Roles of RUNX in B cell immortalisation

Michelle J. West[1] and Paul J. Farrell[2]

[1]School of Life Sciences, University of Sussex, Falmer, Brighton, BN1 9QG, UK.

[2]Section of Virology, Faculty of Medicine, Imperial College London, Norfolk Place, London W2 1PG, UK.

Abstract

RUNX1 and *RUNX3* are the main *RUNX* genes expressed in B lymphocytes. Both are expressed throughout B-cell development and play key roles at certain key developmental transitions. The tumour-associated Epstein-Barr virus (EBV) has potent B-cell transforming ability and manipulates *RUNX3* and *RUNX1* transcription through novel mechanisms to control B cell growth. In contrast to resting mature B cells where *RUNX1* expression is high, in EBV-infected cells *RUNX1* levels are low and *RUNX3* levels are high. Downregulation of *RUNX1* in these cells results from cross-regulation by RUNX3 and serves to relieve RUNX1-mediated growth repression. *RUNX3* is upregulated by the EBV transcription factor (TF) EBNA2 and represses *RUNX1* transcription through RUNX sites in the *RUNX1* P1 promoter. Recent analysis revealed that EBNA2 activates *RUNX3* transcription through an 18 kb upstream super-enhancer in a manner dependent on the EBNA2 and Notch DNA-binding partner RBP-J. This super-enhancer also directs *RUNX3* activation by two further RBP-J-associated EBV TFs, EBNA3B and 3C. Counter-intuitively, EBNA2 also hijacks RBP-J to target a super-enhancer region upstream of *RUNX1* to maintain some *RUNX1* expression in certain cell backgrounds, although the dual functioning EBNA3B and 3C proteins limit this activation. Interestingly, the B-cell genome binding sites of EBV TFs overlap extensively with RUNX3 binding sites and show enrichment for RUNX motifs. Therefore in addition to B-cell growth manipulation through the long-range control of *RUNX* transcription, EBV may also use RUNX proteins as co-factors to deregulate the transcription of many B cell genes during immortalisation.

Keywords: RUNX1, RUNX3, enhancer, transcription, super-enhancer, Epstein-Barr virus, EBNA2, EBNA3B, EBNA 3C, Notch, RBP-J.

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Role of *RUNX* in B cell development

Both *RUNX3* and *RUNX1* are expressed throughout the haemopoietic system including B lymphocytes (Levanon et al. 1994; Bangsow et al. 2001; North et al. 2004) and their tightly regulated expression plays an important role in defining key developmental transitions (Whiteman and Farrell 2006; Imperato et al. 2015). For example, *RUNX1* upregulation is crucial for the endothelial-to-hematopoietic transition (Chen et al. 2009) and *RUNX3* upregulation by TGF-beta1 drives class switching to IgA in splenic B cells through the activation of Ig alpha gene transcription (Shi and Stavnezer 1998).

Studies in mice have confirmed a role for *Runx3* in IgA class-switching (Fainaru et al. 2004) and delineated the role of *Runx1* in embryonic and adult haematopoiesis (Imperato et al. 2015). Homozygous disruption of *Runx1* leads to embryonic lethally and a block to definitive haematopoiesis (Wang et al. 1996) and embryos from *Runx1* knock-out mice lack both myeloid and erythroid cells in their yolk sacs and liver (Okuda et al. 1996). Numerous studies of the role of *Runx1* in adult blood cell development using knock-out mice have led to the conclusion that loss of *Runx1* does not disrupt haematopoiesis completely (as haematopoietic stem cells are maintained), but impairs the development of particular cell lineages. The bone marrow of adult mice lacking functional *Runx1* showed impaired megakaryocytic maturation and defective T- and B-lymphocyte development (Ichikawa et al. 2004). A further similar study documented a reduction in the percentage of mature B cells ($CD19^{+}B220^{+}$) in the bone-marrow, with an almost complete lack of pre-B and pro-B cell precursors (Growney et al. 2005). A reduction in the number of mature B cells expressing IgM and the B-cell marker B220 in the spleens of *Runx1* knock-out adult mice, with no effect on the number of immature B cells was also reported, consistent with an early stage block in

B-cell development (Putz et al. 2006). Taken together these studies indicate that *Runx1* plays a key role in B-cell maturation.

