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## S1PR1 drives a feed forward signalling loop to regulate BATF3 and the transcriptional programme of hodgkin lymphoma cells

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## S1PR1 Drives a Feed Forward Signalling Loop to Regulate BATF3 and the Transcriptional Programme of Hodgkin Lymphoma Cells

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### Abstract

The Hodgkin/Reed-Sternberg (HRS) cells of classical Hodgkin lymphoma (HL) are characterised by the aberrant activation of multiple signalling pathways. Here we show that a subset of HL displays altered expression of sphingosine-1-phosphate (S1P) receptors (S1PR). S1P activates phosphatidylinositol 3-kinase (PI3-K) in these cells that is mediated by the increased expression of S1PR1 and the decreased expression of S1PR2. We also showed that genes regulated by PI3-K signalling pathway in HL cell lines significantly overlap with the transcriptional programme of primary HRS cells. Genes up-regulated by the PI3-K pathway included the basic leucine zipper

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Authorship Contributions:

K.V., M.V, P.G.M. designed research; K.V., M.I., M.V., T.P., S.M., L.L., E.N., D.L., A.L., G.R., M.A., S.S., J.A. performed research and analysed data; R.H., M.I., M.C., D.K., R.T., W.W., C.B.J.W., P.G.M. contributed to the statistical analysis; E.S. contributed clinical samples; K.V., S.S. and P.G.M. wrote the manuscript.

transcription factor, ATF-like 3 (BATF3), which is normally associated with the development of dendritic cells. Immunohistochemistry confirmed that BATF3 was expressed in HRS cells of most HL cases. In contrast, in normal lymphoid tissues, BATF3 expression was confined to a small fraction of CD30-positive immunoblasts. Knockdown of BATF3 in HL cell lines revealed that BATF3 contributed to the transcriptional programme of primary HRS cells, including the up-regulation of S1PR1. Our data suggest that disruption of this potentially oncogenic feed-forward S1P signalling loop could provide novel therapeutic opportunities for patients with HL.

## Introduction

S1P is a bioactive sphingolipid metabolite implicated in cancer growth, survival and invasion.<sup>1,2</sup> S1P is generated by the enzyme, sphingosine kinase 1 (SPHK1), which is over-expressed in different cancer types, including some non-HL.<sup>3</sup> Conversely, sphingosine-1-phosphate phosphatase (SGPP1), which degrades S1P, is down-regulated during tumour development and progression.<sup>4–6</sup> Although the over-production of S1P is a characteristic of many cancers, the biological responses to S1P are governed by binding and activation of five cell surface S1P receptors (S1PR1-5); each coupling to a different repertoire of G proteins. In B cells, S1PR1 mediates mitogenic/pro-survival and chemotactic S1P functions by coupling to Gi to activate Ras/ERK, PI3-K/Akt and Rac,<sup>7–9</sup> whereas S1PR2 couples to G12/13 to inhibit PI3-K/Akt activity leading to reduced cell growth, survival and migration.<sup>10–14</sup> S1PR1 has previously been reported to be over-expressed in HRS cells and to promote their migration *in vitro*.<sup>15</sup> In contrast, S1PR2 may be a tumour suppressor in B cell lymphomas; previous studies show that S1PR2 is mutated in a subset of human diffuse large B-cell lymphoma (DLBCL) and that mice lacking S1PR2 expression develop DLBCL.<sup>16,17</sup>

Here we show that HRS cells are characterised by high expression of SPHK1 and S1PR1, but do not express S1PR2. This leads to enhanced PI3-K activation in response to S1P and to the increased expression of the dendritic cell transcription factor (TF), BATF3 (ref. 18,19). In turn, BATF3 contributes to the transcriptional programme of HL, including the increased expression of S1PR1. Interrupting this BATF3-regulated feed-forward loop, for example by targeting S1PR1, could provide novel therapies for HL patients.

## Materials and Methods

### Cells and tissues

Paediatric tonsils and tumour samples were obtained with informed consent under local ethics committee approval (06/Q2702/50; REC\_RG\_HBRC\_12-071). Germinal center (GC) B cells were isolated and characterised as described earlier.<sup>20–22</sup> Isolation of blood-derived B cells and preparation of lymphoblastoid cell lines (LCL) were described before<sup>21–23</sup> and are detailed in SI Materials and Methods (M&M). KMH2, L428, L1236 and L540 are Epstein Barr virus (EBV)-negative HL cell lines and L591 is an EBV-positive HL cell line. DG75 is an EBV-negative Burkitt lymphoma cell line. HEK293 is derived from human embryonic kidney. Cell lines were cultured at 37°C in 5% CO<sub>2</sub> in either RPMI1640 or DMEM (HEK293) with 10% foetal calf serum (FCS) and 1% penicillin-streptomycin (all ThermoFisher Scientific, Waltham, MA, USA).

## S1P measurements

For measurement of S1P levels in cell lines and B cells, lipids were extracted and sphingolipids quantified by liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS; 4000 QTRAP, AB Sciex, Framingham, MA, USA) as previously described.<sup>24</sup>

## Treatment of cells

Preparation of S1P (Sigma-Aldrich, St. Louis, MI, USA) is detailed in SI M&M. For S1P treatment, KMH2 cells were washed twice, incubated in serum-free media for 1h, followed by addition of 1 $\mu$ M S1P. S1P receptor functional antagonists, Ozanimod (RPC1063, Selleckchem.com, Munich, Germany) or Siponimod (BAF312, Selleckchem.com), were added to cells 1h prior to 15min S1P stimulation. For measurement of viability, cells were stained using FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen™, Franklin Lakes, NJ, USA) and analysed by flow cytometry. For Akt inhibition, cells were cultivated in 10% FCS media with 1 $\mu$ M Ipatasertib (GDC-0068; Selleckchem.com) or vehicle (DMSO) for 6h. Cell-Titer Glo assay (Promega, Fitchburg, WI, USA) showed no cell death at this time point.

For PI3-K inhibition, cells were cultivated in 10% FCS media with 30 $\mu$ M LY294002 or vehicle (DMSO). We have previously shown that LY294002 does not cause significant cell death at the time points and concentration used.<sup>25</sup>

Gene expression in KMH2 cells following 16h of LY294002/vehicle treatment was analysed with Affymetrix HG-Focus arrays (data available from GSE89759). The data was background subtracted and quantile normalized<sup>26,27</sup> using the affy package of Bioconductor. Differentially expressed genes were identified using limma<sup>28</sup> ( $p < 0.05$ , absolute fold change  $> 1.2$ ). Probe sets with "Negative Strand Matching Probes" were removed from the significant gene list. Gene ontology analysis was performed using DAVID 6.7 <http://david.abcc.ncifcrf.gov> using GO-term BP-FAT;  $p < 0.01$ ,  $\geq 1.3$ -fold enrichment.

## BATF3 knockdown

BATF3 knockdown was performed by nucleofection with Silencer® Select s195280 BATF3 siRNA or Silencer® Select Negative Control No.1 siRNA (ThermoFisher Scientific). 48h after BATF3 knockdown in L428 cells, samples were hybridized to Affymetrix PrimeView arrays (data available from GSE89760) and data analysed as described above.

Re-analysis of publicly available datasets is described in SI M&M. Chi-squared test was used to identify significant enrichment or depletion of either PI3-K or BATF3 target genes in these datasets using a threshold for significance of  $p < 0.05$ .

