film (Fuji). A list of antibodies is in the Supplementary information.

Cell Growth Inhibition (GI_{50}) Assay

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

Proliferation assays
$1x10^5$ 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).

Clonogenic Assays in Methylcellulose
Clonogenic potential of HEL and K562 cells was assessed via colony growth in the presence of DMSO or 1μM I-BET151. Cells were plated in methylcellulose supplemented with human cytokines (Methocult H4435, Stemcell Technologies) in duplicate at $1x10^2$ and $1x10^3$ per plate. Colonies were analysed 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 PMNCs from PV5, lineage negative cells enriched for hematopoietic progenitors were obtained by magnetic separation as per manufacturer's instructions (Human Progenitor Enrichment Kit, Easysep, Stem cell Technologies). Cells were incubated in DMEM, 15% FBS, 1Uml EPO and either DMSO, 200nM or 1uM 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.) supplemented with 7-AAD (BD bioscience), according to the manufacturer's instructions. Flow cytometry experiments were performed on a CyAn ADP flow cytometer (Dako), and data were analysed with FlowJo software (Tree Star, Inc.).

Chromatin Immunoprecipitation (ChIP) Assay
HEL cells were treated with 1μM I-BET151 or DMSO for 4 hours. ChIP was carried out as previously described (11).

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

Lentiviral Production and Transduction

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
GFP 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 GFP-positive cell-fraction over a 13-day time-course.

Gene Expression and Bioinformatic Analysis
HEL cell RNA (treated with 1μM I-BET151 or TG101209) was extracted after 4 hours and processed as described before after hybridisation to Illumina Human HT12 v4 BeadChips. Gene expression data were analysed 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 1μM (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 300nM in this assay. In contrast, K562 cells were less effected by I-BET, exhibiting an IC_{50} of 6μM; 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 PARP and active CASPASE 3, are both detectable after 24 hours exposure to I-BET151 and expression of both is significantly up-regulated after 48 hours exposure. A high level of I-BET151 induced apoptosis was also detected via FACS analysis, where 30% of cells were positive for ANNEXIN V staining after a 48h treatment with 1μM I-BET151 (Figure 1D) compared to just 3% positive cells in DMSO treated cells. These data suggest that I-BET151 alters transcriptional programmes in HEL cells that ultimately regulate apoptosis.

To identify the precise transcriptional pathways controlled by I-BET151, global gene-expression analyses were performed in HEL cells after treatment with 1μM I-BET151 or DMSO for 4 hours. 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 cut-off threshold of 2-fold, 125 genes were down-regulated, and 154 genes up-regulated by I-BET151 treatment (Figure 2A and Supplementary Figure 1) Strikingly, the third most down-

regulated 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 (Figure 2C and D). Furthermore, changes in expression of three of the most down-regulated 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 3 transcriptional start sites (26) (TSSs; sites 1, 2 and 3 in Figure 3A). Using chromatin immunoprecipitation (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 dramatically reduced after treatment with I-BET151. ChIP of the elongation competent form of RNA polymerase II, phosphorylated at serine 2 within its CTD 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 (PAFc) to promote transcription. ChIP with antibodies against LEO1, an integral PAFc 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 down-regulated 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 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 out-grown 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 to 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 (Figure 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; Dawson et al, 2009). *LMO2* appeared within the first twenty most down-regulated 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 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 datasets comparing the most differentially expressed genes. We found that 38% (48 out of 125) of I-BET151 down-regulated genes are also down-regulated by JAK2i (Figure 5C). Similarly, 31% (48 out of 154) of I-BET151 up-regulated genes are also up-regulated 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 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 analysed a wide range of concentrations of both inhibitors, both separately and combined, in 72h 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. Since 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 due to 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 (200nM or 1μM) for 4 hours 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 BFU-E colonies in order to identify those that were wildtype (WT) for 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 (1μM) the treatment significantly reduced total colony number (only 47 BFU-Es grew) suggesting cytotoxicity effects at this top concentration. In contrast however, 200nM I-BET151 treatment reduced colonies from *JAK2V617F* containing cells, especially homozygous cells, whilst 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 BFU-E colony-forming assay in three additional patients (Figure 6C). We performed these assays at 200nM I-BET151 since this concentration showed negligible effect on total BFU-E colony numbers (Figure 6B). The data indicated that in all the primary cultures 200nM 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 200nM 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 programmes and that they may function in the same or

highly over-lapping pathways. Therefore, we decided to test the 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 three days. The concentration of inhibitor was progressively increased according to the flow chart in Figure 7A. As expected, HEL cells rapidly developed resistance to JAK2i and they were able to grow in 500nM JAK2i after 30 days of continuous treatment (Figure 7B). The resistant cells were unable to persist at concentrations of JAK2i greater than 500nM, 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. Naïve HEL cells, DMSO chronically treated cells, JAK2i persistent cells and I-BET151 persistent cells were treated with 250nM JAK2i (Figure 7D) or 125nM I-BET151 (Figure 7E). As expected, the JAK2i persistent cells showed no sensitivity to JAK2i whilst 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 125nM I-BET151 compared to naïve 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 human erythroleukemic 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) even though *LMO2* activation, in contrast to T-ALL, does not represent a recognised initiating event in this type of leukemia. Importantly, normal B-cell development does not require *LMO2* as shown elegantly using conditional knock-out 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 AML cell line carrying an MLL translocation, as well as primary mouse bone marrow progenitors transduced with an MLL-ENL retrovirus (25). This study again observed exquisite sensitivity of the MLL-ENL 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 down-regulation of the *LMO2* gene in both cell lines and primary patient samples through the use of specific small molecule inhibitors, the current manuscript 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.

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 the present 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 due to 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. 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 non-specific 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.

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Conflicts of interest

T. Kouzarides is a founder of Abcam Ltd. R. Prinja and I. Rioja are employees and shareholders of GlaxoSmithKline.

Figure Legends

Figure 1
I-BET151 inhibits growth and induces apoptosis in erythroleukemia cells. (**A**) Human erythroleukemia (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 1μM I-BET151. (**C**) Immunoblotting demonstrating an increase in the cleaved PARP and active CASPASE 3 after 24h and 48h of I-BET151 treatment. (**D**) Apoptosis was assessed by FACS analysis after 24h and 48h incubation with DMSO or 1μM I-BET151 by monitoring ANNEXIN V.

Figure 2
I-BET151 potently inhibits the expression of an important hematopoietic regulator, *LMO2*. Messenger RNA was isolated from HEL cells treated for 4h with 1μM I-BET151 or DMSO. Four biological replicates were analysed 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 down-regulated genes are illustrated; shading highlights the position of *LMO2* gene. (**C**) *LMO2* gene expression after treatment with 1μM I-BET151 was validated with qRT-PCR. Data is normalized to *B2M* expression; the expression level of *LMO2* in DMSO was assigned a value of 1 and the error bars reflect standard deviation. (**D**) Immunoblotting demonstrating reduced levels of LMO2 protein after treatment with 1μM I-BET151.

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 chromatin immunoprecipitation 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 standard deviation.

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. 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 PARP cleavage and (**D**) the activation of CASPASE 3 in cells transduced with shLMO2-1 and shLMO2-2.

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 up-regulated by both inhibitors are represented as red hollow dots, while genes significantly down-regulated 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 up-regulated by JAK2i and I-BET151. (**D**) HEL cell 72h proliferation assay using JAK2i and I-BET151 at the indicated concentrations individually or in combination. (**E**) *LMO2* mRNA levels were analysed by RT-qPCR in HEL cells after treatment with DMSO, 125nM I-BET151, 500nM JAK2i or a combination of the two inhibitors.

Figure 6
I-BET151 shows efficacy in samples isolated from patients with Polycythemia Vera (PV). (**A**) Hematopoietic progenitor cells were isolated from whole blood obtained from a PV patient and treated with DMSO, 200nM I-BET151 or 1μM I-BET151. Expression of *LMO2* was examined 4 hours 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 standard deviation 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. BFU-E 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 $1.6X10^6$ 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 BFU-E assay was repeated in a further 3 PV patients in the presence of DMSO or 200nM I-BET151 and the results are presented as described for panel (C).

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 72h proliferation assay. The indicated cells were treated with 250nM JAK2i and counted after 72h proliferation. The indicated values are expressed as % of the DMSO treated control cells. (**E**) HEL cells 72h proliferation assay. The indicated cells were treated with 125nM I-BET151 and counted after 72h proliferation. The indicated values are expressed as % of the DMSO treated control cells.