Consistent with a role for *Runx1* in B cell development, expression of a deregulated *Runx1* transgene in mice results in increased cell survival and an increase in the number of IgM+ and CD19+ cells in the bone marrow (Blyth et al. 2009). Although *Runx1* expression in foetal liver cells *in vitro* led to reduced total cell and B cell survival, an increased proportion of the cells that survived were B220+. These data suggest that, whilst the survival effects of *Runx1* may be context dependent, its expression induces a drive towards the B-cell lineage.

Cross regulation of *RUNX1* by *RUNX3* in B cells

The structure of the mammalian *RUNX* genes is highly conserved and their transcription is controlled from two distinct promoters, P1 (distal) and P2 (proximal), that give rise to different protein isoforms (Figures 1 and 2). These promoters are differentially active in different cell-types and at different stages of differentiation (Bangsow et al. 2001; Levanon et al. 1996). In resting mature B cells *RUNX1* transcripts are derived from the P1 promoter, leading to high-level expression of the RUNX1c isoform. RUNX1c expression drops dramatically when B cells are immortalised though latent infection with the tumour-associated herpesvirus EBV (Spender et al. 2002; Spender et al. 2005). In EBV-immortalised B cells, RUNX1c downregulation occurs concomitantly with the upregulation of *RUNX3* transcription from the P2 promoter (Spender et al. 2002). *RUNX3* is activated by the EBV TF EBNA2 (Spender et al. 2002), the master regulator of latent EBV gene transcription and a key inducer of hundreds of host genes including the growth regulators *MYC*, *CD23* and *FGR* (Kaiser et al. 1999; Wang et al. 1987;

Knutson 1990; Spender et al. 2002; Maier et al. 2006; Zhao et al. 2006) (Table 1). Infection of resting B cells with EBV drives cell proliferation and this depends on the initial and continued expression of EBNA2, indicating a critical requirement for cell gene activation by EBNA2.

The differential expression of *RUNX1* and *RUNX3* distinguished between B-cell lines displaying different patterns of EBV latent gene expression (known as latency 1 and latency III). Only those latency III cell-lines expressing EBNA2 (in association with the other 8 EBV latent proteins) (Table 1) were characterised by high *RUNX3* expression and low *RUNX1* expression (Spender et al. 2002). Latency I cell-lines derived from Burkitt's lymphoma tumour biopsies, that express only the EBV genome maintenance and replication factor EBNA-1, maintained high RUNX1c expression and lacked detectable *RUNX3* expression. A similar induction of *RUNX3* and consequent repression of *RUNX1* occurs when B cells are activated by phorbol ester treatment and an inverse correlation between *Runx3* and *Runx1* expression is observed in the I.29μ mouse B-cell line upon *Runx3* induction by TGF-β (Shi and Stavnezer 1998).

Given this inverse correlation between *RUNX3* and *RUNX1* expression in B cells and the role of RUNX proteins as activators and repressors, a plausible explanation for the downregulation of *RUNX1* was found in direct repression by *RUNX3*. Following the identification of two adjacent RUNX motifs located between positions -2 and +12 in the *RUNX1* P1 promoter, mutagenesis revealed that *RUNX1* transcription was indeed repressed by RUNX3 through these sites (Spender et al. 2005). The ability of the RUNX proteins to activate or repress gene expression is determined by the higher complexes they form with activators or repressors of transcription in a gene- and context-specific

manner (Durst and Hiebert 2004). Mammalian RUNX proteins contain both a transactivation region and a highly-conserved repression domain near the C-terminus, the VWRPY sequence. This VWRPY sequence recruits TLE family proteins, which mediate repression of RUNX target genes through their association with histone deacetylases (Imai et al. 1998; Levanon et al. 1998; McLarren et al. 2001; Wang et al. 2004). The repression of *RUNX1* by RUNX3 in B cells was shown to be dependent on the VWRPY sequence, since its removal prevented RUNX3-mediated repression without affecting RUNX3 binding to the *RUNX1* promoter (Brady et al. 2009). TLE3 was implicated as the co-repressor involved, since it was the only TLE family member expressed in B cell-lines where cross-repression was observed.