## Transfection of cells

GC B cells were nucleofected with pcDNA3.1-PRDM1 or empty vector, cultivated and enriched as before.<sup>20,21</sup> HL cell lines and DG75 cells were transfected by nucleofection (Lonza, Basel, Switzerland) and HEK293 transfected by lipofectamine (ThermoFisher Scientific) using the following vectors and corresponding controls: pcDNA3.1-S1PR2-HA

(UMR cDNA Resource Center University of Missouri-Rolla, MO, USA), pSG5-myc-p110 $\alpha$ -CAAX (Julian Downward, Addgene, Cambridge, MA, USA), Akt1/PKB $\alpha$  cDNA (activated) in pUSEamp containing a MYC tag (Merck Millipore, Billerica, MA, USA), pcDNA3.1-S1PR1 (Dr Ian Paterson, University of Malaya) and pCMV6-SPHK1 (Origene technologies, Rockville, MD, USA).

### Quantitative RT- PCR (Q-PCR)

RNA was extracted with RNeasy Mini/Micro Kit including genomic DNA removal with RNase-Free DNase Set (QIAGEN, Hilden, Germany). cDNA was generated with qScript™ cDNA SuperMix (VWR International, Radnor, PA, USA). Gene transcripts were quantified with commercial assays (Table S1A, ThermoFisher Scientific) as before.<sup>21</sup> The  $2^{-\Delta Ct}$  method was used to quantify target expression relative to GAPDH housekeeping control (unless specified otherwise).

### Immunoblotting

Immunoblotting was performed with the primary antibodies detailed in Table S1B and following standard techniques described in SI M&M.

### Immunohistochemistry (IHC)

IHC was performed as before.<sup>29,30</sup> Slides were blocked in 5x casein followed by primary antibody in 0.05% PBS/Tween (Table S1C). Multiplex immunofluorescence (IF) was performed using Opal™ 7-Plex Kit (NEL791001KT; PerkinElmer, Waltham, MA, USA). For antibody stripping between each step, slides were microwaved in pH=6 citrate buffer, with controls omitting individual antibody steps to ensure adequate stripping. Slides were scanned using wide-range wavelength covering filters available in Vectra system 3.0.3 (For dyes-Cyanine 5, Cyanine 3, DAPI, Texas Red, Fluorescein). Antibodies were optimized with auto-exposure adjustment (100-150ms). Images were taken on Olympus BX-51WI microscope with 10x, 20x and 40x magnification. Multispectral images were analysed using InForm Automated Image Analysis software version 2.2.1 for phenotyping and quantification of co-expressed markers. Analysis was performed with a 20x objective on a minimum of three fields for cytospin preparations and five fields for tissue sections. For assessment of protein expression following S1P treatment the mean intensity and SD were calculated for each of three fields separately and statistical significance measured by Fisher exact test. For image analysis of TMA we used the 1x1 function covering approximately 75% of each core. Positivity in IHC was assessed with 40x objective using the whole TMA area, or ten representative hpf in the case of whole sections. To directly compare our results with those previously reported by Kluk *et al*,<sup>15</sup> S1PR1 was scored as positive if  $\geq 25\%$  of cells expressed S1PR1. The same cut-off was applied for all other markers. Validation of the specificity of SPHK1, S1PR1, S1PR2 and BATF3 antibodies is shown in Figure S1.

## Results

### Expression of SPHK1, SGPP1 and S1P receptors in HRS cells

We first studied the expression of SPHK1 and SGPP1, enzymes important for regulating S1P levels.<sup>3–5</sup> We showed that SPHK1 was expressed in both normal GC B cells and in

HRS cells in most cases (49/58; Figure 1A; Table S2). Owing to the lack of an internal positive control we could not reliably quantify SPHK1 expression by IHC. Therefore, we re-analysed a microarray dataset that compared global gene expression in B cell lymphomas with that in normal B cell subsets.<sup>31</sup> This revealed significantly higher SPHK1 mRNA in micro-dissected HRS cells compared with isolated GC B cells (Figure S2A). Re-analysis of a second independent microarray dataset<sup>32</sup> revealed higher expression of SPHK1 mRNA in both EBV-negative and EBV-positive HRS cells compared to micro-dissected GCs (Figure S2B). Q-PCR showed that SPHK1 mRNA expression was also higher in HL cell lines than in GC B cells (Figure 1B). We next used immunoblotting to show that SPHK1 was constitutively activated in HL cell lines (Figure 1C). Conversely, SGPP1 mRNA was decreased in primary HRS cells and HL cell lines compared with normal GC B cells (Figure S3). Indeed, S1P levels measured by mass spectrometry were higher in HL cells than in normal B cells (Figure 1D). We next studied the expression of S1PR1 and S1PR2. We showed that normal GC B cells expressed S1PR2, but not S1PR1 (Figure 2A, S4). In comparison, HRS cells frequently expressed S1PR1 (36/61), but not S1PR2 (2/61), an observation we confirmed by multiplex staining with CD30 (Figure 2B, S5; Table S2). We conclude that in contrast to their normal counterparts, HRS cells appear to express high levels of SPHK1 and S1PR1 but lack expression of S1PR2 and SGPP1.

### **S1P-induced PI3-K signalling in HRS cells is mediated by the differential expression of S1P receptors**

We next studied the effects of S1P on PI3-K signalling in HRS cells using Akt phosphorylation as a read-out. We chose the KMH2 cell line for these experiments because it expresses S1PR1 and only low levels of S1PR2 in the absence of the other S1P receptors (Figure S6A,B). We observed that the addition of S1P increased Akt phosphorylation in KMH2 cells (Figure 3A). To examine the effect of S1PR1 on Akt phosphorylation, we stimulated KMH2 cells with S1P after targeting S1PR1 (and S1PR5) with their functional antagonists Ozanimod and Siponimod.<sup>33,34</sup> The addition of either drug 1h prior to S1P treatment decreased Akt phosphorylation in a dose-dependent manner (Figure 3B). Furthermore, Ozanimod significantly reduced the viability of KMH2, L428 and L591 cells while Siponimod reduced the viability of KMH2 and L428 cells (Figure S7). L1236 was resistant to both drugs. To study the effect of S1PR2, we transfected KMH2 cells with an S1PR2-HA expression vector and used IF to detect phospho-Akt and the HA tag. Cells ectopically expressing S1PR2 were significantly more likely to be phospho-Akt negative than HA-negative cells in the same transfected population, an effect we observed with ( $p < 0.0001$ ; Figure S8) and without ( $p < 0.0001$ ; not shown) the addition of exogenous S1P. Taken together, our data suggests that binding of S1P to S1PR1 can activate PI3-K signalling in HRS cells.

### **Expression of the transcription factor, BATF3, is regulated by PI3-K signalling in HRS cells**

To identify the transcriptional targets of PI3-K signalling pathway in HL cells, we used the pan-PI3-K inhibitor LY294002. KMH2 cells were treated with LY294002 for 16h under conditions that do not affect viability.<sup>25</sup> The addition of LY294002 for 16h was followed by the up-regulation of 694 genes and the down-regulation of 330 genes (Table S3). We

validated a number of PI3-K targets genes in KMH2, and also in L428, L591 and L1236 cells (Figure S9).

