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ZEB1 and CtBP form a repressive complex at a distal promoter element of the BCL6 locus

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Abstract

BCL6 is essential for normal antibody responses and is highly expressed in germinal centre B-cells. Constitutive expression due to chromosomal translocations or mutations of *cis*-acting regulatory elements contributes to diffuse large B-cell lymphoma. BCL6 expression is, therefore, tightly regulated in a lineage and developmental stage specific manner and disruption of normal controls can contribute to lymphomagenesis. In order to discover potential *cis*-acting control regions we carried out DNase I hypersensitive site mapping. Gel shift assays and chromatin immuno-precipitation of the core region of a hypersensitive site 4.4kb upstream of BCL6 transcription initiation (HSS-4.4) showed an E-box element binding ZEB1 and the co-repressor CtBP. As compared to peripheral blood B-cells, ZEB1, a two-handed zinc finger transcriptional repressor, is expressed at relatively low levels in germinal centre cells whereas BCL6 has the opposite pattern of expression. Transfection of ZEB1 cDNA caused a reduction in BCL6 expression and a mutated ZEB1, incapable of binding CtBP, lacked this effect. siRNA mediated knockdown of ZEB1 or CtBP produced an increase in BCL6 mRNA. We propose that HSS-4.4 is a distal promoter element binding a repressive complex consisting of ZEB1 and CtBP. CtBP is ubiquitously expressed and our results suggest regulation of ZEB1 is required for control of BCL6 expression.

Introduction

BCL6 is a zinc finger transcription factor that is highly expressed in normal germinal centre B-cells[1] and is essential for the production of high affinity antibodies[2, 3]. BCL6 is also involved in chromosomal translocations in 20 to 30% of human diffuse large B-cell lymphomas[4] and is expressed in follicular lymphoma, Burkitt's lymphoma and nodular lymphocyte predominant Hodgkin's lymphoma.

The protein is subject to several post-translational modifications that may promote its degradation[5, 6]. Transcriptional regulation is also important[7-9] especially in the transition from germinal centre B-cell to terminally differentiated plasma cell.

BCL6 inhibits its own transcription through a site in exon 1 of the gene, but the necessary mechanisms to over-ride this effect and allow sustained expression have not been described[7, 9]. BCL6 transcription is responsive to growth factor signalling through STAT5 in primary tonsillar B-cells[10]. The forkhead transcription factor, FoxO3a, which is downstream of PI3K can also drive BCL6 transcription[11] and FoxO3a binding sites have been identified in the BCL6 promoter[12]. Recently we showed that CD40 signalling both represses BCL6 transcription (through NF- κ B and IRF4) and induces it by activating p38 MAPK. We conclude that BCL6 transcription integrates strong repressive signals and weak positive signals such that the balance tends towards reducing BCL6 expression and we suggest that it may only be switched on transiently in small populations within the germinal centre.

In the B-cell lineage, BCL6 is expressed only in germinal centre cells and not in naïve B-cells or plasma cells. BCL6 is also expressed in other cell lineages. It is found in many types of epithelial cell and cardiac myocytes. BCL6 is expressed in normal breast epithelium and breast cancer, and high BCL6 expression correlates with histologically aggressive disease. BCL6 is, therefore, tightly controlled temporally and in a lineage specific manner. We sought to discover cis-acting sites that may contribute to these specific patterns of control.

We implicate the transcriptional repressor, ZEB1, in the control of BCL6. ZEB1 binds DNA through zinc finger DNA binding regions at both the N- and C-termini. ZEB1 recognises a subset of E-box sequences [13] as well as other less defined sequences [14]. ZEB1 accomplishes repression through interactions with the co-repressor, CtBP, which interacts with PLDLS and PLDLS-like sequences [15] as well as other co-repressors [16]. ZEB represses E-cadherin expression, and other proteins required to maintain epithelial cell polarity, and induces an epithelial to mesenchymal transition that may be important in promoting cancer invasiveness and metastasis (17-19). ZEB2 (also called SIP1) has a high degree of homology to ZEB1 and there is some degree of overlap in their expression [17]. ZEB1 is highly expressed in thymus and is required for T-cell differentiation. Its down-regulation accompanies leukaemogenesis in adult T-cell leukaemia/lymphoma (ATLL)[18]. Both ZEB1 and ZEB2 are expressed in B-cells[17], and ZEB1 regulates transcription from the immunoglobulin heavy chain locus [13]. In the model put forward in this work ZEB1 competes for E-box binding sites in the IgH enhancer with the transcriptional activator, E2A.

Using DNase I hypersensitive site mapping we found a distal promoter sequence at the BCL6 locus that has been shown to recruit CtBP [19]. We showed that an E-box at this site binds ZEB1 and that ZEB1 over-expression and siRNA mediated knockdown cause changes in BCL6 expression.

Materials and Methods

Cell lines and tonsils

Germinal centre representative Burkitt's lymphoma cell lines DG75 and Ramos, plasma cell line KMS11, T-cell line Jurkat and HEK293 were grown in RPMI-1640 (Lonza, Basel, Switzerland) supplemented with 10% fetal calf serum (Lonza, Basel, Switzerland), 2% 1-

glutamine and 5% penicillin/streptomycin (Invitrogen, Paisley, UK). DG75 for siRNA mediated knockdown were obtained from Dr. Antonio Parrado (Immunology Unit, University Hospital Virgen de la Arrixaca, Murcia, Spain).

Single cell suspensions were made from tonsils (obtained with permission of the Local Research Ethics Committee). B-cells were separated using Robosep immunomagnetic cell separator with the EasySep Human B cell Enrichment kit (Stem Cell Technologies, Catalogue number 19054). The B-cell population was ~95% pure as assessed by staining with an anti-CD19 antibody.

DNase I hypersensitive site mapping

Approximately 50×10^6 exponentially growing cultured cells or 30×10^6 freshly isolated tonsillar B cells were harvested by centrifugation on the day of the experiment. The cell pellet was washed twice in cold PBS and finally resuspended in 1 ml of ice-cold RSB buffer (hypotonic buffer) per 5×10^6 cells. After DNase I digestion (described in Supplementary Methods) 20 μ g of the resulting DNA was used for each restriction enzyme digest. Digestion was carried out in a total volume of 50 μ l, using 100U of a restriction endonuclease (New England Biolabs, Ipswich, MA, USA). After overnight digestion the samples were loaded on a 0.8 % agarose gel and electrophoresed at 55V overnight. Southern transfer was carried out overnight. Nylon filters were hybridized with probes (Figure 1A and Supplementary Table 1).

Luciferase reporter assays

Genomic fragments of the BCL6 promoter region including HSS-4.4 (Supplementary Table 2) were amplified using *Pfu* DNA polymerase (Promega, Madison, WI, USA) (200ng template DNA, 1x*Pfu* Buffer, 300nM of each primer, 0.06U *Pfu*, 200nM of each dNTP) in a 50 μ l reaction. 30 cycles were carried out at 95°C for 1 minute, 62°C 30 seconds, 72°C 1 minute. In order to mutate the E-box sequence 5'-cttctgcatgggttaacatacaagagggcg was used. PCR product was purified restriction enzyme digested and cloned into the pGL3Promoter vector (Promega, Madison, WI, USA). 4 to 7 kb long fragments (Supplementary Table 2) were amplified using a long template PCR system (Expand Long Template PCR, Roche, Indianapolis, IN, USA) in a 50 μ l reaction (40ng template DNA, 300nM of each primer, 0.4 μ l of DNA polymerase mix and 200nM of each dNTP) and cloned into the pGL3Basic vector. Thermal cycling conditions were 98°C 1 minute 30 cycles of: 98°C 30 seconds, 60°C 30 seconds, 68°C 10 minutes and finally 10 minutes at 68°C.

Chromatin immunoprecipitation

10×10^6 cultured cells (KMS11, DG75) per antibody to be used were cross-linked in their culture medium with 1% formaldehyde at 37 °C for 10 minutes. Cross-linking was stopped with the addition of glycine solution to a final concentration of 0.125M. Cells were then harvested by centrifugation and washed twice with ice cold PBS. Sonication and chromatin precipitation were then carried out as described in Supplementary Methods.