The regulation of B cell growth by *RUNX1*

Given the role of EBNA2 in driving B cell growth, the upregulation of *RUNX3* by EBNA2 could represent an important step in B-cell immortalisation by EBV. Because of the inter-dependence of *RUNX1* and *RUNX3* levels, a key question to address was whether altered levels of RUNX*1*, RUNX3 or both proteins contributed to cell proliferation. The dependence of *RUNX1* repression on *RUNX3* was confirmed under normal physiological conditions through siRNA depletion of endogenous *RUNX3* in EBV transformed lymphoblastoid cell-lines (LCLs) (Spender et al. 2005). In these experiments, knock-down of *RUNX3* expression led to increased *RUNX1* expression as expected. Importantly, this reversal of cross regulation impaired the growth of the LCLs. These growth inhibitory effects were attributed to *RUNX1* when it was shown that ectopic expression of RUNX1c in an LCL blocked cell growth (Brady et al. 2009; Brady et al. 2013). Interestingly, the RUNX1c isoform (expressed in resting B cells) was more potent in inhibiting growth than RUNX1b, suggesting that the extra 30 N-

terminal amino acids in RUNX1c play a role in the growth inhibitory function of this isoform (Brady et al. 2013). These data suggest that a switch from *RUNX1* to *RUNX3* may be a critical step in the activation of resting B cells; *RUNX1* must be removed for B cell proliferation to occur. The effects of *RUNX1* on B cell growth do however seem to be cell-type specific (see later). The experiments conducted to date do not rule out an additional role for *RUNX3* in promoting proliferation. For example, the RUNX3 target gene encoding the survival factor B-cell maturation antigen was induced in RUNX3 expressing B cells (Brady et al. 2009).

Functional differences between RUNX1b and RUNX1c in the regulation of cell proliferation were noted previously when RUNX1b but not RUNX1c was found to delay mitotic arrest in differentiating myeloid cells (Telfer et al. 2004). There are also other examples of RUNX1 and RUNX3 regulating growth differently in the same cell type. RUNX1 and RUNX3 have opposing roles in regulating the survival of cells expressing the BCR-ABL oncoprotein through transcriptional control of the 24p3R cell surface receptor (Sheng et al. 2009). BCR-ABL induces expression of the secreted receptor ligand 24p3, which induces apoptosis in normal cells expressing 24p3R, but in BCR-ABL+ cells 24p3R expression is repressed providing apoptosis resistance. In murine 32D myeloid BCR-ABL expressing cells, *24p3R* gene repression involves the displacement of RUNX3 from the *24p3R* gene promoter by RUNX1 and the recruitment of the Sin3A-HDAC co-repressor complex. This switch in RUNX binding is induced by BCL-ABL signalling through the Ras/MAP kinase pathway resulting in an increase in RUNX1 expression. Inhibition of BCR-ABL with Imatinib reversed this switch, promoting RUNX3 occupancy of the *24p3R* promoter, increased 24p3R expression and increased apoptosis sensitivity in these cells.

The mechanism of *RUNX3* activation by EBV

The original discovery of *RUNX3* as a target gene for the EBV transactivator EBNA2 came from experiments using an EBV transformed LCL expressing a conditionally active EBNA2-oestrogen receptor fusion protein. EBNA2 activation through β-estradiol addition to cells cultured in its absence for 5 days led to an induction of *RUNX3* mRNA (Spender et al. 2002). The upregulation of *RUNX3* by EBV was attributed to the direct actions of EBNA2 rather than the induced synthesis of another TF since the induction was observed in the presence of protein synthesis inhibitors. Subsequent investigations into the mechanism of *RUNX3* activation by EBNA2 using *RUNX3* promoter constructs containing promoter sequences close to the transcription start site failed to detect any reproducible effects of EBNA2 on the *RUNX3* P2 promoter, the promoter active in these cells (Spender et al. 2005; Gunnell et al. 2016).