Re-analysis of GSE12453 (Brune *et al*<sup>31</sup>) identified 743 genes up-regulated and 622 genes down-regulated in micro-dissected HRS cells compared with normal centrocytes (Table S4). Genes up-regulated in HRS cells (OR=2.15;  $p<0.0001$ ), but not those down-regulated in HRS cells (OR=1.00;  $p=0.96$ ), were significantly enriched in genes down-regulated following PI3-K inhibition. Similarly, genes down-regulated in HRS cells (OR=2.95;  $p<0.0001$ ), but not those up-regulated in HRS cells (OR=1.28;  $p=0.056$ ), were significantly enriched in genes up-regulated following PI3-K inhibition (Figure S10; Table S5). These data indicate that the activation of PI3-K signalling pathway contributes to the transcriptional programme of HRS cells. We next wanted to establish if this was also the case for both the EBV-positive and EBV-negative forms of HL. To do this we re-analyzed data from the second published microarray which had compared gene expression in micro-dissected HRS cells with that in micro-dissected normal GCs and which had reported EBV status for a subset of cases GSE39133 (Steidl *et al*<sup>32</sup>). The transcriptional targets of PI3-K signalling we had identified in primary HRS cells were significantly enriched in those genes differentially expressed in both EBV-positive and EBV-negative HRS cells (Figure S10; Tables S6-S9).

A gene ontology (GO) analysis revealed that genes regulated by PI3-K signaling pathway in primary HRS cells were enriched for those with a function in 'transcription initiation from RNA polymerase II promoter', suggesting that PI3-K signalling might contribute to the aberrant expression of TFs observed in HRS cells (Figure 4A). To identify relevant TFs targets we compared genes regulated by PI3-K signalling in primary HRS cells with a comprehensive set of TFs described by Vaquerizas *et al*.<sup>35</sup> This revealed the up-regulation of 12, and the down-regulation of 47 TFs by PI3-K signalling in HRS cells (Figure 4B). They included the dendritic cell TF, BATF3 (ref. 18,19), which was previously reported to be over-expressed at the mRNA level in HRS cells<sup>36,37</sup> and which was also the PI3-K regulated gene most highly up-regulated in the re-analysis of global gene expression in primary HRS cells described above.<sup>31,32</sup> We confirmed that LY294002 down-regulated BATF3 expression in HL cell lines (Figure S11A,B). Furthermore, the pan-Akt inhibitor, Ipatasertib, also decreased BATF3 expression (Figure S11C). To confirm these were not off-target effects, we transfected KMH2 cells with a MYC-tagged plasmid containing a constitutively active p110 or Akt1 gene and performed IF, titrating the MYC antibody to detect only exogenous MYC. We found, that a larger proportion of cells ectopically expressing either p110 (Figure S11D) or Akt1 (not shown) were significantly more likely to be both phospho-Akt- and BATF3-positive ( $p<0.0001$ ) than were un-transfected cells. Taken together, our data show that activation of PI3-K/Akt signalling pathway leads to aberrant BATF3 expression in B cell lymphoma.

### **BATF3 overexpression contributes to the transcriptional programme of HRS cells**

We confirmed that BATF3 mRNA expression was higher in micro-dissected HRS cells compared with normal B cells (Figure S12A) and in both EBV-positive and EBV-negative HRS cells compared with micro-dissected GCs (Figure S12B). IHC of normal lymphoid

tissues revealed that BATF3 protein was not present in GC B cells and only rarely in extrafollicular CD20-positive cells which were also CD30-positive (Figure 5A). In contrast, BATF3 protein was strongly expressed in both EBV-positive (7/10) and EBV-negative (29/42) HRS cells (Table S10) confirmed by CD30 co-staining (Figure 5B), and in HL lines (Figure 5C,D). BATF3 expression was also increased after EBV infection of B cells (Figure S13).

A microarray analysis following BATF3 knockdown in L428 cells revealed the up-regulation of 960 genes and the down-regulation of 1066 genes (Table S11A,B). Taking genes only present on the Primeview platform and also on GSE12453,31 we found that genes down-regulated by BATF3 were significantly enriched in genes down-regulated in primary HRS cells (OR=1.8;  $p<0.0001$ ). A GO analysis of BATF3 target genes revealed the enrichment of a number of HL-related GO terms which included 'lymphocyte differentiation', 'somatic diversification of immune receptors', 'regulation of cell motion', 'positive regulation of transcription from RNA polymerase II promoter' and 'B cell mediated immunity' (Figure 6A). We validated several BATF3 targets within different functional groups including genes down-regulated (JUN, FOS, ATF3, EGR1, EGR2, PTPRC, CASP7, CASP14, RGS1, RGS16) and genes up-regulated (BACH2, STAT5B, CXCL16) by BATF3 (Figure S14). We noted that BATF3 decreased the expression of PRDM1, which encodes BLIMP1 (Figure S14), a TF down-regulated in HRS cells and which is essential for plasma cell differentiation.<sup>21,38–41</sup> Interrogation of our previous microarray dataset<sup>21</sup> which described the transcriptional changes that follow the expression of BLIMP1 in primary GC B cells, also revealed a reciprocal regulation of BATF3 by BLIMP1 (Figure S15A). We confirmed the down-regulation of BATF3 by BLIMP1 by Q-PCR analysis of a further three donors (Figure S15B). These data show that the over-expression of BATF3 contributes to the aberrant transcriptional programme of HRS cells, including the down-regulation of BLIMP1.

### BATF3 up-regulates S1PR1 expression

The knockdown of BATF3 significantly decreased S1PR1 mRNA and protein levels in L428, L1236 and KMH2 cells (all  $p<0.0001$ ; Figure 7A, S16). In control and BATF3-siRNA-treated cells ( $p<0.0001$ ; Figure 7B) as well as in untreated cells (Table S12), BATF3 and S1PR1 levels were also significantly correlated at the single cell level. Next, we wanted to show that this pathway was directly regulated by S1P. To do this we treated the HL cell lines with S1P or control (BSA) for 24h and measured the levels of phospho-Akt, BATF3 and S1PR1 by IF. S1P significantly increased the levels of phospho-Akt, BATF3 and S1PR1 in L428, KMH2 and L591 cells (Figure S17). This regulation could be observed at the single cell level because S1P-treated cells were significantly more likely to co-express phospho-Akt, BATF3 and S1PR1 (all  $p<0.0001$ ; not shown). S1P did not increase the expression of phospho-Akt and BATF3 in L1236 cells; most likely owing to the very high constitutively activated Akt in these cells and in keeping with their resistance to S1PR1 inhibition described above. Finally, we measured BATF3 and S1PR1 expression in primary HRS cells. We found that BATF3-positive primary HL were significantly more likely to be S1PR1-positive than BATF3-negative HL ( $p=0.0006$ ; Figure S18A, Table S10). Single cell image analysis of six representative HL cases showed that BATF3-positive HRS cells within each

case were also significantly more likely to be S1PR1-positive than were BATF3-negative cells (Figure S18B). Taken together our results suggest the existence of a feed-forward S1P signalling loop that drives constitutive BATF3 expression leading to aberrant transcription in HRS cells.

## Discussion

Here we show that HRS cells express BATF3, a TF that is important for the development of CD8 $\alpha$ + classical dendritic cells<sup>18</sup> and related non-lymphoid CD103+CD11b- dendritic cells.<sup>19</sup> Re-analysis of existing global expression datasets and IHC showed that BATF3 expression was significantly higher in primary HRS cells compared with normal B cells. Our data also revealed that BATF3 is regulated by PI3-K/Akt signalling, a pathway we and others have shown is constitutively activated in HRS cells.<sup>25,42,43</sup>

We also showed that PI3-K/Akt signalling pathway is regulated by S1P in HRS cells and that this is dependent upon the increased expression of S1PR1 together with the decreased expression of S1PR2. The up-regulation of S1PR1 in HRS cells has been reported before<sup>15</sup>, albeit in only 7/57 cases, a lower proportion than we have reported here; despite the fact that we used the same cut-off employed by Kluk *et al.* Our observation that BATF3 induces the expression of S1PR1 identifies a feed-forward loop which leads to constitutive S1P signalling, activation of the PI3-K/Akt pathway and sustained BATF3 transcriptional activity (Figure 8). The major source of the S1P necessary to sustain this loop is likely to be the HRS cells, since they express SPHK1 in the absence of the S1P phosphatase, SGPP1. The feed-forward loop described here could be important for the pathogenesis of HL since it has been shown that the knockdown of BATF3 significantly reduces the survival of HL cell lines<sup>44</sup> and that BATF3 can induce B cell lymphomas in a murine model (Weiser *et al.*, personal communication).