Transfection

The adherent HEK293 cell line was plated in 6-well plates in 2.5ml of complete medium at a density of 5×10^5 cells/well one day prior to transfection. On the day of transfection, 5 μ l of Lipofectamine 2000 (Invitrogen, Paisley, UK; catalog number-11668-019) was mixed with the appropriate amount of DNA in 400 μ l of serum-free medium followed by incubation for 30 minutes at room temperature. The transfection mixture was then added to the plated cells (400 μ l of mixture to each well of a 6 well plate) and the lipofected adherent cells were harvested by trypsinization 18 hours post transfection or 36 hours post transfection if an expression plasmid i.e. pCS2-MT-ZEB1 (ZEB1 cDNA), was transfected simultaneously with the reporter plasmids.

DG75 was transfected by means of a Nucleofector (Lonza Amaxa AG, Cologne, Germany) using program O-006 and solution V.

2µg of plasmid with cloned HSS-4.4 was transfected but 4µg of plasmids containing the longer constructs (4 to 7 kb). 1 µg of a Renilla luciferase expression vector, pRL-TK (Promega, Madison, WI, USA) was co-transfected in all experiments.

Gel shift assays

20x10⁶ Ramos or KMS11 cells were harvested and nuclei were prepared as for DNase I hypersensitive site assays. The nuclear pellet was resuspended in 500µl of RSB buffer for 20x10⁶ cells and then brought to a volume of 2 ml by the addition of 25% sucrose/RSB. Cell lysates were produced and binding reactions carried out as described in Supplementary Methods.

Real-time RT-PCR

RNA was extracted using the Qiagen RNeasy kit (Qiagen, Hilden, Germany) and the Qiagen RNase-free DNase I set (Qiagen) or Trizol (Invitrogen, Paisley, Scotland). 2µg of RNA was used per reverse transcription reaction (Omniscript RT kit (Qiagen)) Reactions were incubated at 37°C for 1 hour and then stored as total cDNA sample at -20°C or used immediately for Real-Time PCR as cDNA template. For siRNA knockdown cDNA was generated using Transcriptor High Fidelity cDNA Synthesis kit (Roche, Mannheim, Germany) and real time-PCR was carried out with SYBR Green qPCR Master Mix (Fermentas Inc, Ontario, Canada).

For Real-Time PCR, specific assays were purchased from Applied Biosystems, Foster City, CA, USA: BCL6, Hs00153368_m1; BLIMP1, Hs00153357_m1; HPRT, Hs99999909_m1. The PCR primers used for ZEB1 and CtBP were previously published [20-22]. GAPDH was used as housekeeping control gene to normalize data [23]. Annealing condition for all primers were 60°C for 45 seconds. Each sample was analyzed in triplicate. Expression levels were normalized to GAPDH. Relative values are represented.

siRNA knockdown

DG75 was transfected with siRNA oligonucleotides at 50nM using Lipofectamine RNAiMAX (Invitrogen). siRNA oligos were ordered from Invitrogen using sequences described in the literature: ZEB1 [24], and CtBP [25]. As a negative control we used Low GC-Stealth RNAi Negative Control (Invitrogen).

Results

A DNase I hypersensitive site 4.4kb upstream of the transcription start site

In order to discover cis-acting sites responsible for these specific patterns of control we carried out DNase I hypersensitive site mapping of the BCL6 locus. 35kb of the locus (from 19.5kb upstream of the transcription start site to 15.1kb downstream) were mapped (Figure 1A) and strong DNase I hypersensitive sites were found in DG75 human EBV negative Burkitt's lymphoma cells at +0.3kb and +0.5kb from the transcription start site and at -4.4kb (HSS-4.4) (Figure 1B and Supplementary Figure 1).

Jurkat human T-cell line possesses a weak hypersensitive site (HSS-1.9) within the *Swa* I restriction fragment but do not have HSS-4.4 demonstrating that this site is B-cell specific (Figure 1B). Purified human tonsillar B-cells also demonstrate HSS-4.4 (Figure 1C).

We conclude that there is a DNase I hypersensitive site at the BCL6 locus in primary human B-cells 4.4kb upstream of the BCL6 transcription start site. A sequence of 356bp flanked by 5'-polyT and 3'-polyA (forming a partial inverted repeat) is present at this location, of which the core sequence of 240bp shows homology to the dog and mouse sequences (Figure 2A and 2B).

We carried out genomic comparison[26], between human, mouse and dog, across a 30kb region comprising intron 1, exon 1 and approximately 20kb of upstream sequence. Pairwise comparisons (Figure 2B) reveal sections of non-coding sequence homology. HSS-4.4

corresponds to a site of >50% sequence homology, separated from the proximal promoter region by sequence showing no homology between species. Other hypersensitive sites - HSS+3.8 and HSS+5.8 - reported in this work also appear to correspond with regions of sequence homology.

Single stranded sections of promoter regions can be recognized by transcription factors and contribute to recruitment of specific proteins and tight gene regulation [27, 28]. The partial inverted repeat sequences suggested that DNA at this site could form a secondary structure. Utilizing M-fold (<http://mfold.bioinfo.rpi.edu/>) [29, 30] we found that the 356bp sequence can be folded (Figure 2C) and the lowest free energy form (Figure 2D) appears to form a cruciate structure. HSS-4.4 is, therefore, a potential site for a *cis*-acting control region and we focused on this for further investigation.

ZEB1 binds at HSS-4.4

ChIP-chip data has shown that the co repressor CtBP is recruited to exon 1 of BCL6 and also to a site upstream of the coding sequence at approximately the location of HSS-4.4 [19]. CtBP associates with several transcription factors including the two-handed zinc finger, ZEB1 [15]. This protein binds to E-boxes and its binding is facilitated by the additional consensus sequence GTTTC[14], both of which are present in HSS-4.4 (Figure 2A).

In order to analyze potential protein binding sites we carried out gel shift assays using overlapping oligonucleotides across the core sequence. Oligos 3,4,6 and 7 showed shifted bands. We chose to focus on Oligo7, which showed two strong shifted bands and possessed an E-box sequence (CAGGTGT) (Figure 3A). In gel shift assays protein binding was competed less well by an oligonucleotide bearing mutations in the E-box sequence demonstrating its importance in forming the protein/DNA complex. We, therefore, chose to test the hypothesis that HSS-4.4 is the site of binding of a ZEB1/CtBP complex.

The ZEB1 protein complex involves CtBP and E2A

ZEB1 was cloned from a B-cell line and found to compete with E2A to regulate transcription at the immunoglobulin H-chain locus, although subsequently most work has been carried out on its role in epithelial cells and T-cells. Using supershift antibodies (Figure 3B) we showed that in the Burkitt's lymphoma cell line, Ramos, (which does not express ZEB1, but does express E2A), an anti-E2A antibody produced a supershifted band with concomitant reduction in intensity of the upper band obtained with oligo7 (grey arrowhead Figure 3B). E2A is not expressed in the plasma cell line, KMS11, which does express ZEB1. Anti-ZEB1 antibody produced a decrease in intensity of the upper shifted band. Both ZEB1 and E2A can, therefore, bind to oligonucleotide-7.

Next we used ChIP to analyze *in vivo* protein binding to HSS-4.4 (Figure 3C). We again compared a germinal centre representative, DG75, with the plasma cell line, KMS11. DG75 expressed ZEB1 and we were, therefore, able to immuno-precipitate the endogenous protein. CtBP and ZEB1 were immuno-precipitated from both cell lines, and E2A was only present at HSS-4.4 in DG75. We conclude that HSS-4.4 is the previously identified site of CtBP recruitment and that it is also capable of binding ZEB1 and E2A.

Transcriptional activity of HSS-4.4

Luciferase reporter assays were carried out in order to measure the transcriptional activity of the isolated HSS-4.4 sequence. An 800bp fragment that included HSS-4.4 showed a 12-fold increase in transcription (as compared to empty vector) in DG75 but not in Jurkat cells or the human embryonic kidney line, HEK293 (Figure 3D) demonstrating that this sequence responds to B-cell specific transcription factors. Next we analyzed position and orientation dependence of transcription (Figure 3E) and showed that only when this sequence was upstream of the luciferase reporter gene and in the correct orientation did it have transcriptional activity. We conclude that HSS-4.4 is a distal element of the BCL6 promoter.

Role of HSS-4.4 in the BCL6 promoter

Elements regulating BCL6 transcription have been found in the proximal part of intron 1[8, 31], exon 1[7, 9] and about 1.3kb upstream of the transcription start site[12]. CtBP is recruited to the BCL6 binding site in exon 1 and, therefore, we used luciferase reporter assays to assess the transcriptional effects of HSS-4.4 in the context of the whole BCL6 promoter in a B-cell line expressing BCL6 (Figure 3F).