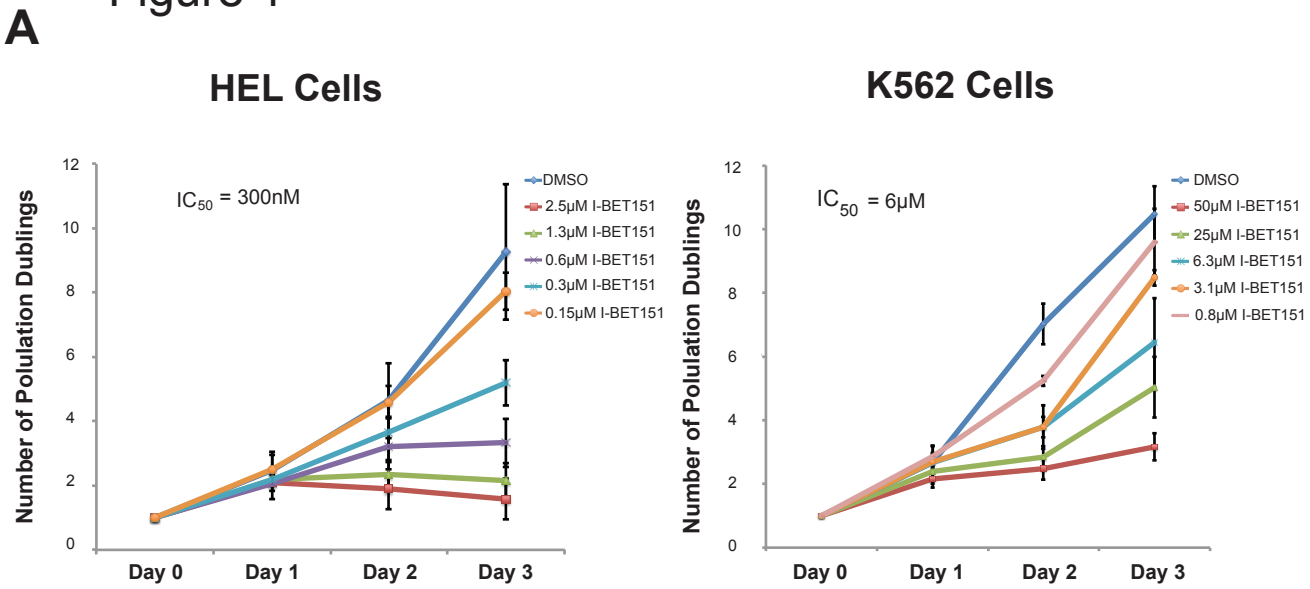
References

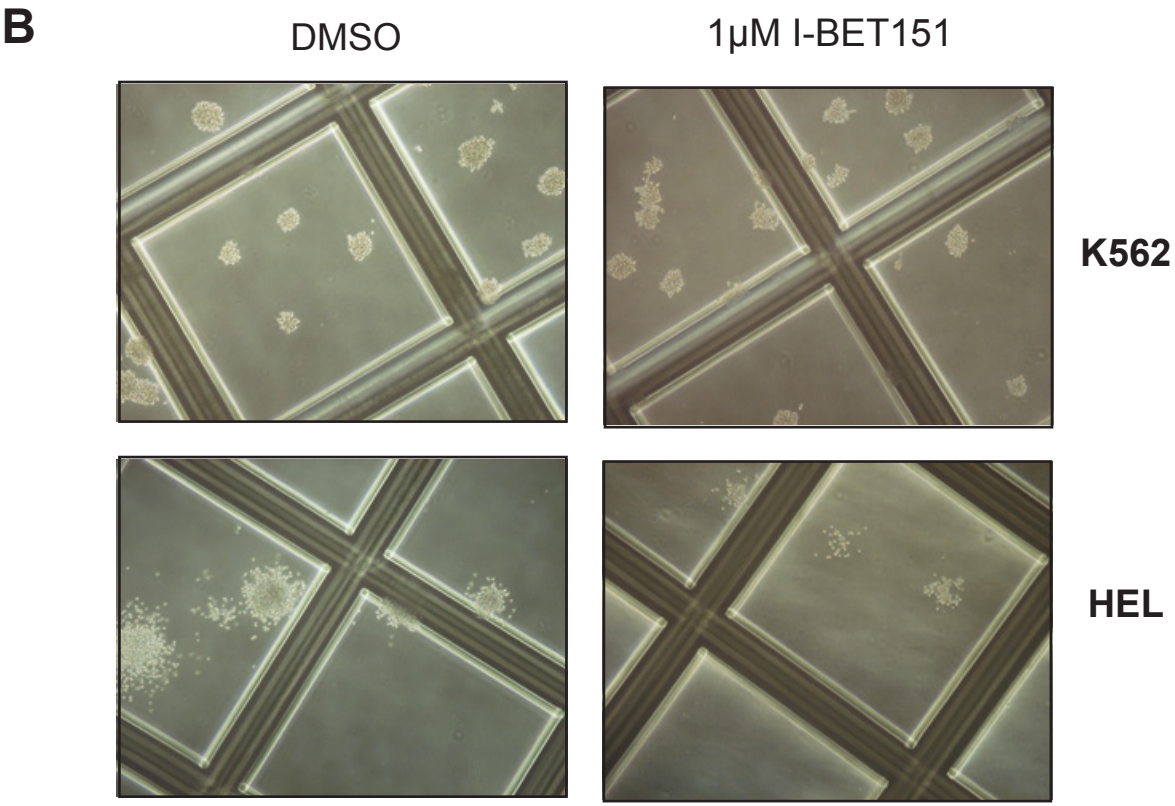
1. Adamson JW, Fialkow PJ, Murphy S, Prchal JF, Steinmann L. Polycythemia vera: stem-cell and probable clonal origin of the disease. N Engl J Med. 1976;295(17):913-6.
2. Fialkow PJ, Faguet GB, Jacobson RJ, Vaidya K, Murphy S. Evidence that essential thrombocythemia is a clonal disorder with origin in a multipotent stem cell. Blood. 1981;58(5):916-9.
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(10):3548-55.
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(9464):1054-61.
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(17):1779-90.
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(4):387-97.
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(24):22788-92.
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(7037):1144-8.
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(6):584-96.
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(9):1528-38.
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(7265):819-22.
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(1):81-8.
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(9):787-98.
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(9):799-807.

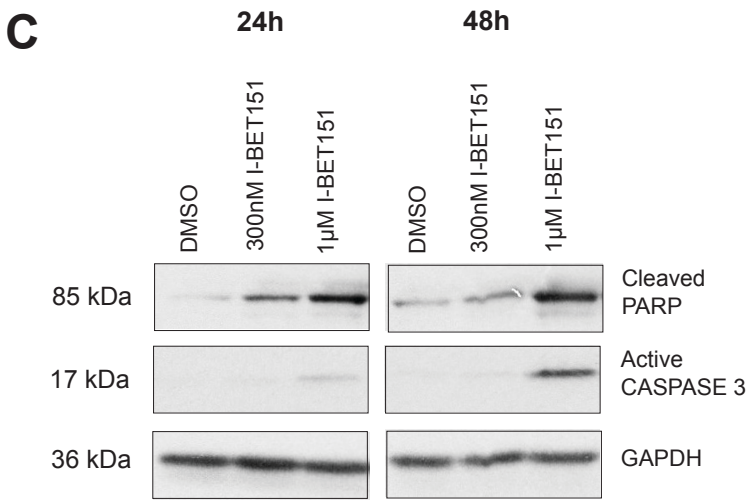
15. Verstovsek S, Kantarjian H, Mesa RA, Pardanani AD, Cortes-Franco J, Thomas DA, et al. Safety and efficacy of INCB018424, a JAK1 and JAK2 inhibitor, in myelofibrosis. N Engl J Med. 2010;363(12):1117-27.
16. Koppikar P, Bhagwat N, Kilpivaara O, Manshouri T, Adli M, Hricik T, et al. Heterodimeric JAK-STAT activation as a mechanism of persistence to JAK2 inhibitor therapy. Nature. 2012;489(7414):155-9.
17. Barbieri I, Cannizzaro E, Dawson MA. Bromodomains as therapeutic targets in cancer. Brief Funct Genomics. 2013.
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(4):535-45.
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(7370):529-33.
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(7370):524-8.
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(6):904-17.
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 U S A. 2011;108(40):16669-74.
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(13):2704-7.
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(6):962-70.
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.
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(43):5796-808.
27. Ma AC, Ward AC, Liang R, Leung AY. The role of jak2a in zebrafish hematopoiesis. Blood. 2007;110(6):1824-30.
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(7):789-96.
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(1):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(24):9003-13.

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(5967):879-83.
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(1):46-57.

Figure 1


A
HEL Cells
K562 Cells
Number of Polulation Dublings
IC50 = 300nM
IC50 = 6μM
DMSO
2.5μM I-BET151
1.3μM I-BET151
0.6μM I-BET151
0.3μM I-BET151
0.15μM I-BET151
50μM I-BET151
25μM I-BET151
6.3μM I-BET151
3.1μM I-BET151
0.8μM I-BET151
Day 0
Day 1
Day 2
Day 3


B
DMSO
1μM I-BET151
K562
HEL


C
24h
48h
DMSO
300nM I-BET151
1μM I-BET151
85 kDa
17 kDa
36 kDa
Cleaved PARP
Active CASPASE 3
GAPDH