Our studies revealed that BATF3 down-regulated expression of BLIMP1, a TF required for plasma cell differentiation and which is either mutated or transcriptionally down-regulated in B cell lymphomas, including HL.<sup>21,38–41</sup> BLIMP1 also decreased the expression of BATF3, indicating a reciprocal regulation of BATF3 and BLIMP1. Furthermore, BATF3 increased the expression of BACH2 which has been shown to delay BLIMP1 expression and plasma cell differentiation in mouse lymphocytes.<sup>45,46</sup> These data suggest that the constitutive expression of BATF3 could be important for the suppression of terminal B cell differentiation in HRS cell progenitors.

In keeping with the reported down-regulation of AP-1 signalling by BATF3 (ref. 47), we observed that BATF3 decreased the transcription of the key AP-1 components, JUN, FOS and ATF3. Furthermore, we showed that BATF3 regulated many other transcriptional changes characteristic of HRS cells. These included the down-regulation of PTPRC (CD45), an essential regulator of BCR signalling<sup>48,49</sup> and a number of TFs, including STAT5B, NOTCH1 and EGR1, known to be aberrantly expressed in HRS cells.<sup>50–53</sup>

In a re-analysis of the BATF3 targets reported by Lollies *et al.*<sup>44</sup> (using the same cut-off as in our array, but using shRNA and different time point), we found that genes up-regulated (OR=1.62; p=0.003), but not those down-regulated (OR=0.96; p=0.89) by BATF3 in our

array were significantly enriched in those genes up-regulated by BATF3 in Lollies *et al.* Similarly, genes down-regulated (OR=2.7;  $p<0.00001$ ), but not those up-regulated (OR=0.82;  $p=0.37$ ), by BATF3 in our array were significantly enriched in those genes down-regulated by BATF3 in Lollies *et al.* It is noteworthy that S1PR1 was among those genes significantly up-regulated by BATF3 in Lollies *et al.*

Both EBV-positive and EBV-negative HRS cells expressed BATF3, consistent with our observation that BATF3 expression was increased by *in vitro* EBV infection. Because the EBV lytic cycle has been shown to be induced upon terminal B cell differentiation<sup>54</sup> leading to viral replication and cell death, the increased BATF3 expression observed in EBV-infected tumor cells could be important for suppression of the lytic cycle, in turn preventing replication-induced cell death. In keeping with this, several of the BATF3 targets we identified (e.g. AP-1, EGR1, BLIMP1), are known to induce the EBV lytic cycle.<sup>21,55–59</sup>

Our data also suggest that the therapeutic blockade of S1P signalling could inhibit the oncogenic effects of BATF3. The two functional antagonists of S1PR1, Ozanimod and Siponimod, which we showed can block the S1P-mediated activation of Akt, are already in phase II and III clinical trials of patients with inflammatory and autoimmune diseases. These and other S1PR1 modulators should be investigated for their therapeutic potential in HL.

## Supplementary Material

Supplementary information is available at Leukemia's Website.

## Acknowledgments

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We wish to dedicate this work to the memory of an excellent scientist, a wonderful colleague and a kind friend, Professor Ciaran BJ Woodman, we had the privilege to work with.

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**Figure 1. Expression of SPHK1 in normal lymphoid tissues and HRS cells**

A) SPHK1 expression in GC B cells and primary HL. Black arrows show HRS cells. GC=germinal center, MZ=mantle zone B) SPHK1 mRNA and C) protein expression in HL cell lines. T1-4 are GC B cells isolated from 4 donors. Note, SPHK1 is phosphorylated in all HL cell lines. D) S1P levels in HL cell lines and B cells (mean of three different donors) measured by mass spectrometry.

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**Figure 2. Expression of S1PR1 and S1PR2 in normal lymphoid tissues and HRS cells**

A) Normal tonsil. Upper panels: S1PR1 expression in MZ B cells, but not in GC B cells, confirmed by co-staining for CD20. Lower panels: S1PR2 was expressed in normal GC B cells, confirmed by co-staining with CD20 (white arrow) and BCL6 (Figure S4). B) HRS cells expressed S1PR1 but not S1PR2 (white arrows) confirmed by CD30 co-staining. Endothelial cells and red blood cells were strongly positive for S1PR1 and S1PR2, respectively (black arrows). Further examples of S1PR1 staining in HRS cells indicating the presence of both membrane and cytoplasmic staining are shown in Figure S5.

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**Figure 3. S1P activates Akt in HRS cells mediated by differential expression of S1P receptors**  
A) Immunoblot of KMH2 cells following S1P stimulation and B) following 1h pre-treatment with increasing concentrations of Ozanimod or Siponimod.

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**Figure 4. PI3-K signalling up-regulates BATF3 in HRS cells**

A) GO analysis of genes regulated by PI3-K signalling in primary HRS cells (GSE1245331 or GSE3913332 data or both; total of 137 up-regulated and 443 down-regulated genes) revealed enrichment of several functions including ‘transcription initiation from RNA polymerase II promoter’ B) Comparison of genes regulated by PI3-K signalling in primary HRS cells with a comprehensive set of TF revealed the up-regulation of 12 TF and the down-regulation of 47 TF by PI3-K signalling in HRS cells.

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**Figure 5. BATF3 is overexpressed in HRS cells**

A) IHC of normal tonsil. BATF3-positive cells were mainly located outside the GC some of which expressed CD20 and CD30 but not BCL6 (arrowed). MZ=mantle zone, IF=interfollicular. B) BATF3 expression in CD30-positive HRS (arrowed) C) BATF3 mRNA and D) protein expression in HL cell lines. T1-T5 are GC B cells isolated from 5 donors.

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**Figure 6. BATF3 over-expression contributes to the transcriptional programme of HRS cells**  
GO analysis of BATF3 targets in L428 cells. Immunoblotting shows knockdown of BATF3  
in L428 cells.

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**Figure 7. BATF3 regulates S1PR1 expression**

Lower levels of S1PR1 protein were observed following BATF3 knockdown in L428 cells ( $p < 0.0001$ ) shown here by IF (A) and confirmed by single cell image analysis (B). BATF3 knockdown also significantly decreased S1PR1 protein in L1236 and KMH2 cells (both  $p < 0.0001$ ; not shown).