Deletion of the HSS-4.4 sequence up to the E-box (Figure 3Fii) produced a non-significant increase in transcription. However, mutation of the E-box sequence almost abolished transcription. Changing the mutated site to the wild-type sequence restored transcriptional activity demonstrating that this specific change was responsible for the effect observed. Binding to the E-box sequence is, therefore, essential for activity.

Expression of ZEB1 and BCL6

Previous work suggested a reciprocal relationship between ZEB1 and E2A in B-cells[13]. We, therefore, analyzed protein expression in cell lines (Figure 4A). ZEB1 is highly expressed in the human T-cell line, Jurkat, and not expressed in HEK293 cells. It is expressed at low levels in the human plasma cell line, KMS11 and the Burkitt's lymphoma cell line, DG75. However, Ramos, another Burkitt's line showed absent ZEB1 and high expression of E2A. CtBP was detectable in all lines and BCL6 was only expressed in Ramos and DG75. In purified human tonsillar B-cells BCL6 and E2A associated with low ZEB1. Therefore, the germinal centre representative cell line, Ramos, and primary tonsillar cells support the association of E2A with BCL6 and absent or reduced ZEB1.

To further analyze the correlation between ZEB1, E2A and BCL6 we turned to gene expression microarray data utilizing primary cells [32] (www.llmpp.nih.gov/lymphoma/) (Figure 4B and 4C). We found a reciprocal relationship between ZEB1 expression (high in peripheral blood B-cells and low in germinal centre B-cells) and BCL6. ZEB1 expression was higher in peripheral blood B-cells (median mRNA level relative to the whole microarray=1.5) than in germinal centre cells (median=0.74). BCL6 expression is associated with the germinal centre (GC) type of human diffuse large B-cell lymphoma in a "cell of origin" classification[32], and with the BCR cluster in a functional classification[33]. ZEB1 expression is suppressed to normal germinal centre levels (median=0.69) in GC-DLBCL, and is higher in ABC-DLBCL (median=1.15), however the difference is not significant (Mann-Whitney U-test) and there is no correlation with overall survival. E2A expression is low in resting B-cells and higher in normal germinal centre B-cells (similar to that of BCL6), but in the cell lines tested on the microarray there seems to be high E2A with low BCL6.

Over-expression of ZEB1 represses BCL6 transcription

Utilizing the Burkitt's cell line, DG75, we next tested whether over-expression of ZEB1 can regulate BCL6 (Figure 5A). Both 1µg and 5µg of ZEB1 cDNA produced reduction in BCL6 mRNA. Statistically significant reduction ($p=0.03$, Mann Whitney U-test) was obtained with 5µg ZEB1 cDNA. Westerns showed that the reduction in BCL6 mRNA was sufficient to produce a decrease in BCL6 protein expression (Figure 5B). BLIMP1 is a transcription factor that is required for B-cell terminal differentiation to plasma cells, and is also directly repressed by BCL6. Therefore, in order to show that the reduction in BCL6 protein was functionally important we measured BLIMP1 mRNA (Figure 5C). Transfection of 5µg of ZEB1 cDNA produced a 5-fold increase in BLIMP1 mRNA demonstrating an effect on downstream BCL6 targets. Finally, in order to show that the ZEB1 effect on BCL6 was mediated through an interaction with CtBP we employed a mutated form of ZEB1, ZEB1-CID, in which the CtBP interaction sites in ZEB at residues 705, 734 and 767 were mutated from PLDLS/T to ASASA thus preventing CtBP binding. The ZEB1 effect was reverted by mutation of the CtBP binding sites as ZEB1-CID failed to repress BCL6 or induce BLIMP1 mRNA expression (Figure 5B). We conclude that ZEB1 repressed BCL6 transcription in a mechanism that involved binding of the CtBP co-repressor.

siRNA knockdown of ZEB1 or CtBP increases BCL6 mRNA expression

As a further test of the repressive effects of the ZEB1/CtBP complex on BCL6 expression we used siRNA mediated knockdown (Figure 5D) in DG75. Reduction of either ZEB1 or CtBP produced a ~2.5-fold increase in BCL6 mRNA. We conclude that under basal conditions the ZEB1/CtBP complex contribute to repression of transcription at the BCL6 locus.

Discussion

BCL6 represents a model gene for analyzing lineage and stage specific regulation of gene expression. It is expressed in primary epithelial cells[34] and in breast cancer[35], although most work has been carried out on its targets and function in germinal centre B-cells and lymphomas derived from these cells.

In this initial study we used DNase I hypersensitive site assays to discover potential *cis*-acting control regions. By this means we discovered HSS-4.4, a site that appeared to correspond to a site of CtBP recruitment, whose relevance was unknown[19]. CtBP is an important co-repressor for ZEB1[15], a transcription factor that was cloned by its ability to bind to the delta 1-crystallin core enhancer [36] and subsequently from a λ -phage expression library through its ability to bind to E-box sequences at the IgH enhancer in B-cells [13]. Most work has been carried out on the role of ZEB1 in the epithelial-mesenchymal transition (EMT), a process in which polarized epithelial cells lose expression of some epithelial markers including E-cadherin, and acquire a more motile (mesenchymal) phenotype. An EMT-like process is believed to take place in carcinomas as they invade surrounding normal tissues. ZEB1 represses E-cadherin and is one of several transcription factors (together with ZEB2, SNAI1 and SNAI2) that is up-regulated during EMT [37]. Target genes of ZEB1, or those that prevent EMT, are anticipated to be down-regulated during EMT. It is interesting that in one study of gene expression changes in EMT in epithelial cells, BCL6 mRNA was down-regulated [38] consistent with increased levels of ZEB1 in such a process. BCL6 is expressed in diverse epithelial tissues [34] and may have a role in epithelial development as well as B-cell development. A function for ZEB1 in the regulation of BCL6 in B-cells may represent a further link between BCL6 and epithelial cell development.

Others have shown that CtBP is a co-repressor for BCL6 at its binding site within exon1[19]. This binding site has a powerful inhibitory effect on BCL6 transcription, and whilst mutations of the BCL6 binding site are associated with constitutive BCL6 expression in lymphomas [7], it is not clear how the consequences of binding are normally avoided to allow sustained BCL6 expression. We suggest a model (Figure 5E and 5F) in which repressive complexes involving both the BCL6 binding site in exon 1 and HSS-4.4 regulate BCL6 transcription. Both ZEB1 and BCL6 are known to use CtBP as a co-repressor and we speculate that a complex is formed that involves binding of transcription factors to HSS-4.4 and exon 1 (respectively ZEB1 and BCL6) that are linked through binding of a common co-repressor, CtBP. In the absence of ZEB1 the HSS-4.4 site may be occupied by E2A, which forms part of an activator complex.

We carried out most of our experiments using the Burkitt's lymphoma cell line DG75. This line expresses both ZEB1 and E2A and may potentially be able to assemble both activating and repressive complexes, although the activating E2A complex is present in basal conditions (because BCL6 protein is expressed). Evidence for the presence of an activating complex under basal conditions comes from the luciferase reporter assays (Figure 3A) which demonstrate an activating effect of HSS-4.4. However, transient transfection of ZEB1 cDNA (Figure 5B) show repression of BCL6 expression. This result is compatible with our model: over-expression of ZEB1 caused the repressive complex to form and the consequent decrease in BCL6 expression.

ZEB1 binds to a subset of E-boxes. Does the E-box site at HSS-4.4 conform to a consensus ZEB1 site? A systematic approach to discovering the sequence requirements for ZEB1 binding showed that the zinc fingers at the N- and C-terminals can bind separately to a

consensus CACCTGT. The entire protein can also bind to this sequence together with "additional consensus sequence" GTTTC/G that is spaced and oriented variably with respect to the consensus[14]. More recently it has been demonstrated that each zinc finger domain of ZEB1 binds to separate and inverted target sites[39] such as are found in several ZEB1 target promoters. However, ZEB1 also binds to single CACCT sites [13, 40-44] and exerts transcriptional repression suggesting that context dependent factors have a role. A CACCTGT motif is present at HSS-4.4 (Figure 3), but there is no related inverted site although 150bp upstream there is a GTTTC. Although the single CACCTGT is sufficient for *in vitro* binding these two sites may co-operate *in vivo*.