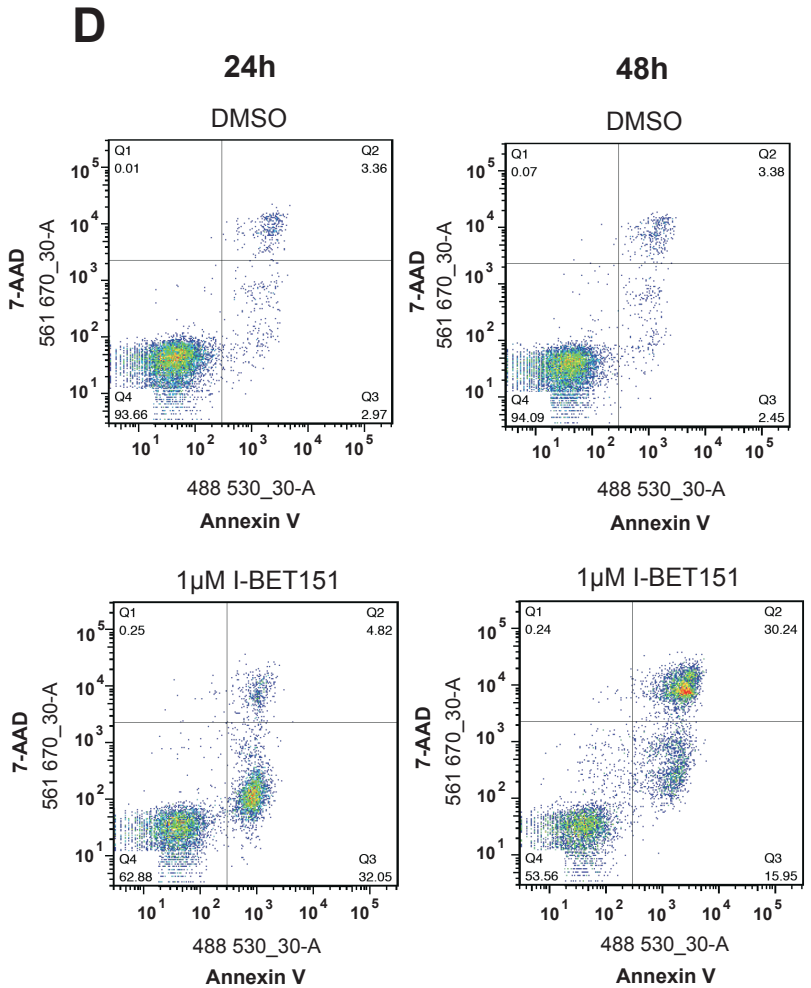
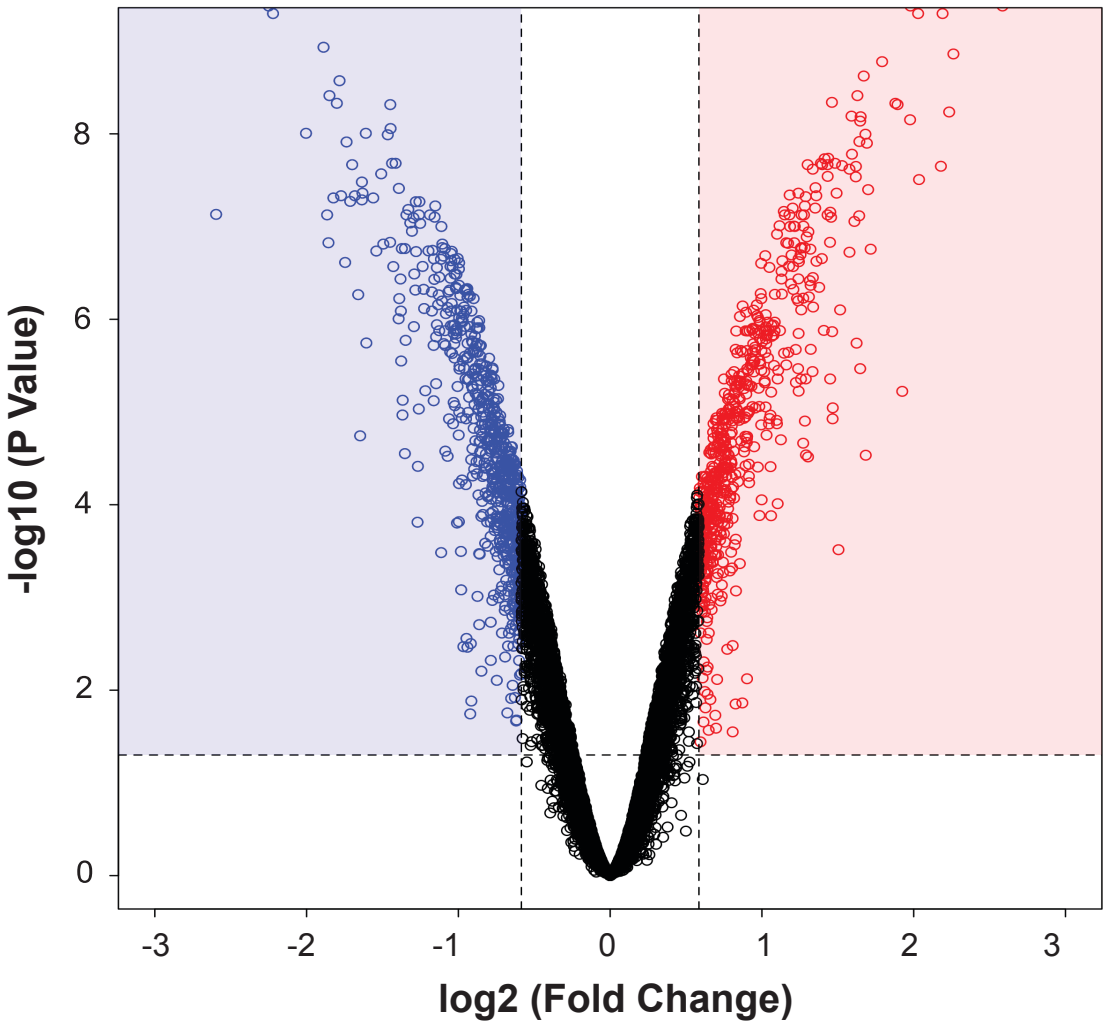

D
24h
48h
DMSO
1μM I-BET151
7-AAD
561 670_30-A
488 530_30-A
Annexin V
Q1 0.01
Q2 3.36
Q4 93.66
Q3 2.97
Q1 0.07
Q2 3.38
Q4 94.09
Q3 2.45
Q1 0.25
Q2 4.82
Q4 62.88
Q3 32.05
Q1 0.24
Q2 30.24
Q4 53.56
Q3 15.95

Figure 2

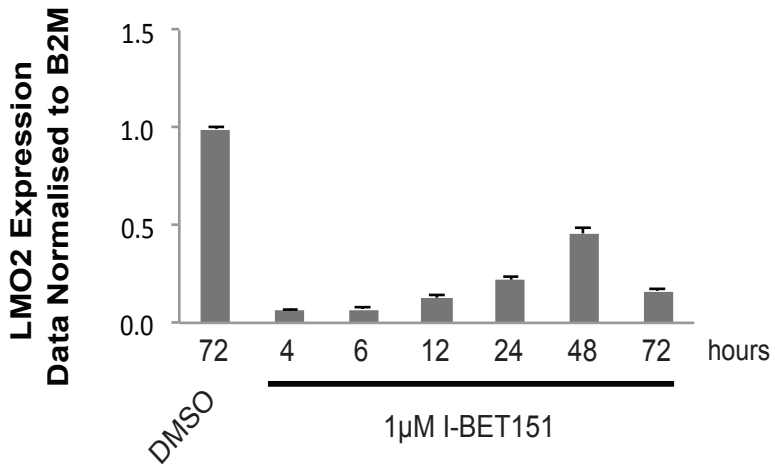
A



B

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

C



D

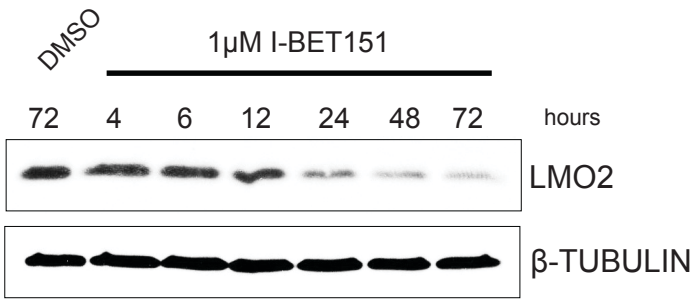


Figure 3

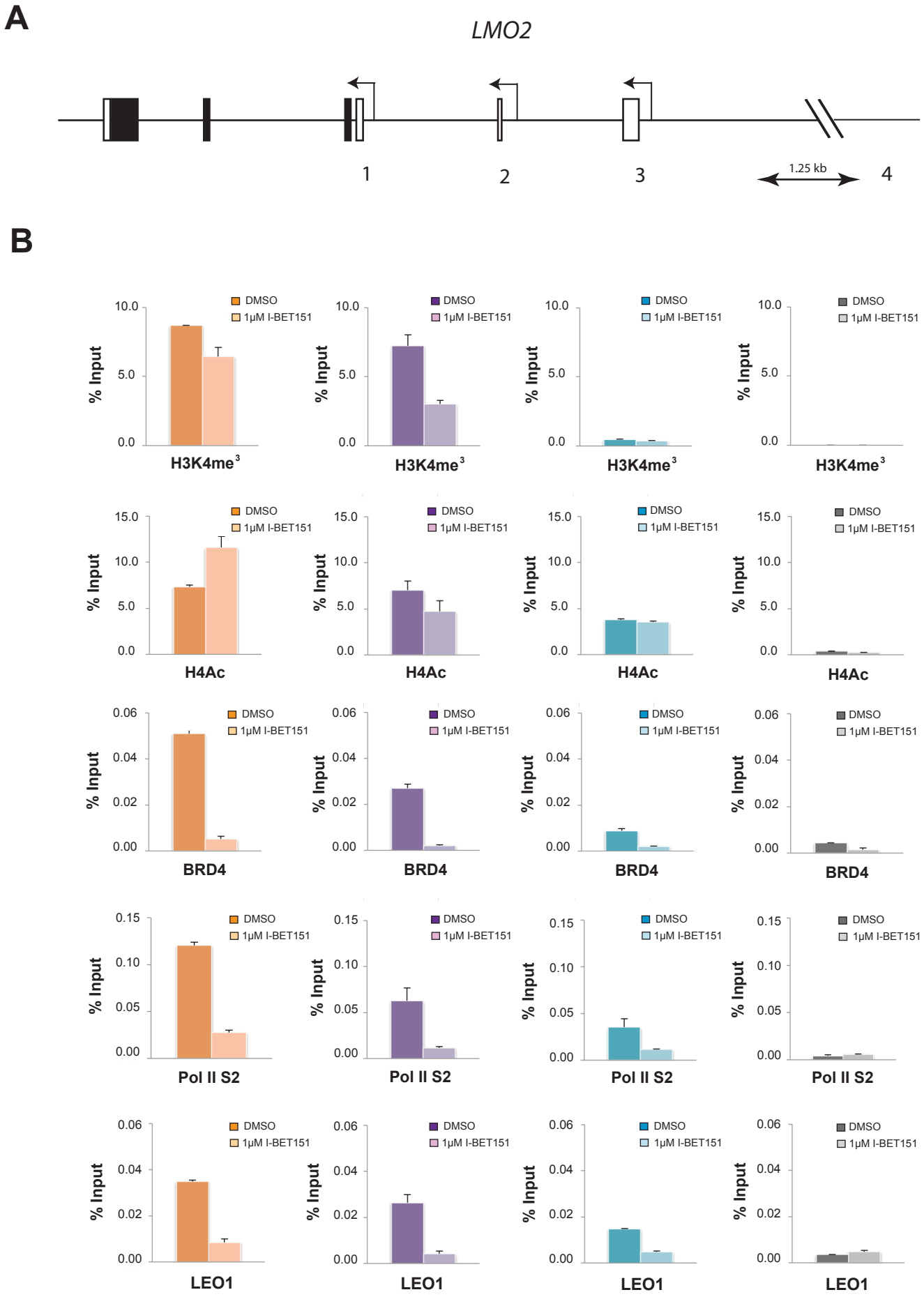

A
LMO2
1
2
3
1.25 kb
4
B
DMSO
1μM I-BET151
% Input
H3K4me3
H4Ac
BRD4
Pol II S2
LEO1
1
2
3
4