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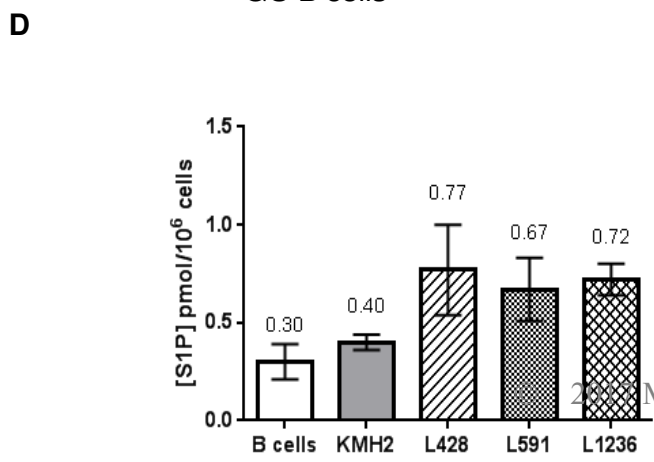
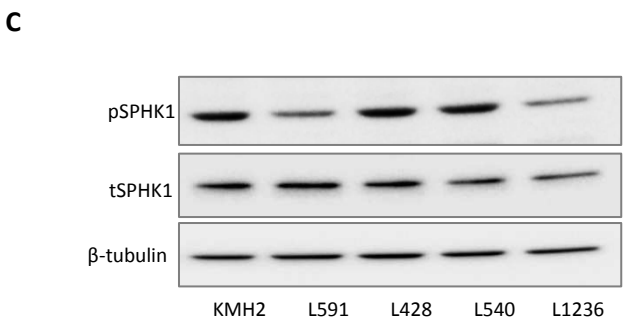
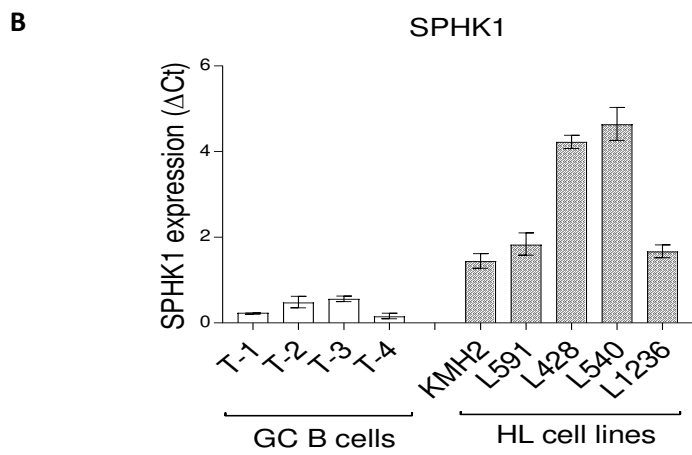
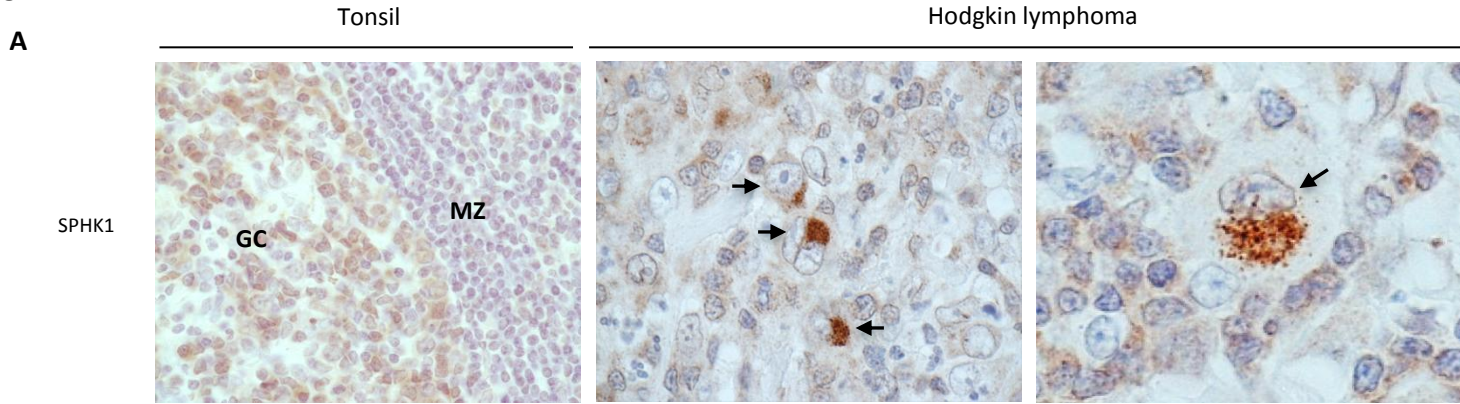
**Figure 8. Proposed model for the regulation of BATF3 by S1P signalling in HRS cells**

S1P levels are regulated by SPHK1, which is upregulated in HRS cells and catalyses S1P production, and SGPP1, which is down-regulated in HRS cells and degrades S1P. S1P acts on the S1P receptor, S1PR1 and in the absence of S1PR2 this drives PI3-K/Akt signalling pathway leading to increased BATF3 expression and downstream aberrant transcription. S1PR1 expression is increased by BATF3 leading to a feed-forward loop to sustain S1P signalling and constitutive BATF3 transcription.

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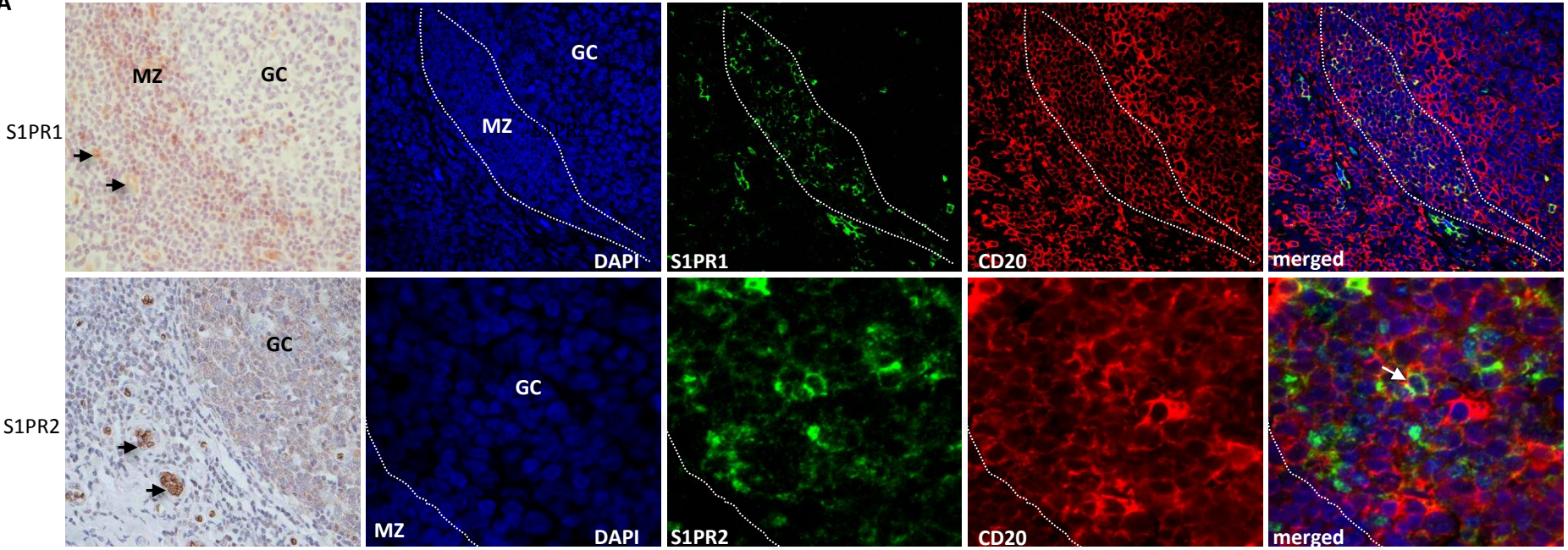
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**Figure 1**