We found higher ZEB1 expression in ABC-DLBCL than in GC-DLBCL with BCL-6 showing the opposite association. This is consistent with a role of ZEB1 as a repressor of BCL-6. There was no correlation of ZEB1 expression with overall survival but, in this study, and unlike others[45], there is also no correlation of BCL-6 expression with survival. Therefore, a larger study to analyze expression of ZEB1 in DLBCL is required. Another factor reducing the impact of ZEB1 is that a proportion of these lymphomas will have mutation or translocation of the BCL6 locus to cause constitutive expression and, therefore, bypass regulation by ZEB1.

This is the first report suggesting a germinal centre B-cell function for ZEB1 through repression of BCL6, a gene essential for germinal centre formation. Previous work showed a B-cell role for ZEB1 is repression of transcription at the IgH enhancer[13] and ZEB1 in B-cells has also been implicated in repression of BZLF1, an EBV immediate early gene, that controls the switch between latent and lytic infection [41, 46]. In epithelial cells ZEB1 expression is regulated by TGF β signalling[47] through SMAD-dependent and independent pathways. The miR-200 family also has a controlling role in EMT[48, 49], in part, through the regulation of ZEB1. There are major differences in ZEB1 expression between peripheral blood and germinal centre B-cells but the factors regulating this protein in B-cell development are unknown and will give insights into germinal centre formation and lymphomagenesis.

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Figure Legends

Figure 1

A DNase I hypersensitive site 4.4kb upstream of the BCL6 transcription start site. (a) Restriction map of the BCL6 locus from 19.5kb upstream of the transcription start site to 15.1kb downstream and including exons 1 to 4. Arrow indicates the transcription start site. Numbers are the distance upstream (-) or downstream (+) of the transcription start site. Numbered grey bars represent the position of the probes used. (b) DNase I hypersensitive site mapping of a *Swa* I restriction fragment in DG75 and Jurkat cells with probe 2 (Pr2). (c) DNase I hypersensitive site mapping of a *Swa* I restriction fragment in purified human tonsillar B-cells. Black arrowhead indicates the germline band and labelled grey arrows the hypersensitive sites. Black wedges above the autorad indicate the use of increasing amounts of DNase I.

Figure 2

Sequence at HSS-4.4. (a) Comparison of human (Hs), mouse (Mm) and dog (Cf) sequence at HSS-4.4. Shared bases are shaded in grey. The oligonucleotide used in the gel shift assay (Figure 3C) is indicated by a black line. (b) Pairwise comparison of mouse/human (upper panel) and mouse/dog (lower panel). The position of exon 1 is indicated by a black rectangle above the panels with intron 1 to the left and the proximal promoter (grey bar) to the right. Sites of sequence homology corresponding to HSS+5.8, HSS+3.8 and HSS-4.4 are indicated. (c) Dot plot shows base pairing for the optimal energy for folding of 356bp at HSS-4.4 (lower triangle) and base pairing for all four predicted structures (upper triangle). (d) Structure for optimal energy folding of the 356bp sequence. Sequences outlined in red are the consensus (CAGGTGT) and additional consensus ZEB1 binding site (GTTTC).

Figure 3

Protein binding and transcriptional activity of HSS-4.4. (a) Gel shift assay using wild-type and E-box mutated oligonucleotide as a probe and lysates from DG75. Lane 1-wild type probe without lysate, Lane 2-mutated oligonucleotide without lysate, Lane 3-wild-type probe with lysate, Lane 4-mutated probe with lysate, Lane 5-wild-type probe with lysate, Lanes 6, 7 and 8-labelled wild-type probe with 2, 5 and 10pmol unlabelled competitor wild-type probe, Lanes 9, 10 and 11- labelled wild-type probe with 2, 5 and 10pmol unlabelled competitor mutated probe. (b) Gel shift assay. Left hand panel-Wild-type oligonucleotide and lysates from DG75 (lane 1) with increasing amounts of anti-E2A antibody (lanes 2 and 3) Black arrowhead indicates the super-shifted band and the grey arrowhead the band of reduced intensity. Right hand panel-wild type oligonucleotide and lysate from KMS11 (lane 1) with increasing amounts of anti-ZEB1 antibody (lanes 2 and 3). Black arrowhead indicates band of decreased intensity. (c) Chromatin immunoprecipitation. Lane 1-100bp molecular weight markers, Lane 2-PCR for HSS-4.4 in the input material, PCR from material immunoprecipitated with an anti-E2A antibody (Lane 3), an anti-ZEB1 antibody (Lane 4), and an anti-CtBP antibody (Lane 5), and also from a control with normal rabbit serum (Lane 6). (d) Luciferase reporter assays. 800bp of genomic sequence centring on HSS-4.4 was cloned upstream of a reporter gene and transcriptional activity was measured in HEK293, DG75 and Jurkat cells. (e) Position and orientation dependence of this sequence was tested in DG75. The placement of the test sequence relative to the luciferase gene is indicated in the diagrams to the left. The black bar represents the E-box sequence at the 3' end of HSS-4.4, and * indicates the E-box mutation described in Materials and Methods. (f) Luciferase constructs are shown diagrammatically to the right and include HSS-4.4 (with the E-box) shown as a black line) and exon1. (f) Effects of deletion or mutation of HSS-4.4. (i) 6.9kb including HSS-4.4, exon 1 and 1.5kb of intron 1 (ii) is a deletion of the 5' portion of HSS-4.4 up to the E-box and (iii) is mutation of the E-box alone.

Figure 4

Expression of ZEB1 and BCL6. (a) Westerns of ZEB1, BCL6, E2A, CtBP and GAPDH (loading control) in Burkitt's cell lines (Ramos and DG75), plasma cell line (KMS11), T-cell line (Jurkat) and HEK293 cells. In addition expression of these proteins in purified human tonsillar B-cells is shown. (b) Scatter plot showing gene expression analysis (using data from www.llmpp.nih.gov/lymphoma/) [32] of BCL6 mRNA in normal peripheral blood B-cells (PBL), normal germinal centre cells (GC) and the two subtypes of DLBCL (GC-DLBCL and ABC-DLBCL). Average across the whole microarray=1. (c) Scatter plot showing gene expression analysis of ZEB1 mRNA levels. Left-hand panel shows expression in peripheral blood and right-hand panel expression in GC and GC-DLBCL and ABC-DLBCL. (d) Scatter plot showing gene expression analysis of E2A mRNA levels in peripheral blood, GC and GC-DLBCL and ABC-DLBCL.