Figure 4

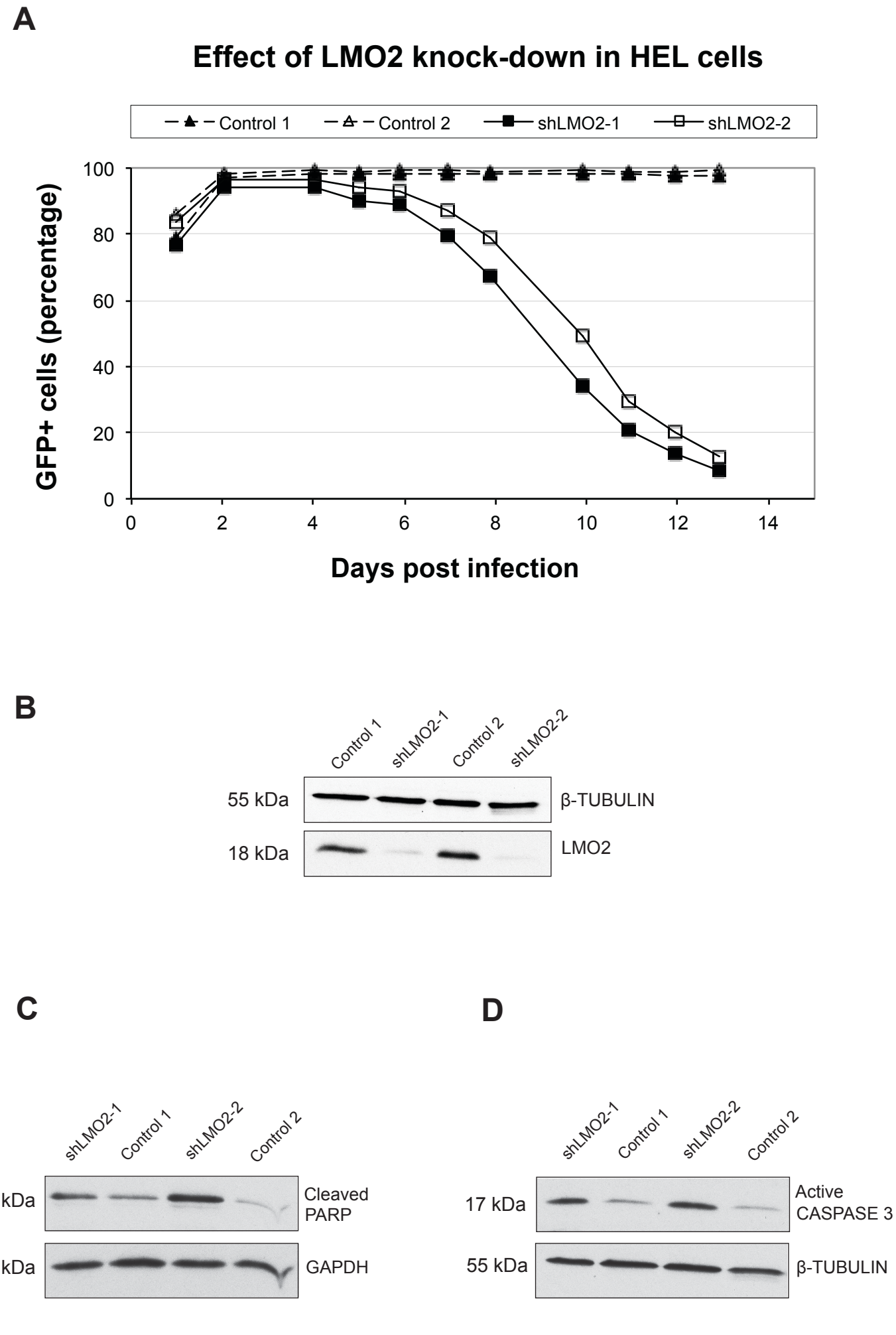

A
Effect of LMO2 knock-down in HEL cells
Control 1
Control 2
shLMO2-1
shLMO2-2
GFP+ cells (percentage)
100
80
60
40
20
0
0
2
4
6
8
10
12
14
Days post infection
B
Control 1
shLMO2-1
Control 2
shLMO2-2
55 kDa
β-TUBULIN
18 kDa
LMO2
C
shLMO2-1
Control 1
shLMO2-2
Control 2
85 kDa
Cleaved PARP
36 kDa
GAPDH
D
shLMO2-1
Control 1
shLMO2-2
Control 2
17 kDa
Active CASPASE 3
55 kDa
β-TUBULIN

Figure 5

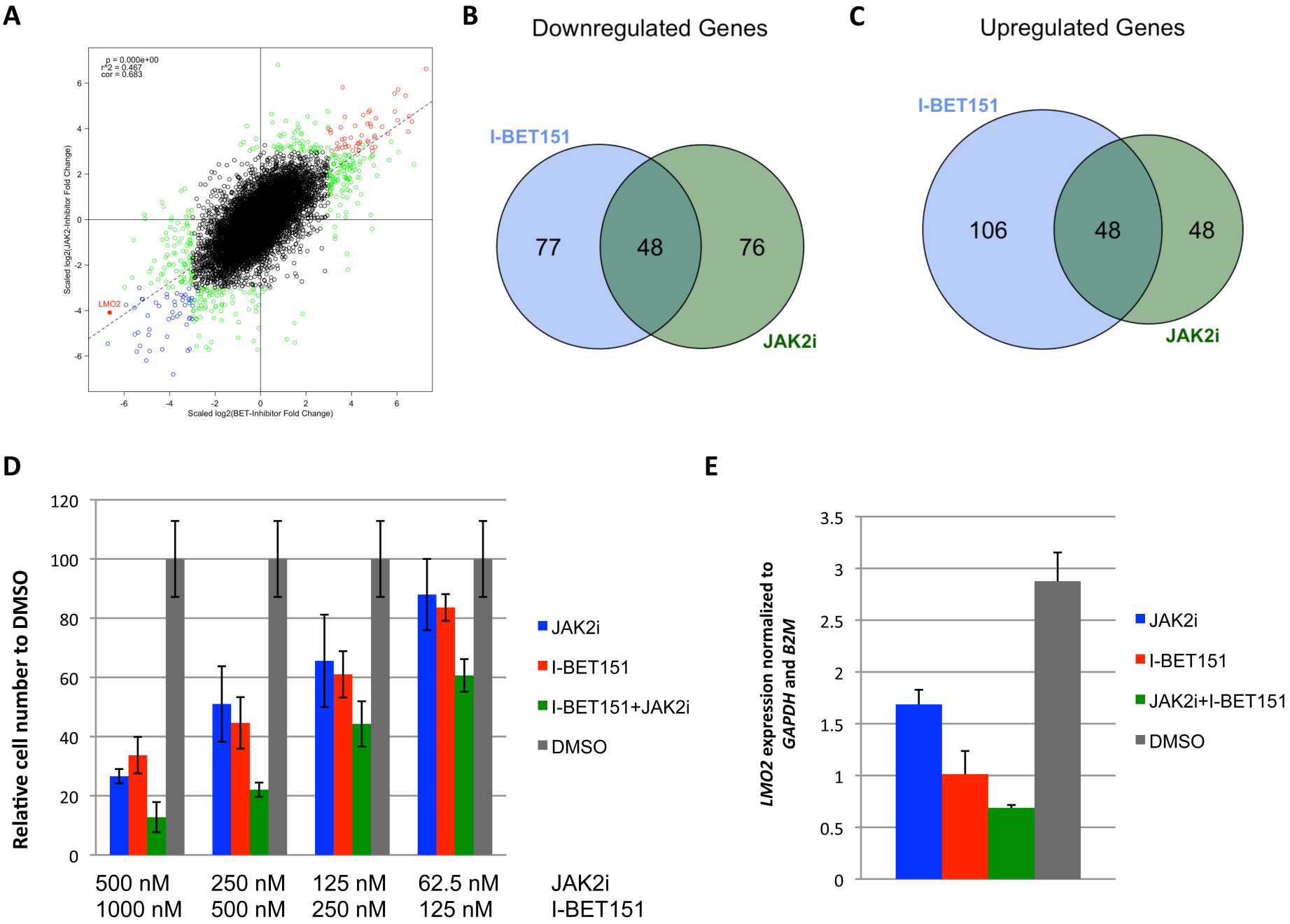
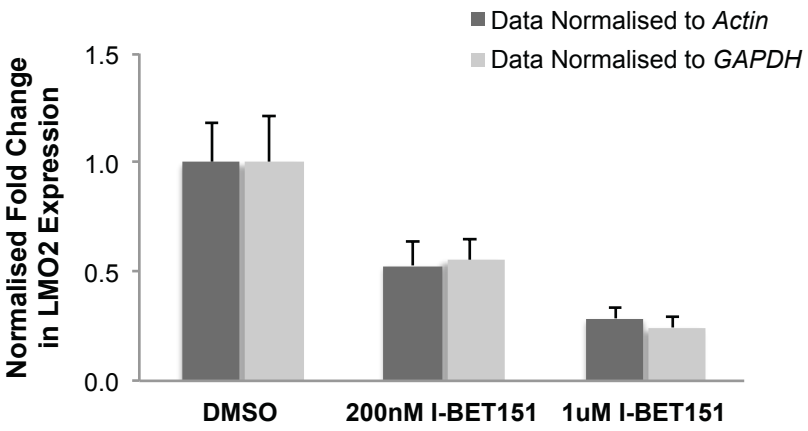
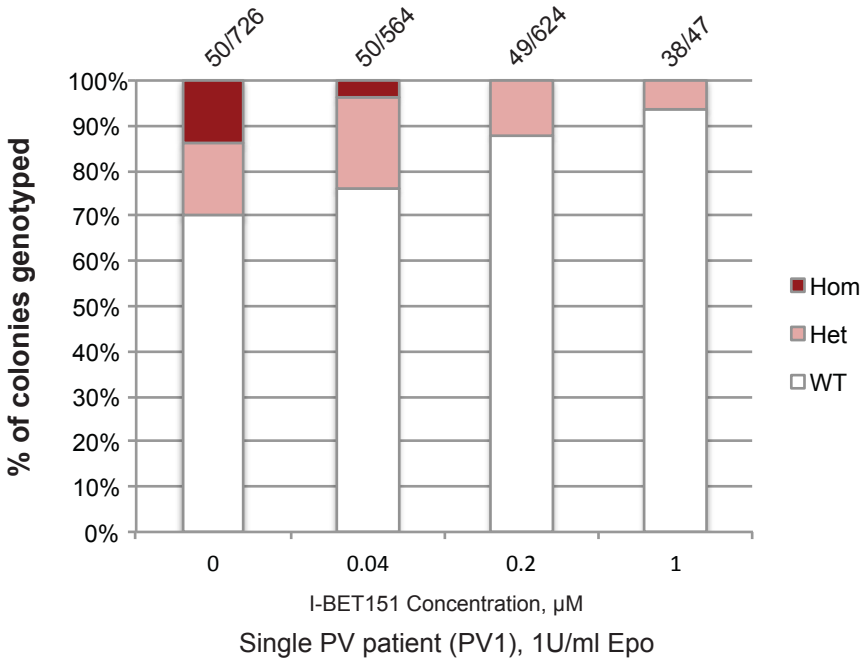