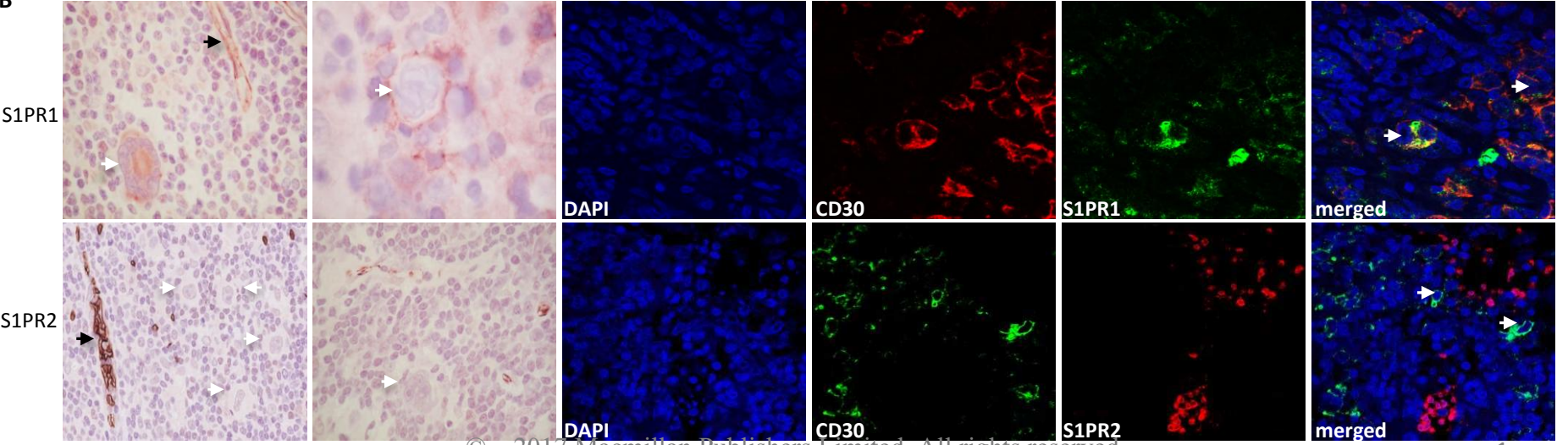


**Figure 2**

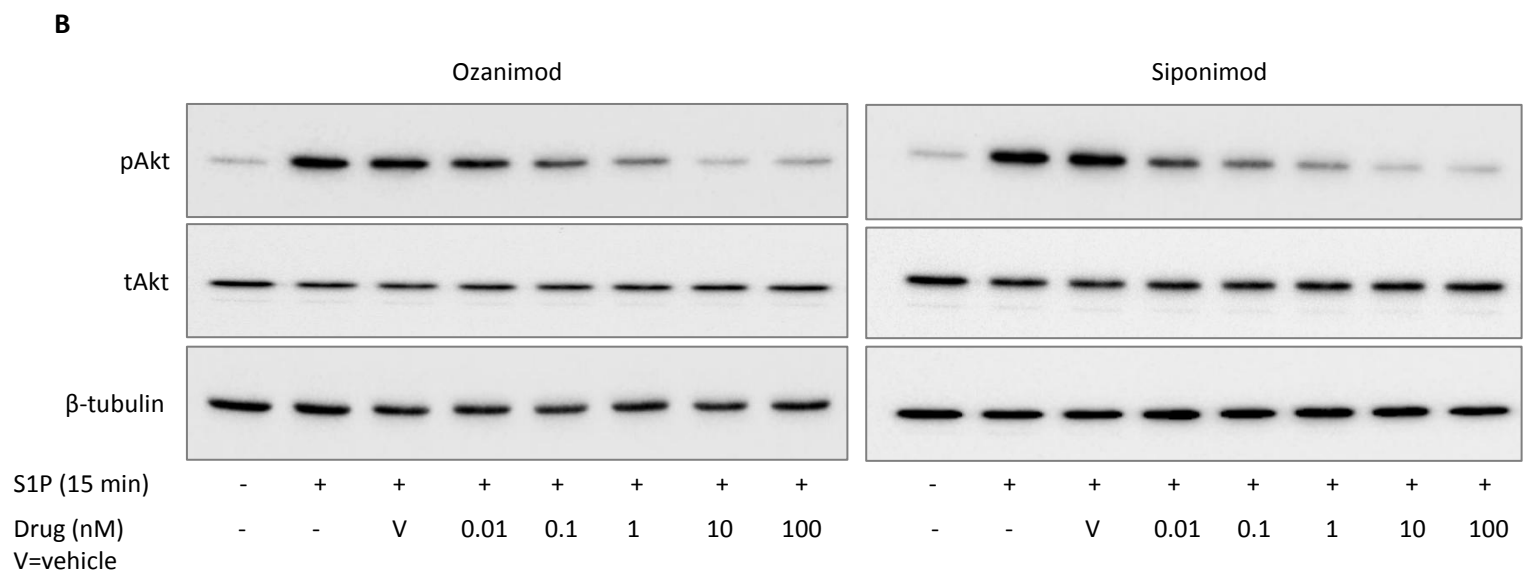
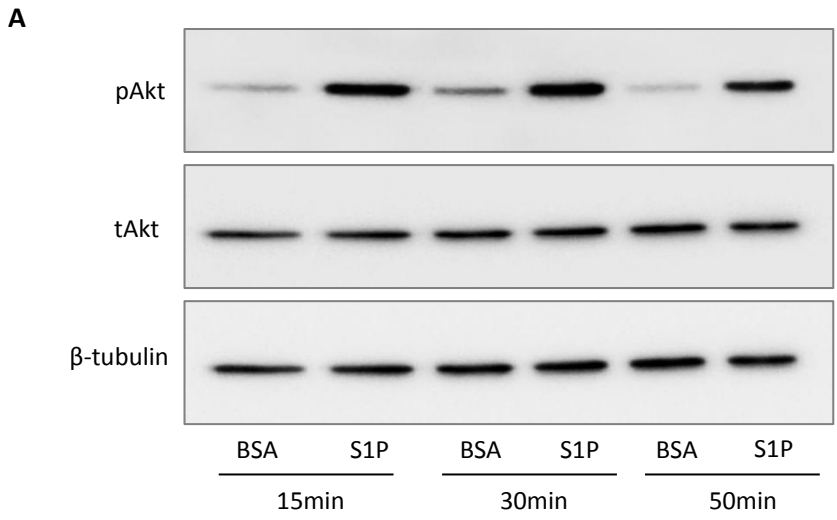
**A**