Figure 5

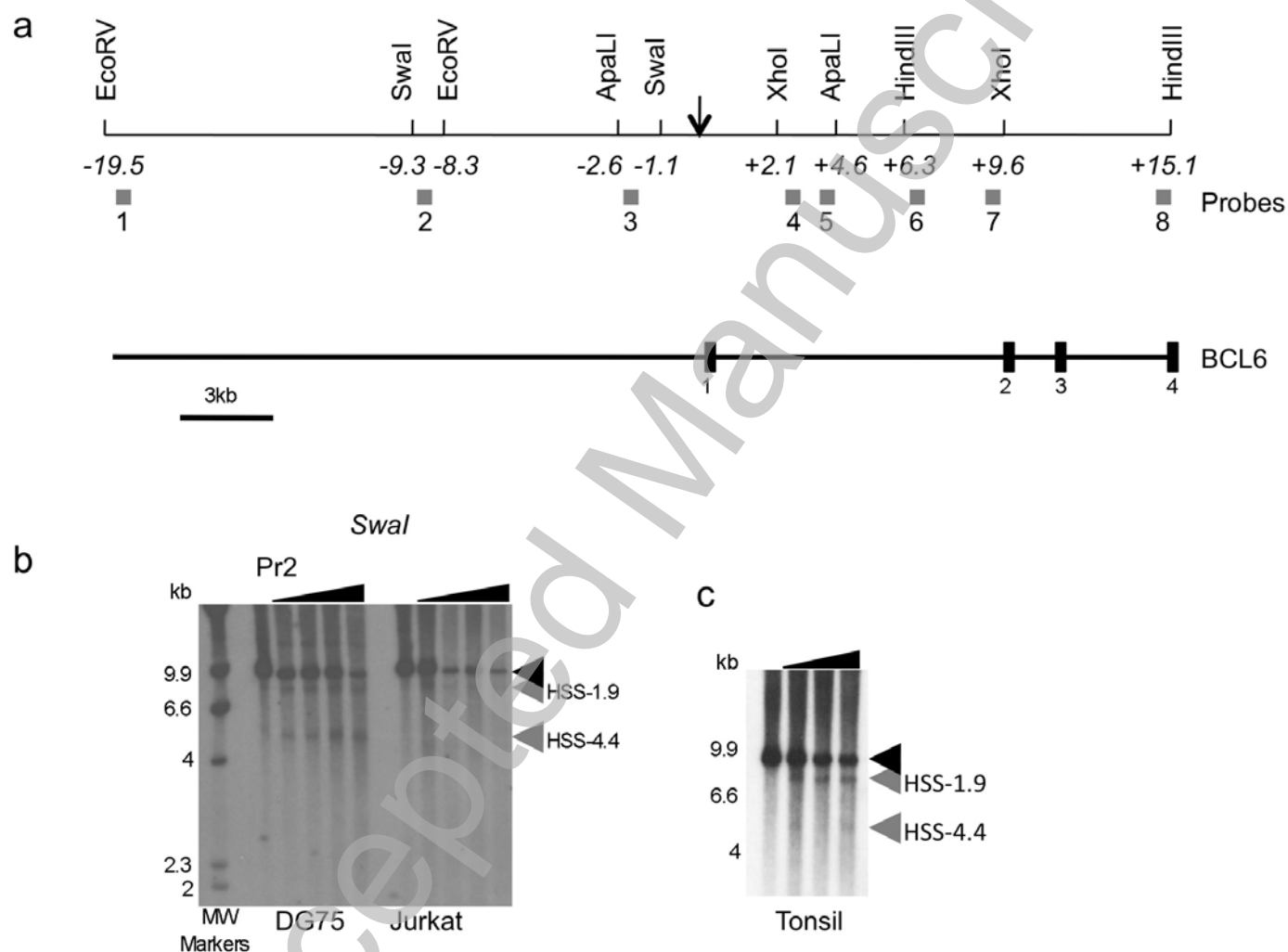
ZEB1 represses BCL6 mRNA and protein and requires its CtBP sequence. (a) Taqman RT-PCR (representative of 3 experiments) showing BCL6 mRNA expression following transfection of DG75 with wild-type ZEB1 cDNA (ZEB1) or ZEB1 bearing mutations in its CtBP binding site (ZEB1-CID). 5µg of ZEB1 cDNA produces a significant ($p=0.03$, Mann-Whitney U-test) reduction in BCL6 mRNA. (b) Western (representative of 3 experiments) showing effect of transfection of ZEB1 or ZEB1-CID cDNA on expression of endogenous BCL6 protein. ZEB1 and ZEB1-CID transfection was consistently associated with the appearance of lower molecular weight material (grey arrowhead), which may represent degradation. A non-specific band on the BCL6 western is indicated by ns. (c) Taqman RT-PCR (representative of 3 experiments) showing BLIMP1 mRNA expression following transfection with ZEB1 and ZEB1-CID. (d) DG75 cells were transfected with negative control siRNA or siRNA against ZEB1 or CtBP. ZEB1, CtBP or BCL6 mRNA was then measured and results are expressed relative to expression using the control siRNA. Results are representative of three experiments. (e) Model of ZEB1 complex at the BCL6 locus. BCL6 transcription is associated with E2A expression and E2A is capable of binding the HSS-4.4 E-box (Figure 5). Other transcription factors e.g. STAT5, induce BCL6 expression and this model does not imply that E2A is either necessary or sufficient for transcription. (f) BCL6 transcription is repressed. CtBP recruitment to exon 1 and HSS-4.4 has been proven [19], as has BCL6 binding to exon 1 [9]. We have shown ZEB1 binding to HSS-4.4. We speculate that these proteins form a repressive complex at the BCL6 locus.

References

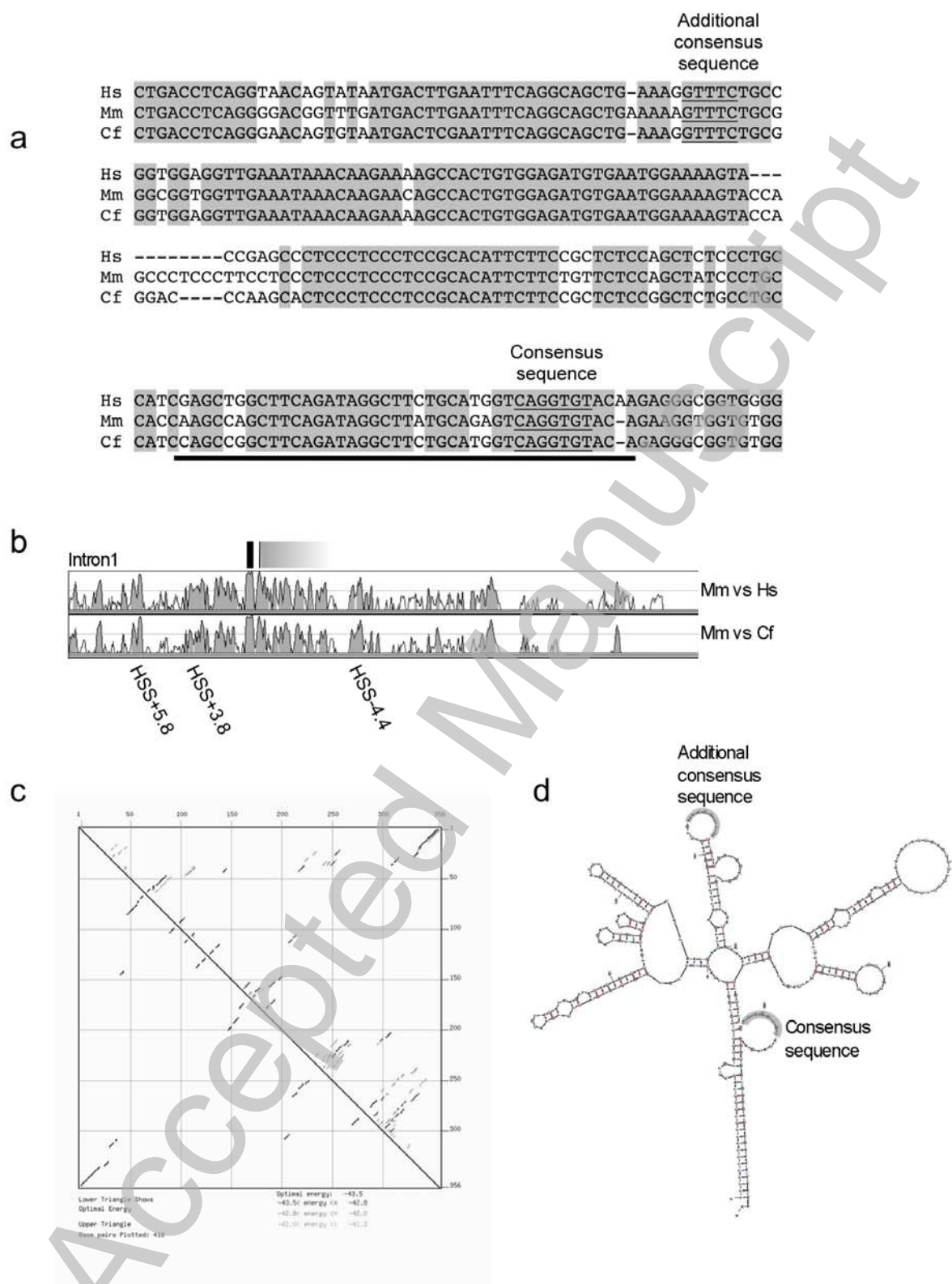
- 1 Cattoretti, G., Chang, C. C., Cechova, K., Zhang, J., Ye, B. H., Falini, B., Louie, D. C., Offit, K., Chaganti, R. S. and Dalla-Favera, R. (1995) BCL-6 protein is expressed in germinal-center B cells. *Blood*. **86**, 45-53
- 2 Dent, A., Shaffer, A., Yu, X., Allman, D. and Staudt, L. (1997) Control of inflammation, cytokine expression and germinal centre formation by BCL-6. *Science*. **276**, 589-592
- 3 Ye, B. H., Cattoretti, G., Shen, Q., Zhang, J., Hawe, N., de Waard, R., Leung, C., Nouri-Shirazi, M., Orazi, A., Chaganti, R. S., Rothman, P., Stall, A. M., Pandolfi, P. P. and Dalla-Favera, R. (1997) The BCL-6 proto-oncogene controls germinal-centre formation and Th2-type inflammation. *Nat Genet*. **16**, 161-170
- 4 Offit, K., Lo Coco, F., Louie, D., Parsa, N., Leung, D., Portlock, C., Ye, B., Lista, F., Filippa, D., Rosenbaum, A. and al., e. (1994) Rearrangement of the bcl-6 gene as a prognostic marker in diffuse large-cell lymphoma. *N Engl J Med*. **331**, 74-80
- 5 Bereshchenko, O. R., Gu, W. and Dalla-Favera, R. (2002) Acetylation inactivates the transcriptional repressor BCL6. *Nat Genet*. **32**, 606-613
- 6 Niu, H., Ye, B. H. and Dalla-Favera, R. (1998) Antigen receptor signaling induces MAP kinase-mediated phosphorylation and degradation of the BCL-6 transcription factor. *Genes Dev*. **12**, 1953-1961
- 7 Pasqualucci, L., Migliazza, A., Basso, K., Houldsworth, J., Chaganti, R. S. and Dalla-Favera, R. (2003) Mutations of the BCL6 proto-oncogene disrupt its negative autoregulation in diffuse large B-cell lymphoma. *Blood*. **101**, 2914-2923
- 8 Saito, M., Gao, J., Basso, K., Kitagawa, Y., Smith, P. M., Bhagat, G., Pernis, A., Pasqualucci, L. and Dalla-Favera, R. (2007) A signaling pathway mediating downregulation of BCL6 in germinal center B cells is blocked by BCL6 gene alterations in B cell lymphoma. *Cancer Cell*. **12**, 280-292
- 9 Wang, X., Li, Z., Naganuma, A. and Ye, B. H. (2002) Negative autoregulation of BCL-6 is bypassed by genetic alterations in diffuse large B cell lymphomas. *Proc Natl Acad Sci U S A*. **99**, 15018-15023
- 10 Scheeren, F. A., Naspetti, M., Diehl, S., Schotte, R., Nagasawa, M., Wijnands, E., Gimeno, R., Vyth-Dreese, F. A., Blom, B. and Spits, H. (2005) STAT5 regulates the self-renewal capacity and differentiation of human memory B cells and controls Bcl-6 expression. *Nat Immunol*. **6**, 303-313
- 11 Fernandez de Mattos, S., Essafi, A., Soeiro, I., Pietersen, A. M., Birkenkamp, K. U., Edwards, C. S., Martino, A., Nelson, B. H., Francis, J. M., Jones, M. C., Brosens, J. J., Coffey, P. J. and Lam, E. W. (2004) FoxO3a and BCR-ABL regulate cyclin D2 transcription through a STAT5/BCL6-dependent mechanism. *Mol Cell Biol*. **24**, 10058-10071
- 12 Tang, T. T., Dowbenko, D., Jackson, A., Toney, L., Lewin, D. A., Dent, A. L. and Lasky, L. A. (2002) The forkhead transcription factor AFX activates apoptosis by induction of the BCL-6 transcriptional repressor. *J Biol Chem*. **277**, 14255-14265
- 13 Genetta, T., Ruezinsky, D. and Kadesch, T. (1994) Displacement of an E-box-binding repressor by basic helix-loop-helix proteins: implications for B-cell specificity of the immunoglobulin heavy-chain enhancer. *Mol Cell Biol*. **14**, 6153-6163
- 14 Ikeda, K. and Kawakami, K. (1995) DNA binding through distinct domains of zinc-finger-homeodomain protein AREB6 has different effects on gene transcription. *Eur J Biochem*. **233**, 73-82
- 15 Postigo, A. A. and Dean, D. C. (1999) ZEB represses transcription through interaction with the corepressor CtBP. *Proc Natl Acad Sci U S A*. **96**, 6683-6688
- 16 Postigo, A. A. and Dean, D. C. (1999) Independent repressor domains in ZEB regulate muscle and T-cell differentiation. *Mol Cell Biol*. **19**, 7961-7971
- 17 Postigo, A. A. and Dean, D. C. (2000) Differential expression and function of members of the zfh-1 family of zinc finger/homeodomain repressors. *Proc Natl Acad Sci U S A*. **97**, 6391-6396
- 18 Hidaka, T., Nakahata, S., Hatakeyama, K., Hamasaki, M., Yamashita, K., Kohno, T., Arai, Y., Taki, T., Nishida, K., Okayama, A., Asada, Y., Yamaguchi, R., Tsubouchi, H., Yokota, J., Taniwaki, M., Higashi, Y. and Morishita, K. (2008) Down-regulation of TCF8 is involved in the leukemogenesis of adult T-cell leukemia/lymphoma. *Blood*. **112**, 383-393
- 19 Mendez, L. M., Polo, J. M., Yu, J. J., Krupski, M., Ding, B. B., Melnick, A. and Ye, B. H. (2008) CtBP is an essential corepressor for BCL6 autoregulation. *Mol Cell Biol*. **28**, 2175-2186
- 20 Pena, C., Garcia, J. M., Garcia, V., Silva, J., Dominguez, G., Rodriguez, R., Maximiano, C., Garcia de Herreros, A., Munoz, A. and Bonilla, F. (2006) The expression levels of the transcriptional regulators p300 and CtBP modulate the correlations between SNAIL, ZEB1, E-cadherin and vitamin D receptor in human colon carcinomas. *Int J Cancer*. **119**, 2098-2104