A
p = 0.000e+00
r^2 = 0.467
cor = 0.683
Scaled log2(JAK2-Inhibitor Fold Change)
Scaled log2(BET-Inhibitor Fold Change)
LMO2
B
Downregulated Genes
I-BET151
77
48
76
JAK2i
C
Upregulated Genes
I-BET151
106
48
48
JAK2i
D
Relative cell number to DMSO
120
100
80
60
40
20
0
500 nM
1000 nM
250 nM
500 nM
125 nM
250 nM
62.5 nM
125 nM
JAK2i
I-BET151
JAK2i
I-BET151
I-BET151+JAK2i
DMSO
E
LMO2 expression normalized to GAPDH and B2M
3.5
3
2.5
2
1.5
1
0.5
0
JAK2i
I-BET151
JAK2i+I-BET151
DMSO

Figure 6

A


Data Normalised to Actin
Data Normalised to GAPDH
Normalised Fold Change in LMO2 Expression
1.5
1.0
0.5
0.0
DMSO
200nM I-BET151
1uM I-BET151

B


50/726
50/564
49/624
38/47
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
% of colonies genotyped
Hom
Het
WT
0
0.04
0.2
1
I-BET151 Concentration, μM
Single PV patient (PV1), 1U/ml Epo

C

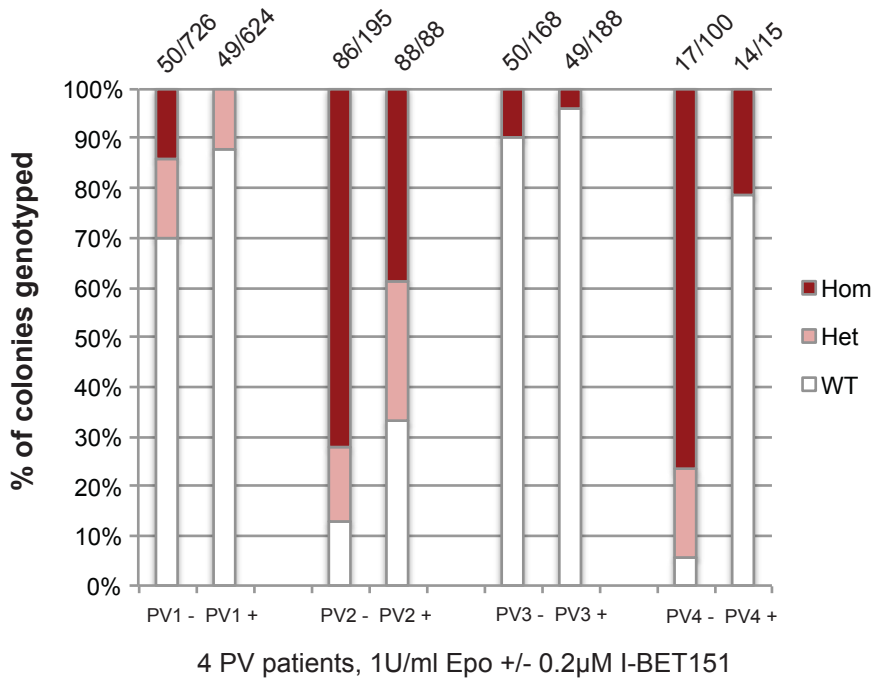
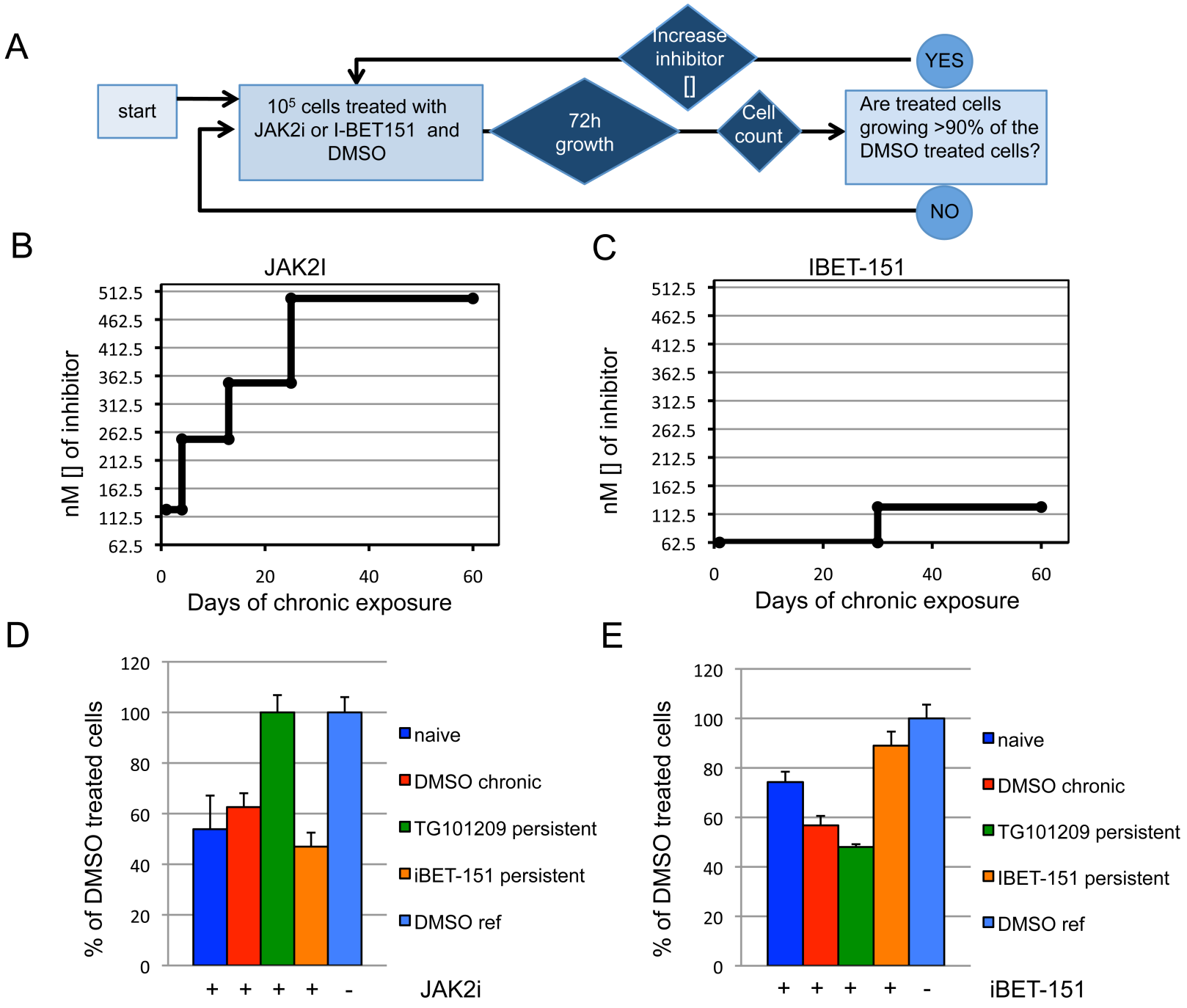

50/726
49/624
86/195
88/88
50/168
49/188
17/100
14/15
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
% of colonies genotyped
Hom
Het
WT
PV1 - PV1 +
PV2 - PV2 +
PV3 - PV3 +
PV4 - PV4 +
4 PV patients, 1U/ml Epo +/- 0.2μM I-BET151

Figure 7


A
start
10^5 cells treated with JAK2i or I-BET151 and DMSO
72h growth
Cell count
Are treated cells growing >90% of the DMSO treated cells?
YES
Increase inhibitor []
NO
B
JAK2I
nM [] of inhibitor
512.5
462.5
412.5
362.5
312.5
262.5
212.5
162.5
112.5
62.5
0
20
40
60
Days of chronic exposure
C
IBET-151
nM [] of inhibitor
Days of chronic exposure
D
% of DMSO treated cells
120
100
80
60
40
20
0
naive
DMSO chronic
TG101209 persistent
iBET-151 persistent
DMSO ref
+ + + + -
JAK2i
E
% of DMSO treated cells
iBET-151