**B**

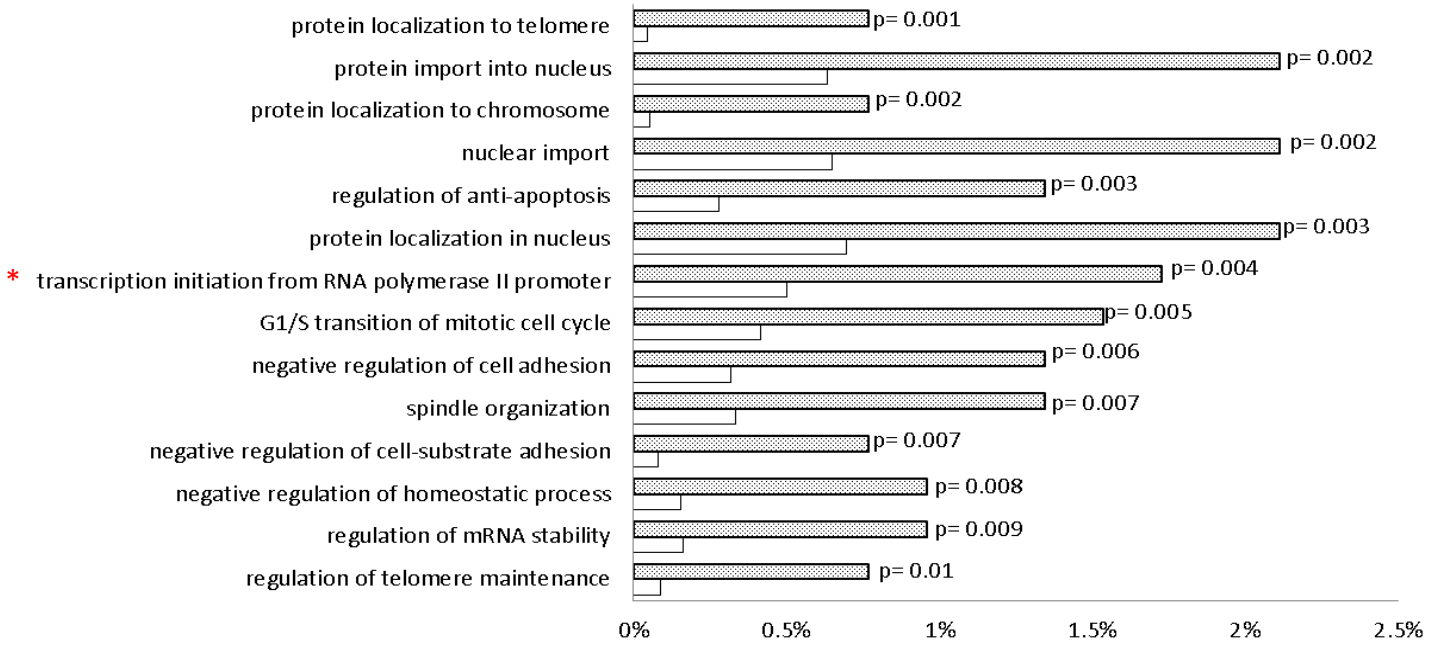


**Figure 3**



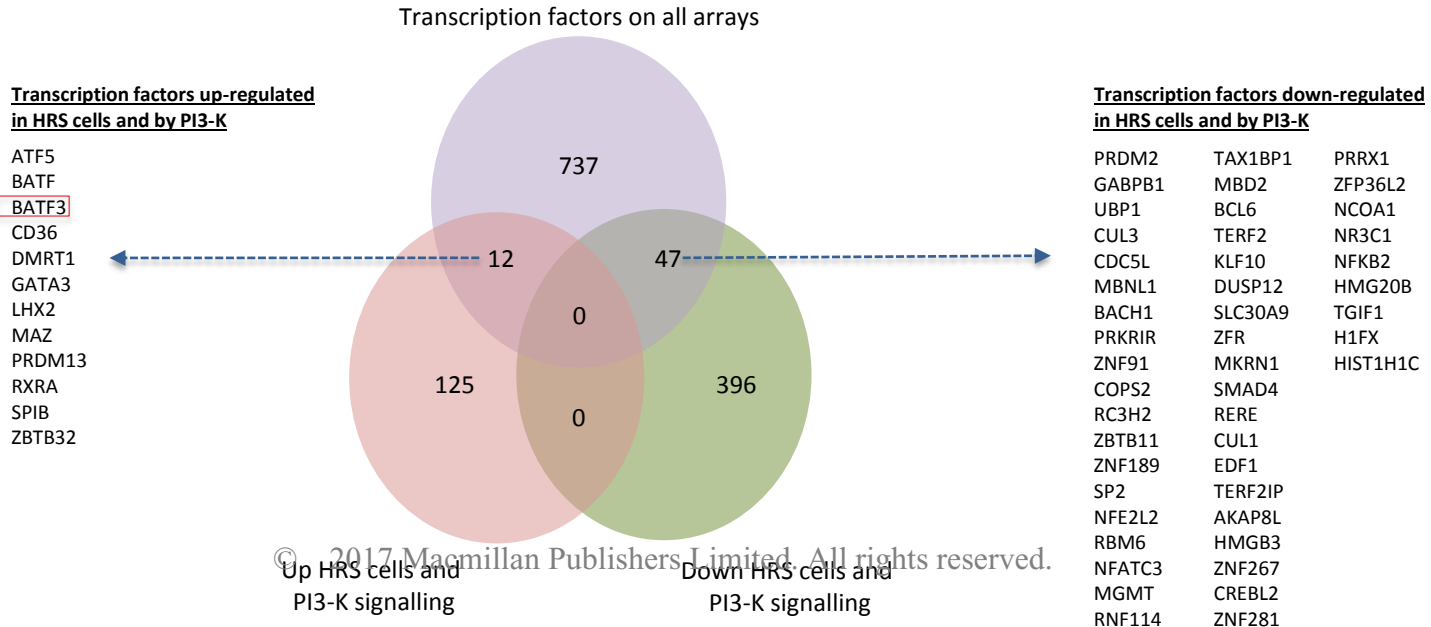
**Figure 4**

**A**



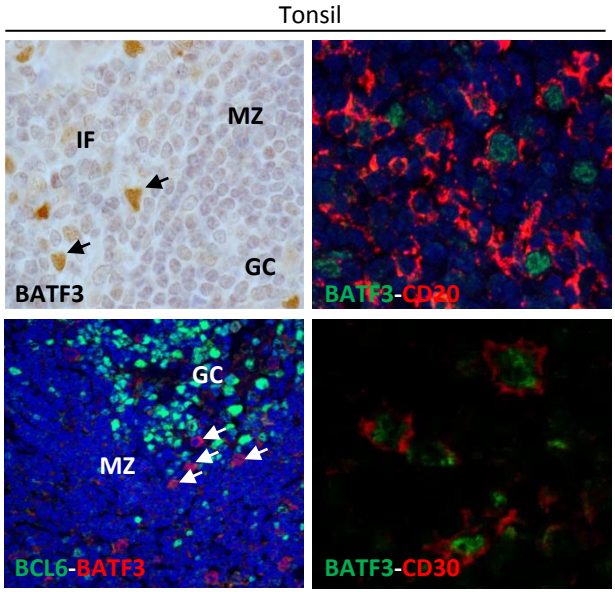
**B**

■ % of genes in regulated list with given GO term (Observed) □ % of genes in regulated list with given GO term (Expected)