- 21 Pena, C., Garcia, J. M., Silva, J., Garcia, V., Rodriguez, R., Alonso, I., Millan, I., Salas, C., de Herreros, A. G., Munoz, A. and Bonilla, F. (2005) E-cadherin and vitamin D receptor regulation by SNAIL and ZEB1 in colon cancer: clinicopathological correlations. *Hum Mol Genet.* **14**, 3361-3370
- 22 Yoon, S. O., Zhang, X., Berner, P. and Choi, Y. S. (2009) IL-21 and IL-10 have redundant roles but differential capacities at different stages of Plasma Cell generation from human Germinal Center B cells. *J Leukoc Biol.* **86**, 1311-1318
- 23 Dohadwala, M., Yang, S. C., Luo, J., Sharma, S., Batra, R. K., Huang, M., Lin, Y., Goodglick, L., Krysan, K., Fishbein, M. C., Hong, L., Lai, C., Cameron, R. B., Gemmill, R. M., Drabkin, H. A. and Dubinett, S. M. (2006) Cyclooxygenase-2-dependent regulation of E-cadherin: prostaglandin E(2) induces transcriptional repressors ZEB1 and snail in non-small cell lung cancer. *Cancer Res.* **66**, 5338-5345
- 24 Eger, A., Aigner, K., Sonderegger, S., Dampier, B., Oehler, S., Schreiber, M., Berx, G., Cano, A., Beug, H. and Foisner, R. (2005) DeltaEF1 is a transcriptional repressor of E-cadherin and regulates epithelial plasticity in breast cancer cells. *Oncogene.* **24**, 2375-2385
- 25 Zhang, Q., Wang, S. Y., Fleuriel, C., Leprince, D., Rocheleau, J. V., Piston, D. W. and Goodman, R. H. (2007) Metabolic regulation of SIRT1 transcription via a HIC1:CtBP corepressor complex. *Proc Natl Acad Sci U S A.* **104**, 829-833
- 26 Brudno, M., Do, C. B., Cooper, G. M., Kim, M. F., Davydov, E., Green, E. D., Sidow, A. and Batzoglou, S. (2003) LAGAN and Multi-LAGAN: efficient tools for large-scale multiple alignment of genomic DNA. *Genome Res.* **13**, 721-731
- 27 Braddock, D. T., Louis, J. M., Baber, J. L., Levens, D. and Clore, G. M. (2002) Structure and dynamics of KH domains from FBP bound to single-stranded DNA. *Nature.* **415**, 1051-1056
- 28 Michelotti, E. F., Sanford, S. and Levens, D. (1997) Marking of active genes on mitotic chromosomes. *Nature.* **388**, 895-899
- 29 Mathews, D. H., Sabina, J., Zuker, M. and Turner, D. H. (1999) Expanded sequence dependence of thermodynamic parameters improves prediction of RNA secondary structure. *J Mol Biol.* **288**, 911-940
- 30 Zuker, M. (2003) Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res.* **31**, 3406-3415
- 31 Kikuchi, M., Miki, T., Kumagai, T., Fukuda, T., Kamiyama, R., Miyasaka, N. and Hirose, S. (2000) Identification of negative regulatory regions within the first exon and intron of the BCL6 gene. *Oncogene.* **19**, 4941-4945
- 32 Alizadeh, A., Eisen, M., Davis, R., Ma, C., Lossos, I., Rosenwald, A., Boldrick, J., Sabet, H., Tran, T., Yu, X., Powell, J., Yang, L., Marti, G., Moore, T., Hudson, J., Lu, L., Lewis, D., Tibshirani, R., Sherlock, G., Chan, W., Greiner, T., Weisenburger, D., Armitage, J., Warnke, R. and Staudt, L. (2000) Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. *Nature.* **403**, 503-511
- 33 Monti, S., Savage, K. J., Kutok, J. L., Feuerhake, F., Kurtin, P., Mihm, M., Wu, B., Pasqualucci, L., Neuberg, D., Aguiar, R. C., Dal Cin, P., Ladd, C., Pinkus, G. S., Salles, G., Harris, N. L., Dalla-Favera, R., Habermann, T. M., Aster, J. C., Golub, T. R. and Shipp, M. A. (2005) Molecular profiling of diffuse large B-cell lymphoma identifies robust subtypes including one characterized by host inflammatory response. *Blood.* **105**, 1851-1861
- 34 Bajalica-Lagerkrantz, S., Piehl, F., Farnebo, F., Larsson, C. and Lagerkrantz, J. (1998) Expression of the BCL-6 gene in the pre- and postnatal mouse. *Biochem. Biophys. Res. Commun.* **247**, 357-360
- 35 Logarajah, S., Hunter, P., Kraman, M., Steele, D., Lakhani, S., Bobrow, L., Venkitaraman, A. and Wagner, S. (2003) BCL-6 is expressed in breast cancer and prevents mammary epithelial differentiation. *Oncogene.* **22**, 5572-5578
- 36 Funahashi, J., Sekido, R., Murai, K., Kamachi, Y. and Kondoh, H. (1993) Delta-crystallin enhancer binding protein delta EF1 is a zinc finger-homeodomain protein implicated in postgastrulation embryogenesis. *Development.* **119**, 433-446
- 37 Moreno-Bueno, G., Portillo, F. and Cano, A. (2008) Transcriptional regulation of cell polarity in EMT and cancer. *Oncogene.* **27**, 6958-6969
- 38 Jechlinger, M., Grunert, S., Tamir, I. H., Janda, E., Ludemann, S., Waerner, T., Seither, P., Weith, A., Beug, H. and Kraut, N. (2003) Expression profiling of epithelial plasticity in tumor progression. *Oncogene.* **22**, 7155-7169
- 39 Remacle, J. E., Kraft, H., Lerchner, W., Wuytens, G., Collart, C., Verschueren, K., Smith, J. C. and Huylebroeck, D. (1999) New mode of DNA binding of multi-zinc finger transcription factors: deltaEF1 family members bind with two hands to two target sites. *EMBO J.* **18**, 5073-5084