Supplementary Figure S1

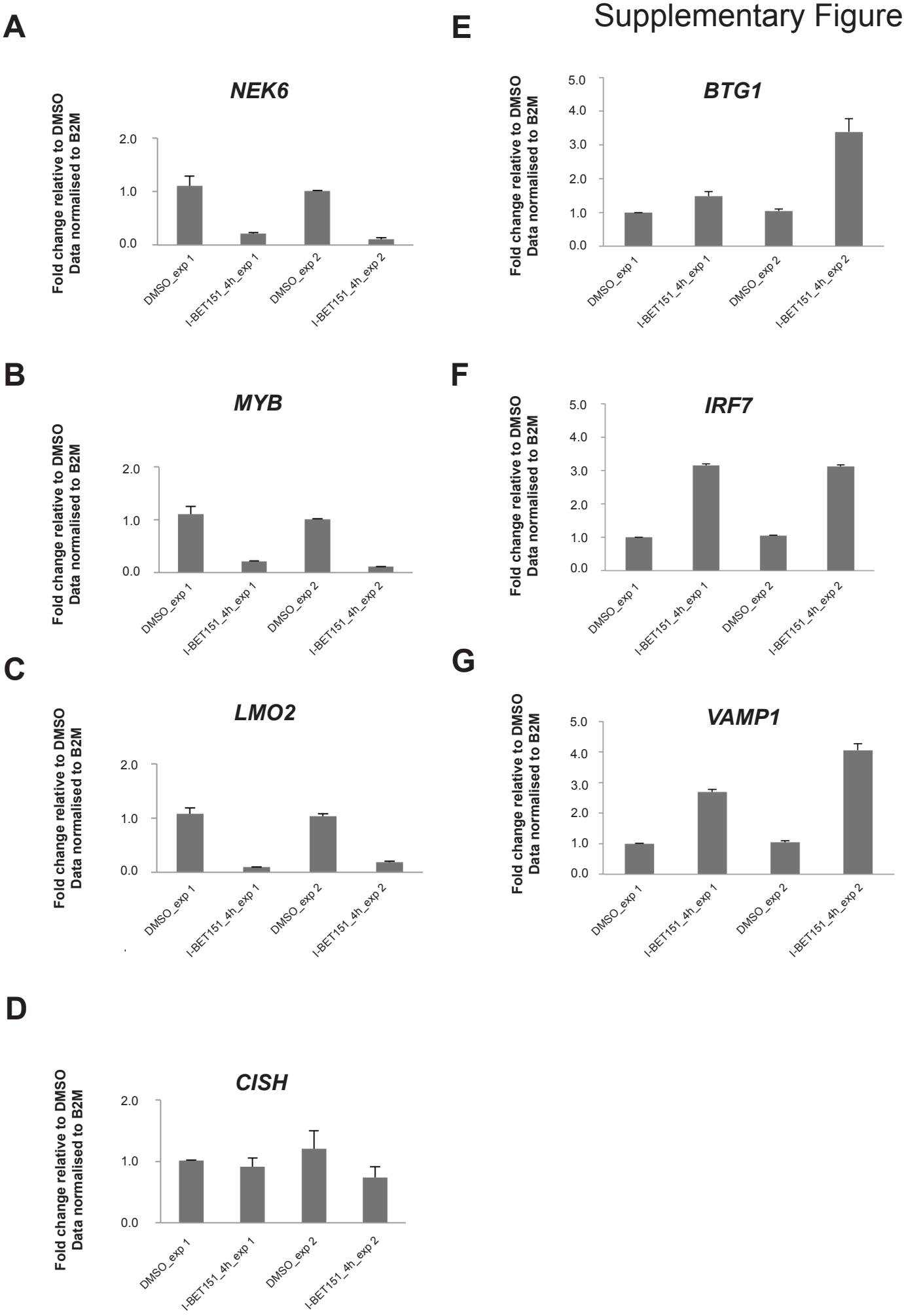
A

	Gene Symbol	Fall Change		Gene Symbol	Fall Change
1	APLNR	0.17	51	ZMYND8	0.40
2	ARHGAP22	0.21	52	ZMYND8	0.40
3	LMO2	0.21	53	SLC19A1	0.41
4	NFIB	0.25	54	NRXN2	0.41
5	NA	0.25	55	NA	0.41
6	TNS3	0.27	56	UBASH3B	0.41
7	ITLN2	0.27	57	PRDM10	0.41
8	IL1B	0.28	58	NA	0.41
9	ARHGAP39	0.28	59	F2RL2	0.41
10	ANKRD55	0.28	60	IGFBP5	0.42
11	NEK6	0.29	61	NA	0.42
12	RFESD	0.29	62	GAPT	0.42
13	NA	0.29	63	MLKL	0.42
14	TUBAL3	0.30	64	MGAT3	0.42
15	KCNH2	0.30	65	PSKH2	0.42
16	GYPE	0.31	66	GRAP2	0.43
17	GYPE	0.31	67	GJA1	0.43
18	MOBKL2B	0.31	68	RGS18	0.43
19	IGFBP5	0.32	69	GAPT	0.44
20	TGM5	0.32	70	PLCH1	0.44
21	WNT5B	0.32	71	PRR5	0.44
22	*MARCH4	0.32	72	SH3PXD2A	0.44
23	PLD6	0.32	73	ERMAP	0.44
24	RFESD	0.33	74	ADORA2B	0.45
25	C1orf186	0.33	75	RAB3IL1	0.45
26	PRICKLE1	0.34	76	LAT	0.45
27	DARC	0.34	77	GDF3	0.45
28	F2R	0.35	78	TOMM40L	0.45
29	TRIM15	0.35	79	NA	0.45
30	RFESD	0.36	80	PIM1	0.45
31	HEMGN	0.37	81	ARHGAP25	0.45
32	GFRA2	0.37	82	FEZ1	0.45
33	LTB	0.37	83	ADCYAP1	0.45
34	VAV3	0.37	84	ANGPT1	0.46
35	HEMGN	0.37	85	RSAD2	0.46
36	DHRS3	0.38	86	MTSS1	0.46
37	AMHR2	0.38	87	CHCHD4	0.46
38	F2RL3	0.38	88	TUBB1	0.46
39	LIN28B	0.38	89	TGM5	0.46
40	NA	0.38	90	ITGA4	0.46
41	TRIM15	0.38	91	ZNF692	0.46
42	RASGRP3	0.39	92	*MARCH2	0.46
43	LRRC32	0.39	93	RBM12	0.46
44	NA	0.39	94	TMEM223	0.47
45	HMBS	0.39	95	RAB33A	0.47
46	SLC45A3	0.39	96	KAZN	0.47
47	TRIB2	0.39	97	MMACHC	0.47
48	TRIM10	0.39	98	PTPN22	0.47
49	ICAM2	0.39	99	TRIB1	0.47
50	MYB	0.40	100	NFE2	0.47

B

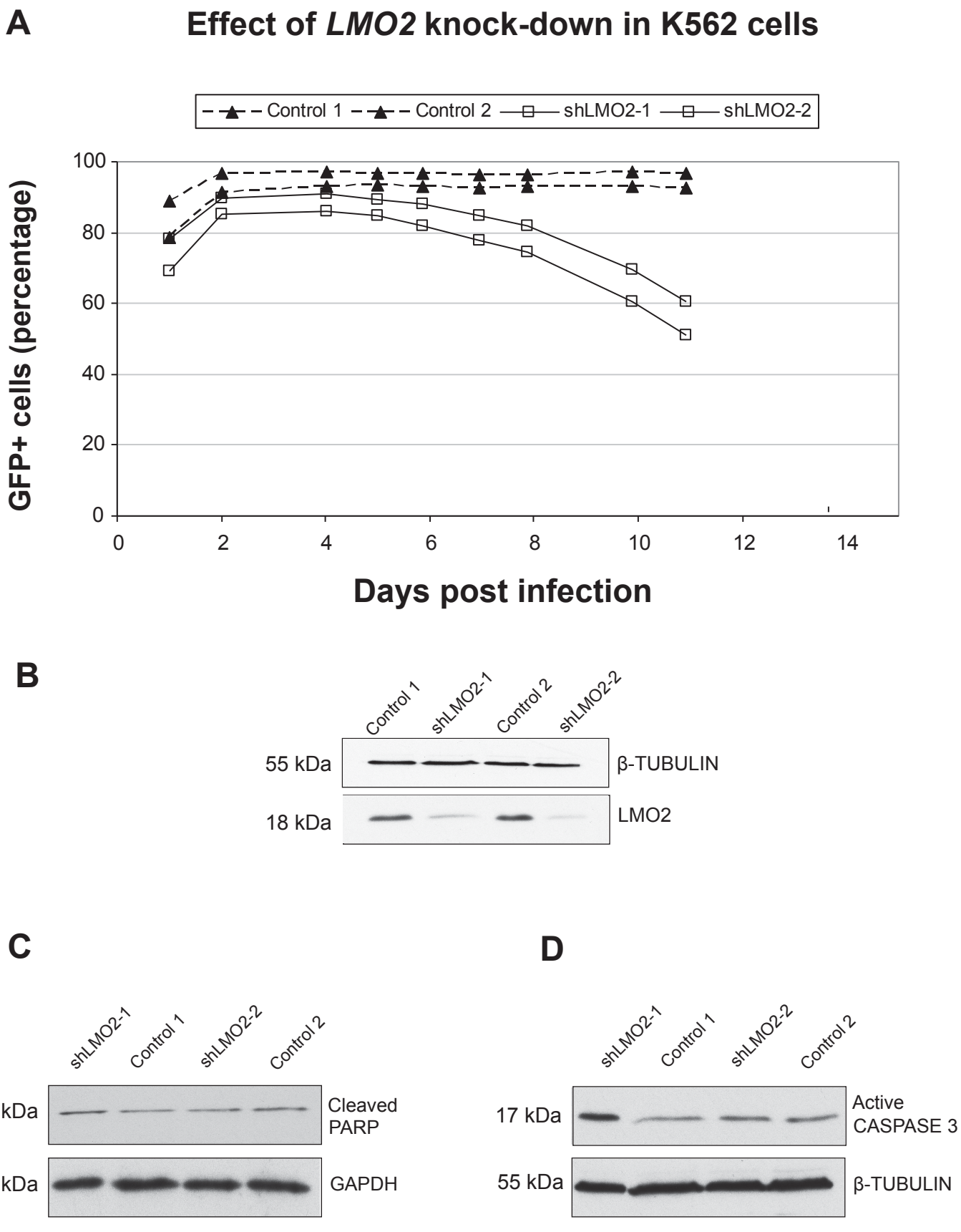
	Gene Symbol	Fall Change		Gene Symbol	Fall Change
1	TUBB3	6.00	51	ERRFI1	2.70
2	HIST1H2BD	5.43	52	CSRNP2	2.70
3	SERTAD1	4.80	53	C12orf34	2.70
4	IRF7	4.70	54	PELI1	2.66
5	IRF7	4.56	55	SERPINE2	2.65
6	HES6	4.53	56	PRDM2	2.63
7	TUFT1	4.40	57	MT1A	2.63
8	MARCKSL1	4.10	58	CHCHD7	2.62
9	BTG1	4.08	59	CHCHD7	2.60
10	HIST2H2BE	4.06	60	HIST2H4A	2.57
11	SERPINI1	3.94	61	KIF1B	2.56
12	HIST1H2AC	3.93	62	KLHL7	2.55
13	HIST2H2AA3	3.80	63	CDO1	2.55
14	CSRP2	3.72	64	PLEKHO2	2.53
15	NEU1	3.68	65	DGAT1	2.52
16	NDRG1	3.46	66	IER5	2.52
17	ZCCHC12	3.29	67	MT2A	2.52
18	MT1X	3.25	68	NA	2.50
19	RGS2	3.23	69	ATP1B1	2.50
20	HIST2H2AA4	3.21	70	TUBB2A	2.50
21	SERPINI1	3.21	71	HIST1H2BG	2.49
22	CSRNP2	3.18	72	HIST1H2BD	2.48
23	MKNK2	3.14	73	RELL1	2.47
24	LFNG	3.13	74	H2AFJ	2.47
25	MKNK2	3.13	75	FBXO33	2.46
26	RHPN2	3.12	76	RFC3	2.45
27	CDO1	3.12	77	EGR1	2.45
28	NXF1	3.09	78	ABHD3	2.44
29	CDKN1A	3.08	79	FGFRL1	2.44
30	VAMP1	3.07	80	RPPH1	2.43
31	NXF1	3.07	81	TAX1BP3	2.43
32	MT1E	3.05	82	ELOVL4	2.43
33	TESK2	3.02	83	NFKBIZ	2.42
34	NA	3.01	84	NA	2.42
35	TM7SF2	2.98	85	WDR47	2.42
36	MT1G	2.98	86	HIST2H2AC	2.41
37	TOB1	2.88	87	OPN3	2.40
38	HIST3H2A	2.86	88	NA	2.40
39	HIST2H2AA3	2.84	89	HNRNPA2B1	2.39
40	NA	2.81	90	HIST1H2BK	2.39
41	TUBB2C	2.79	91	JUP	2.39
42	TEX14	2.76	92	MIDN	2.39
43	HIST1H2BK	2.76	93	TXNIP	2.37
44	GLS	2.76	94	HIST1H4H	2.37
45	SLC9A1	2.75	95	SLC30A3	2.36
46	SAT1	2.74	96	EPB41L5	2.36
47	FAM107B	2.73	97	DACT3	2.36
48	HIST1H2BJ	2.73	98	EPB41L5	2.36
49	NA	2.73	99	MXD4	2.36
50	MT1H	2.71	100	CCDC92	2.36

Supplementary Figure S1
The top 100 genes (A) down-regulated, and (B) up-regulated in HEL cells after treatment with 1μM I-BET151 for 4 hours.