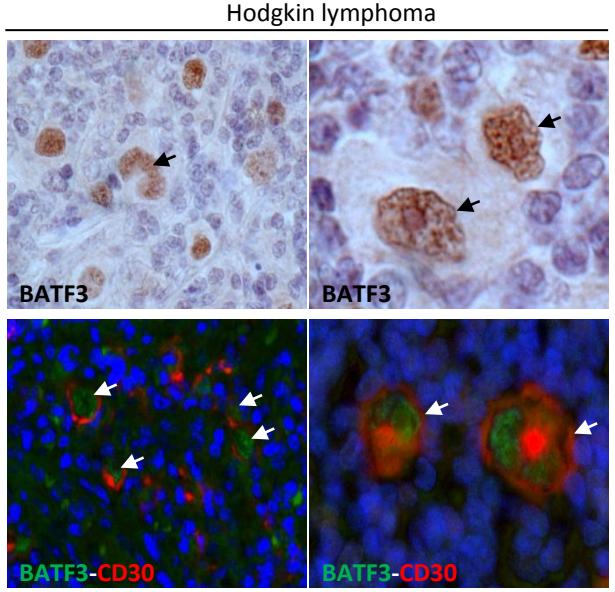


**Figure 5**

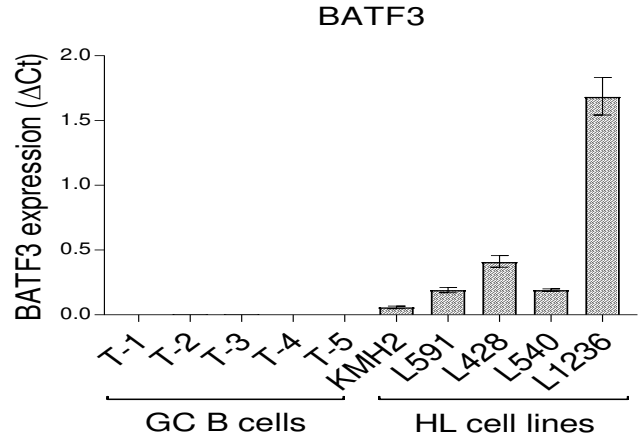
**A**



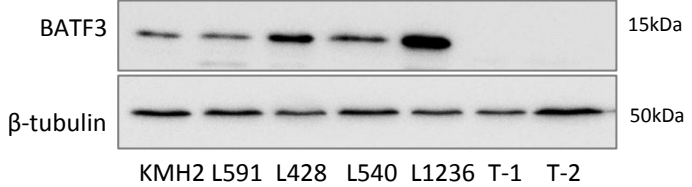
**B**



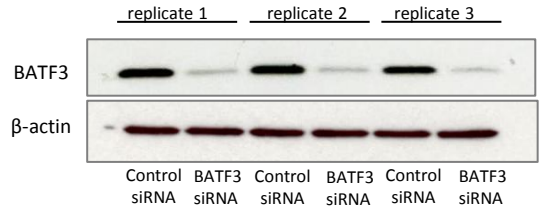
**C**



**D**

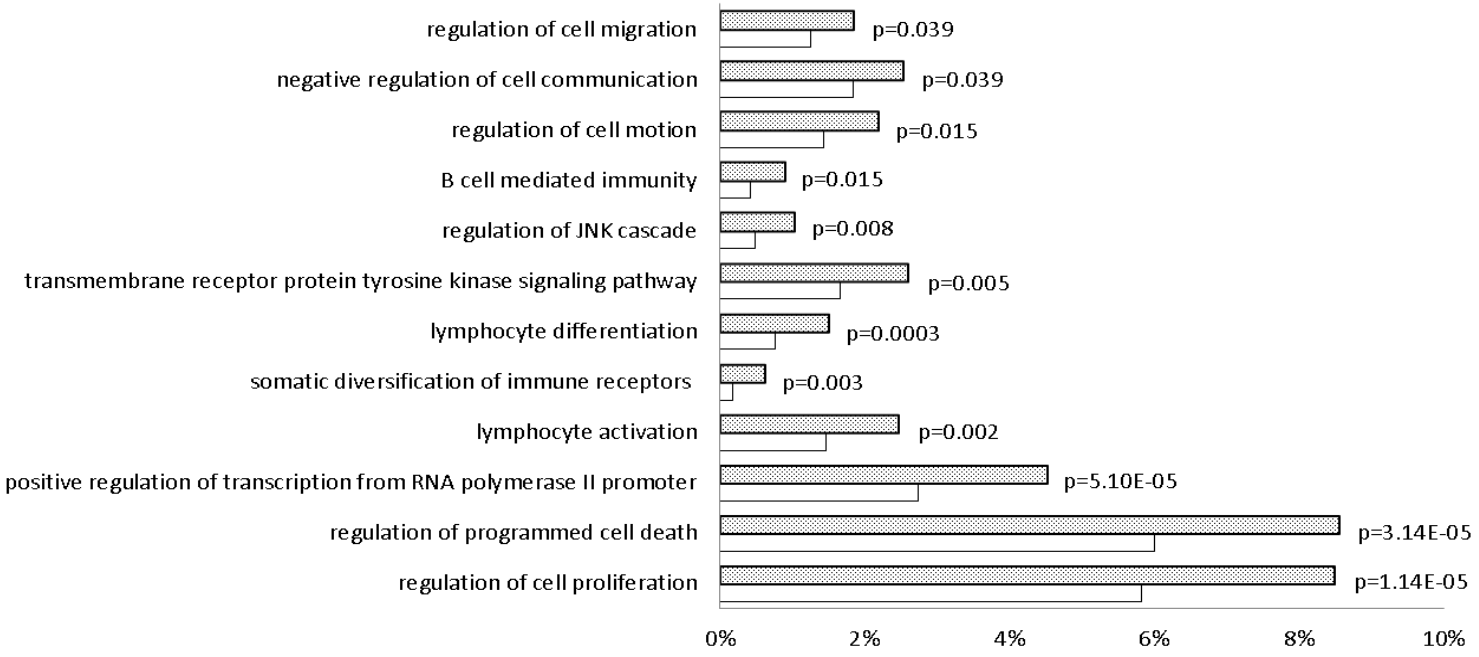


**Figure 6**



$p < 0.05$

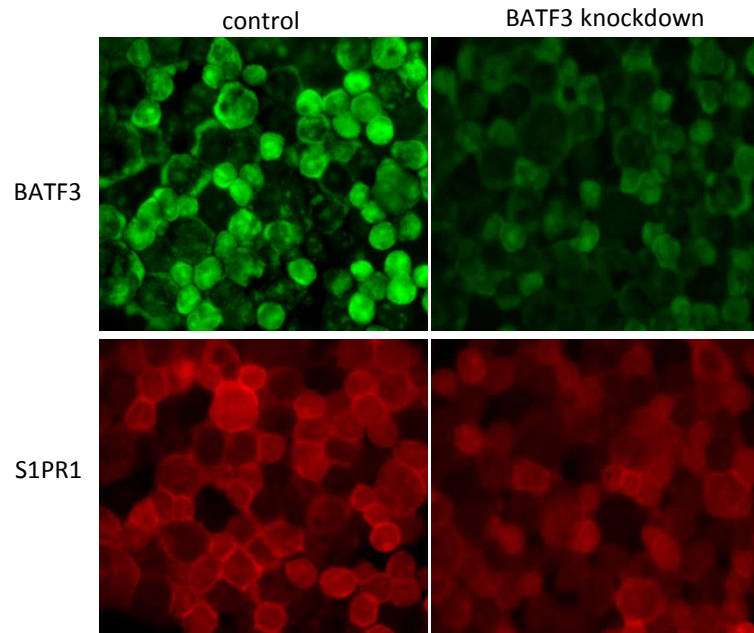
**BATF3 knockdown L428 cells**



■ % of genes in regulated list with given GO term (Observed)    □ % of genes in regulated list with given GO term (Expected)

Figure 7

A



B

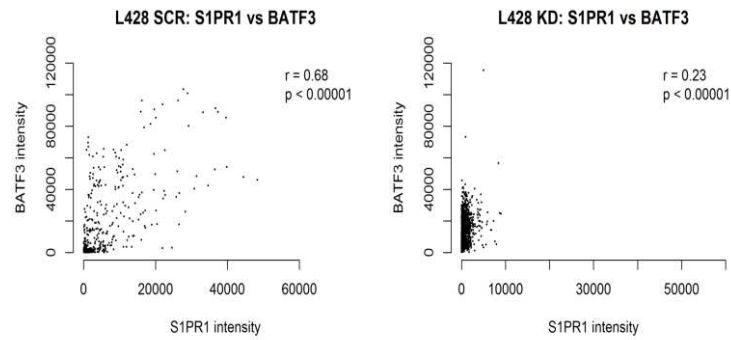


Figure 8