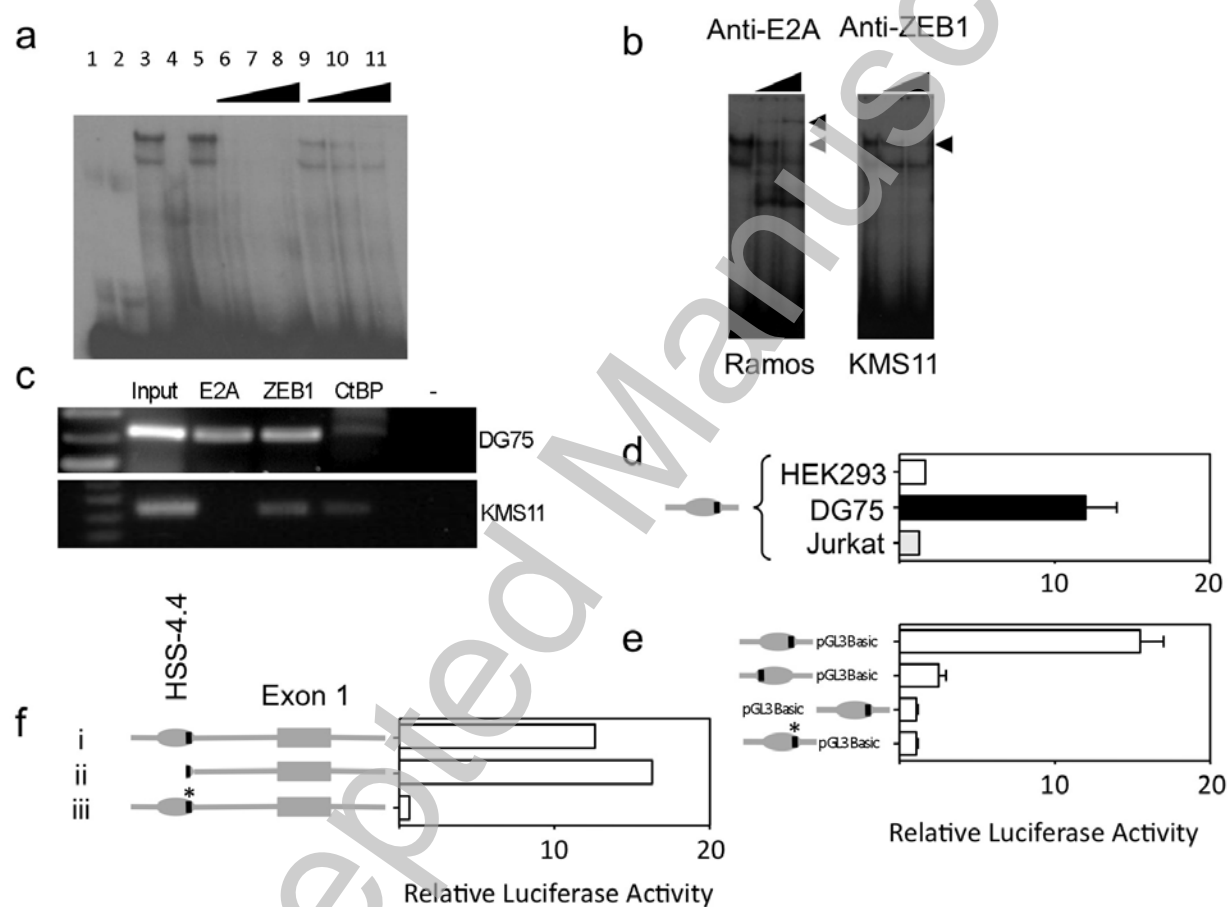
- 40 Aigner, K., Dampier, B., Descovich, L., Mikula, M., Sultan, A., Schreiber, M., Mikulits, W., Brabletz, T., Strand, D., Obrist, P., Sommergruber, W., Schweifer, N., Wernitznig, A., Beug, H., Foisner, R. and Eger, A. (2007) The transcription factor ZEB1 (deltaEF1) promotes tumour cell dedifferentiation by repressing master regulators of epithelial polarity. *Oncogene*. **26**, 6979-6988
- 41 Kraus, R. J., Perrigoue, J. G. and Mertz, J. E. (2003) ZEB negatively regulates the lytic-switch BZLF1 gene promoter of Epstein-Barr virus. *J Virol*. **77**, 199-207
- 42 Miller, M. M., Jarosinski, K. W. and Schat, K. A. (2008) Negative modulation of the chicken infectious anemia virus promoter by COUP-TF1 and an E box-like element at the transcription start site binding deltaEF1. *J Gen Virol*. **89**, 2998-3003
- 43 Sekido, R., Murai, K., Kamachi, Y. and Kondoh, H. (1997) Two mechanisms in the action of repressor deltaEF1: binding site competition with an activator and active repression. *Genes Cells*. **2**, 771-783
- 44 Wang, J., Lee, S., Teh, C. E., Bunting, K., Ma, L. and Shannon, M. F. (2009) The transcription repressor, ZEB1, cooperates with CtBP2 and HDAC1 to suppress IL-2 gene activation in T cells. *Int Immunol*. **21**, 227-235
- 45 Lossos, I. S., Jones, C. D., Warnke, R., Natkunam, Y., Kaizer, H., Zehnder, J. L., Tibshirani, R. and Levy, R. (2001) Expression of a single gene, BCL-6, strongly predicts survival in patients with diffuse large B-cell lymphoma. *Blood*. **98**, 945-951
- 46 Yu, X., Wang, Z. and Mertz, J. E. (2007) ZEB1 regulates the latent-lytic switch in infection by Epstein-Barr virus. *PLoS Pathog*. **3**, e194
- 47 Zavadil, J. and Bottinger, E. P. (2005) TGF-beta and epithelial-to-mesenchymal transitions. *Oncogene*. **24**, 5764-5774
- 48 Gibbons, D. L., Lin, W., Creighton, C. J., Rizvi, Z. H., Gregory, P. A., Goodall, G. J., Thilaganathan, N., Du, L., Zhang, Y., Pertsemlidis, A. and Kurie, J. M. (2009) Contextual extracellular cues promote tumor cell EMT and metastasis by regulating miR-200 family expression. *Genes Dev*. **23**, 2140-2151
- 49 Korpala, M., Lee, E. S., Hu, G. and Kang, Y. (2008) The miR-200 family inhibits epithelial-mesenchymal transition and cancer cell migration by direct targeting of E-cadherin transcriptional repressors ZEB1 and ZEB2. *J Biol Chem*. **283**, 14910-14914