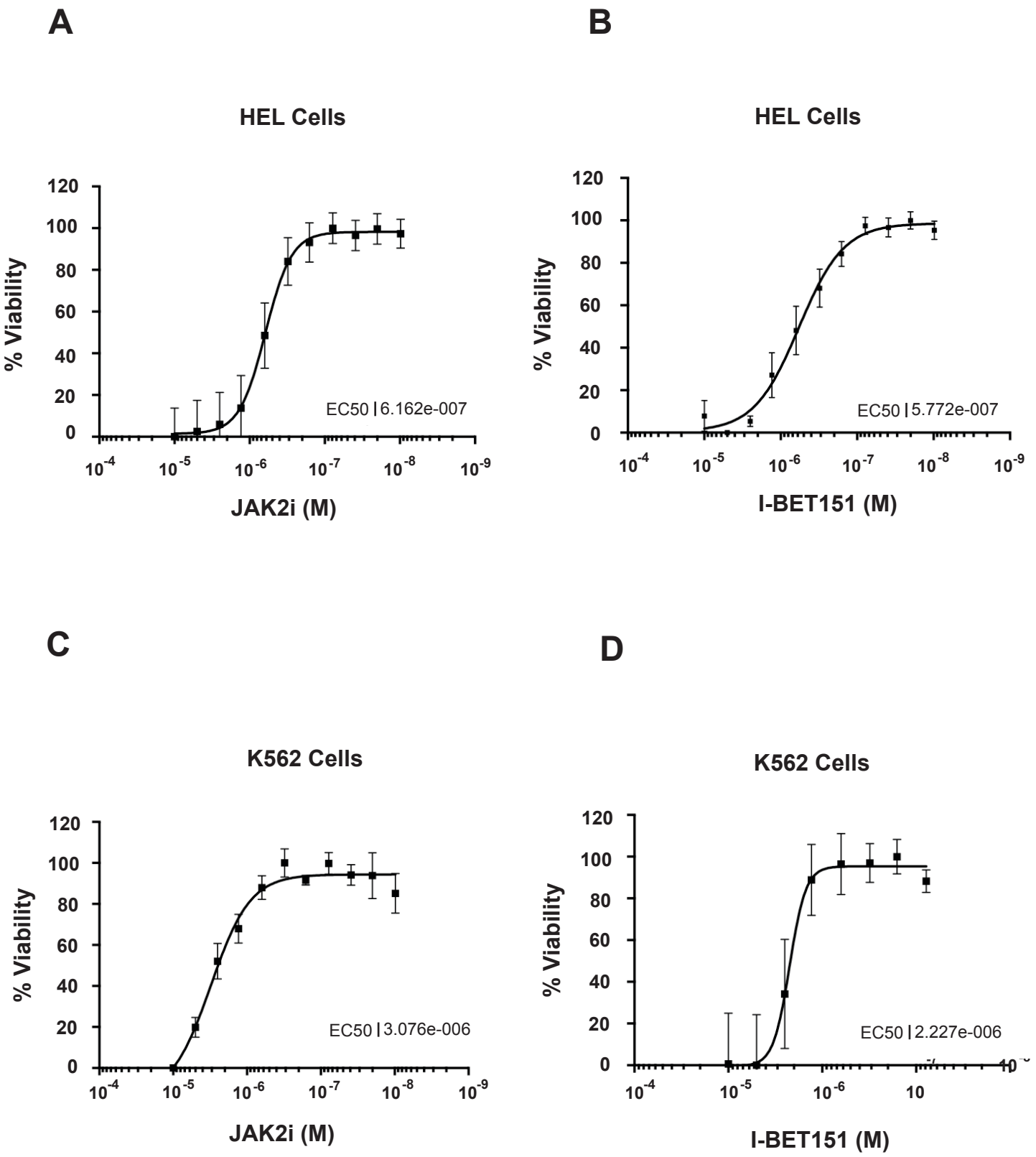
Supplementary Figure S2

The expression changes of seven genes in response to 4 hours treatment with 1μM I-BET151 were validated by qRT-PCR. Three down-regulated genes (A) *NEK6*, (B) *MYB*, (C) *LMO2*, one gene unresponsive to I-BET151 treatment (D) *CISH*, and three up-regulated genes (E) *BTG1*, (F) *IRF7*, (G) *VAMP1*, were analysed.



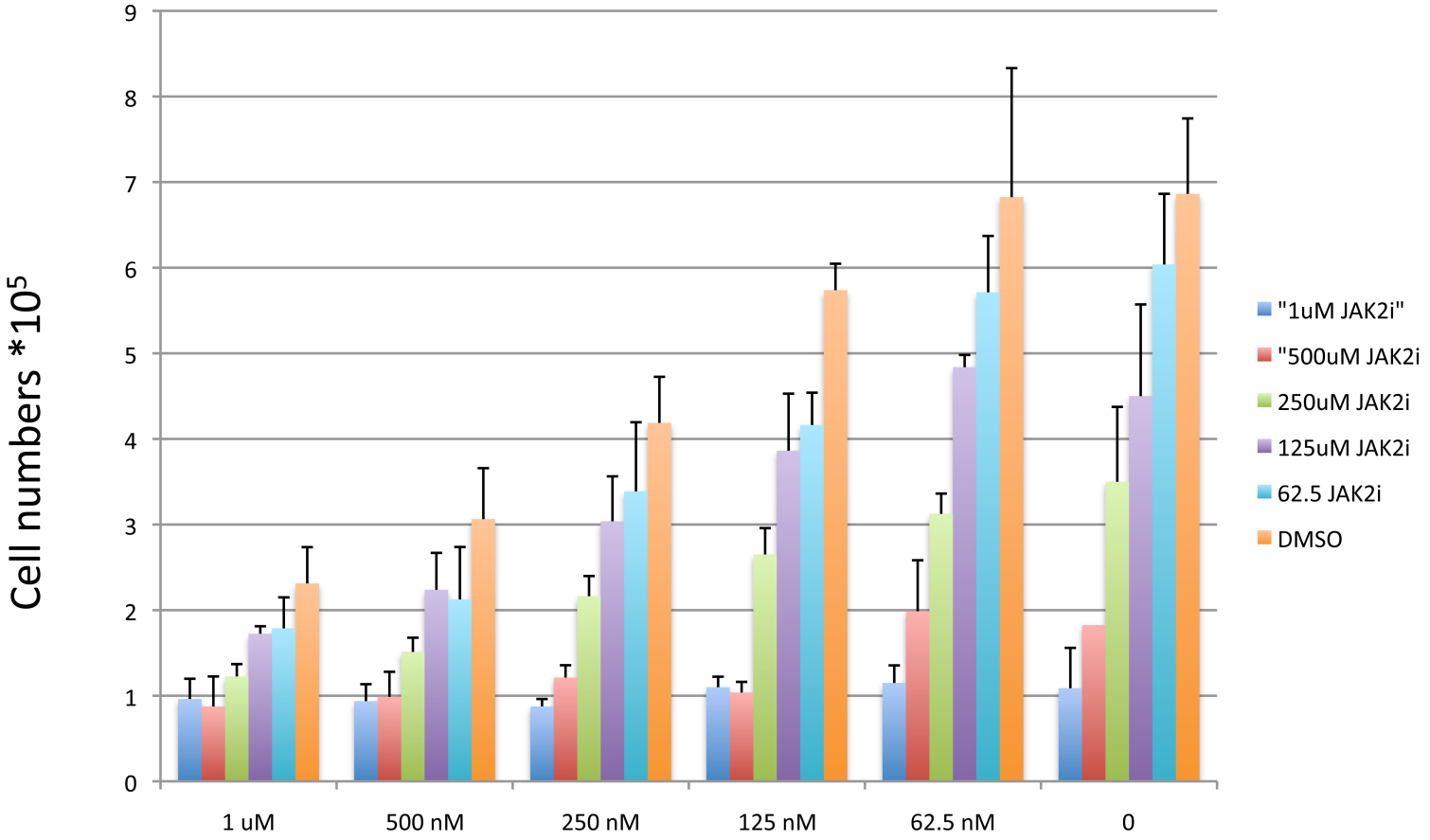
Supplementary Figure S3
Knockdown of *LMO2* in K562 cells (A) K562 cells were transduced with constructs containing shRNA against *LMO2* or the empty vector as a control. GFP presence was monitored over 13 days after infection and percentages of GFP positive cells are indicated. Shown are the results from a representative experiment performed in duplicate, (B) shRNA knockdown validation by immunoblotting showing LMO2 protein disappearing at day 5 post-infection with shLMO2-1 and shLMO2-2, (C) Immunoblotting demonstrating levels of cleaved PARP in cells transduced with shLMO2 and control virus, (D) Immunoblotting showing levels of active caspase 3.

Supplementary Figure S4



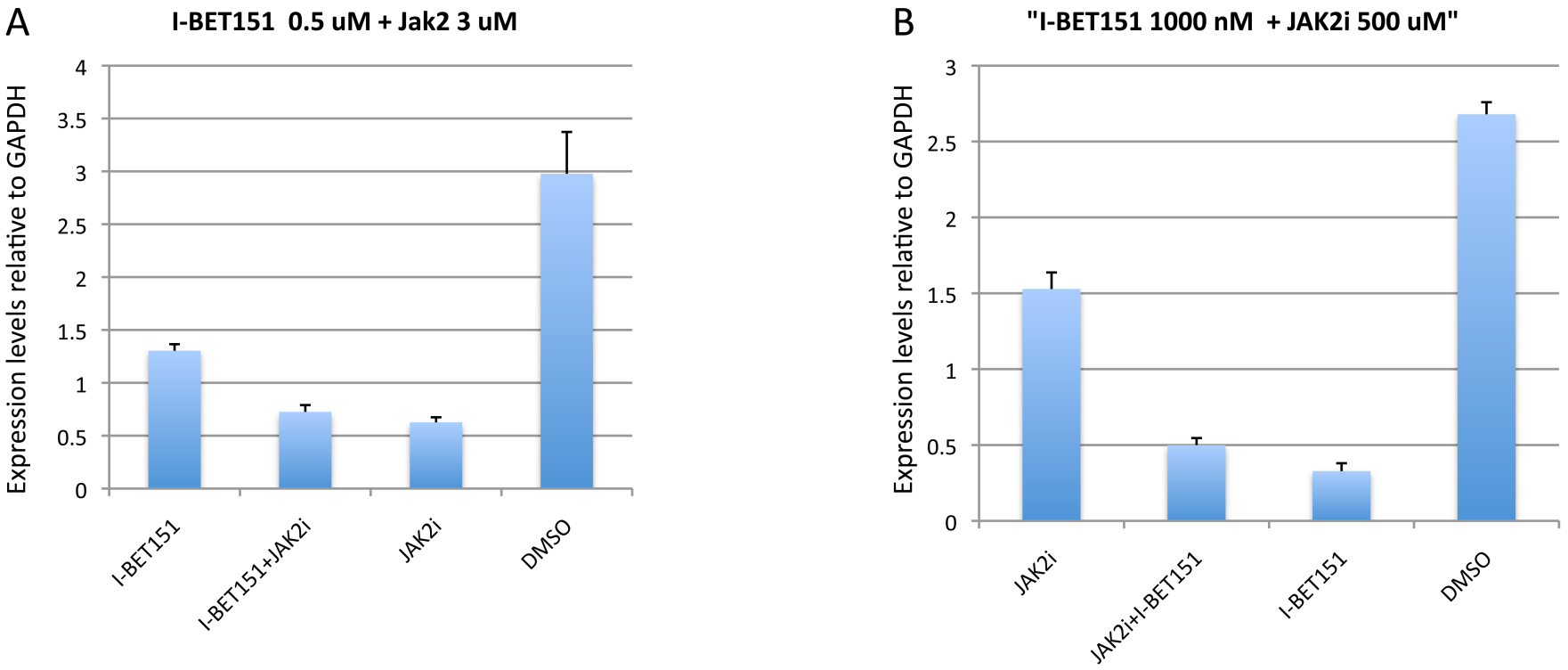
Supplementary Figure S4
The effect of I-BET151 and JAK2i (TG101209) inhibitors on HEL cell and K562 cell growth. (A) JAK2i (TG101209) in HEL cells (B) I-BET151 in HEL cells, (C) JAK2i (TG101209) in K562 cells (D) I-BET151 in K562 cells. Data are represented as mean of three independent experiments. Error bars represent standard deviation.

Supplementary Figure S5



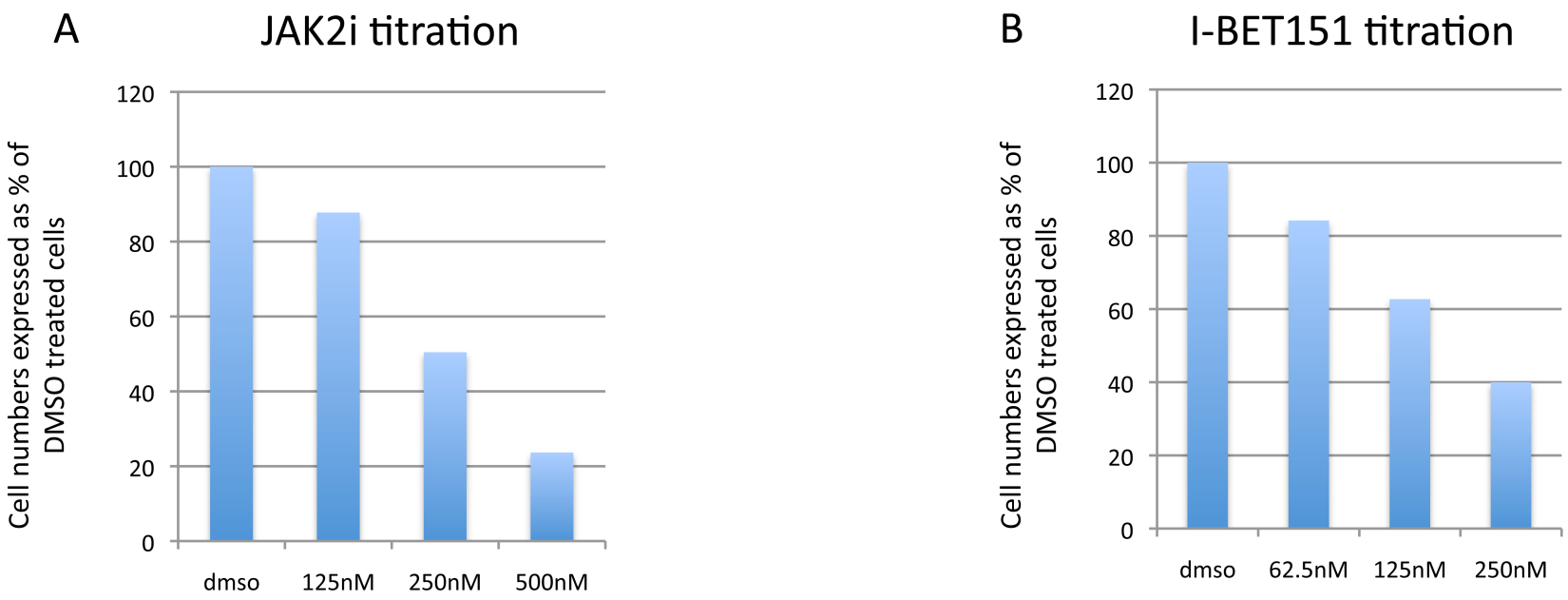
Supplementary Figure S5
Cooperation of I-BET151 and JAK2i on HEL cells. HEL cells 72h proliferation assay. 10^5 cells were plated and treated with the indicated concentration of the two inhibitors or DMSO as negative control.

Supplementary Figure S6



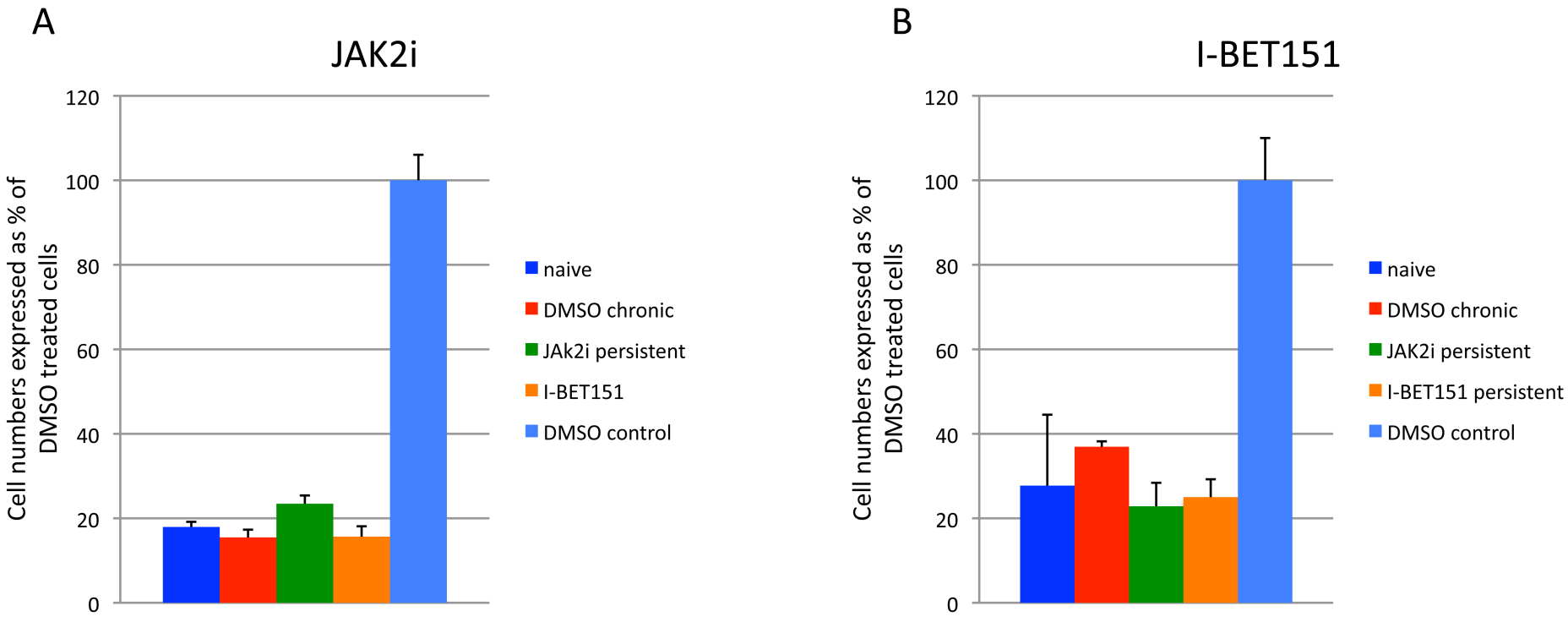
Supplementary Figure S6
JAK2i and I-BET151 effects on *LMO2* repression. *LMO2* expression levels were analyzed by RT-qPCR in HEL cells treated for 4h with the indicated concentration of I-BET151 and JAK2i or DMSO. Expression levels relative to *GAPDH* are shown.

Supplementary Figure S7



Supplementary Figure S7
HEL cells 72h proliferation assay. HEL cells were treated with the indicated concentrations of (A) JAK2i or (B) I-BET151 and counted after 72h proliferation. The indicated values are expressed as % of the DMSO treated control cells.

Supplementary Figure S8



Supplementary Figure S8
(A) HEL cells 72h proliferation assay. The indicated cells were treated with 1000nM JAK2i and counted after 72h proliferation. The indicated values are expressed as % of the DMSO treated control cells. (B) HEL cells 72h proliferation assay. The indicated cells were treated with 1000nM I-BET151 and counted after 72h proliferation. The indicated values are expressed as % of the DMSO treated control cells.