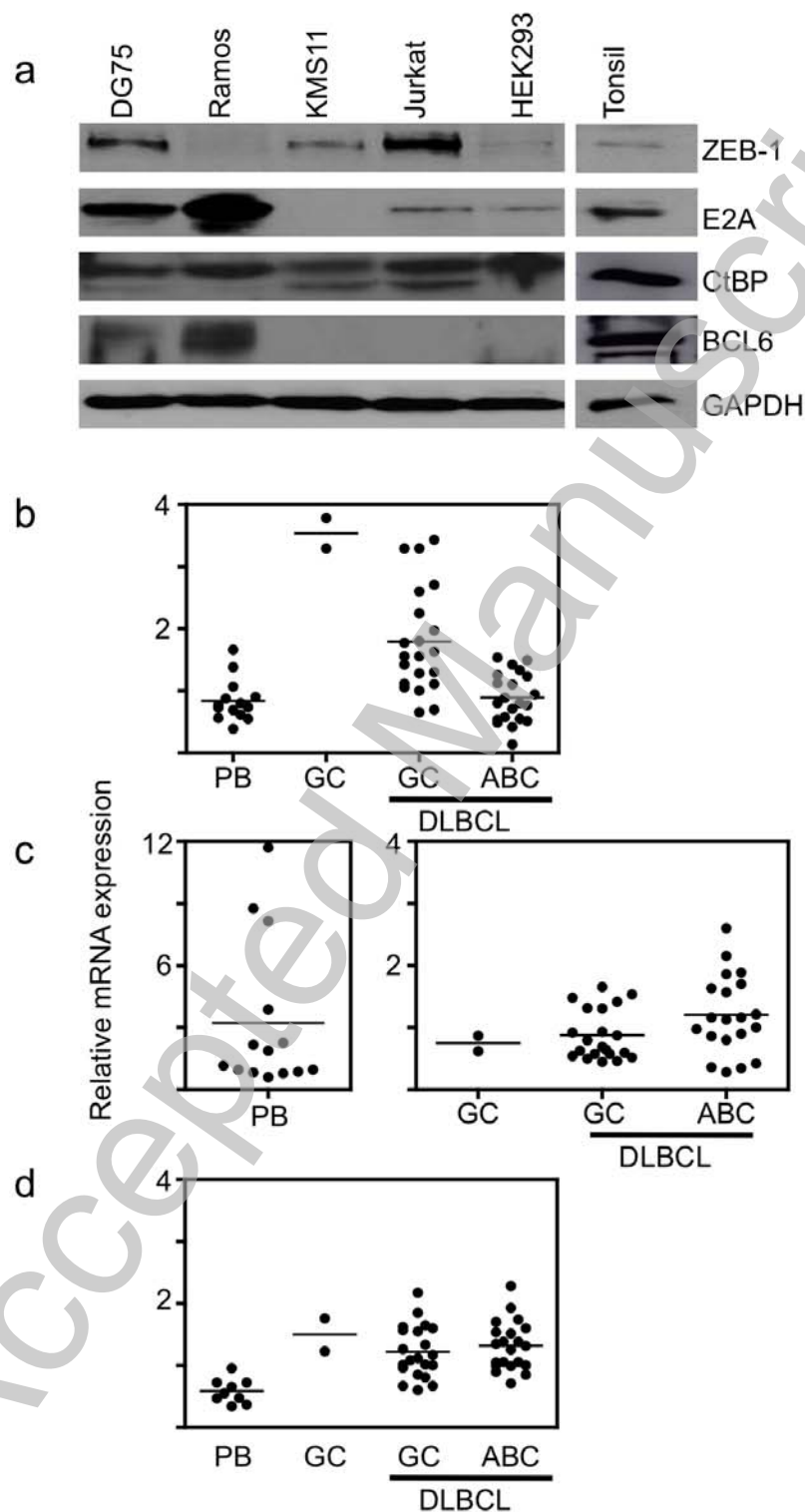
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Figure 1



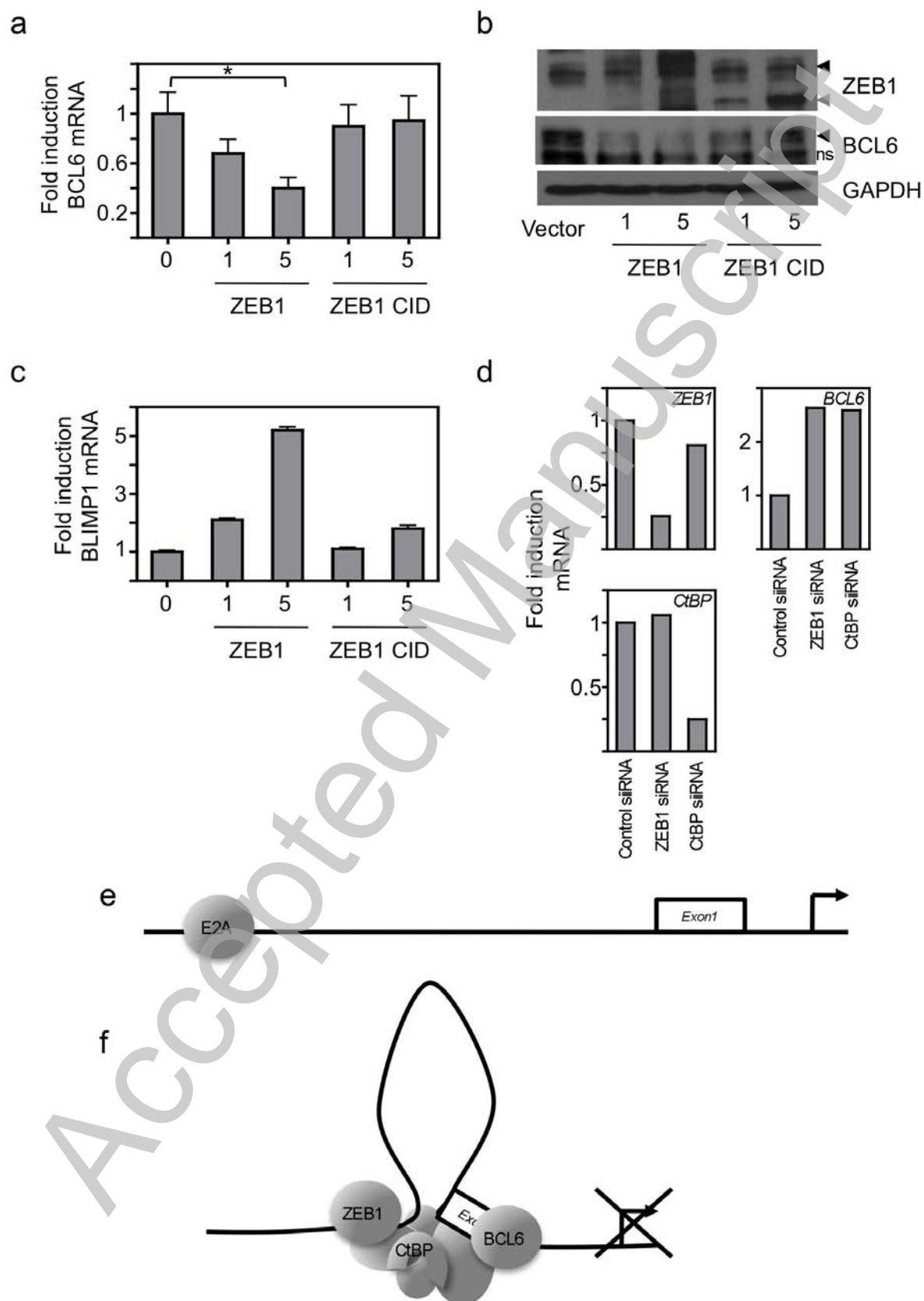
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Figure 2**



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Figure 3



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Figure 4



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Figure 5