The development of ChIP-sequencing assays for EBNA2 has shed considerable light on the potential mechanism of EBNA2 activation of a plethora of cellular genes, including many whose promoters had proved to be EBNA2 unresponsive (McClellan et al. 2013; Zhao et al. 2011). These genome-wide binding analyses revealed that a significant proportion (75% or 86%) of EBNA2 binding sites were located distal to gene transcription start sites and coincided with regions of the genome that display the chromatin signature of active or poised enhancers (H3K4 monomethylation with or without H3K27 acetylation)(McClellan et al. 2013). EBNA2 binding data generated in the latency III Burkitt's lymphoma (BL) cell-line Mutu III and new data from the GM12878 EBV transformed LCL revealed the presence of a large 18 kb enhancer region centred -97 kb upstream of the *RUNX3* P2 promoter (chr1:25,344,250-

25,363,078, GRCh37/hg19) (Gunnell et al. 2016). This region displays high-level H3K27 acetylation in GM12878 and chromatin landscape analysis (dbSUPER, http://bioinfo.au.tsinghua.edu.cn/dbsuper/ (Khan and Zhang 2015)) classifies this region as a super-enhancer, a highly-active and characteristically large TF binding site cluster that drives transcription of lineage-specific genes (Whyte et al. 2013). An independent study also reported the super-enhancer characteristics of this EBNA2 binding region in EBV-infected cells (Zhou et al. 2015). Interestingly, based on H3K27 acetylation spread, this region is classified as a super-enhancer in GM12878 cells, early haematopoietic and T cell lineages but not CD19+ primary and CD20+ B cells. This suggests that the binding of EBNA2 drives its activation in EBV-infected B cells.

EBNA2 binding was also detected at the *RUNX3* P2 promoter, but the lack of EBNA2 responsiveness of this region in reporter assays indicates that these peaks may represent 'shadow' peaks of EBNA2 binding as a result of looping interactions between the EBNA2-bound super-enhancer region and the P2 promoter. This would result in the capturing of promoter DNA in cross-linked EBNA2 ChIP assays. In fact an adaptation of the genome-wide chromosome conformation capture technique that enriches for chromosome interactions involving promoters (capture Hi-C) (Mifsud et al. 2015) demonstrated that the *RUNX3* EBNA2 super-enhancer interacts with *RUNX* promoters in GM12878 cells supporting its regulatory function (Gunnell et al. 2016).

The main six EBNA2 binding peaks from the *RUNX3* super-enhancer were cloned upstream of the *RUNX3* P2 promoter and used in reporter assays to determine which regions of the super-enhancer were responsible for directing EBNA2 activation. These experiments identified the 1.3 kb super-enhancer region 2 as the main EBNA2-

responsive component of the super-enhancer. Since EBNA2 does not bind DNA directly and relies on the hijacking of cellular TFs to control both viral and cell genes, cellular TFs bound to this region must be responsible for mediating the effects of EBNA2 on *RUNX3* transcription. As expected, this region contained binding sites for multiple cell TFs, but the use of a knock-out B cell line identified the Notch DNA binding partner RBP-J as the main TF responsible (Gunnell et al. 2016). This is consistent with the known association of EBNA2 with RBP-J via a conserved WϕP motif found in Notch family members and the essential nature of the EBNA2-RBPJ interaction for B-cell immortalisation (Ling and Hayward 1995; Yalamanchili et al. 1994).

A separate study identified a role for NF-κB binding to the *RUNX3* super-enhancer in maintaining *RUNX3* mRNA levels in EBV-infected cells, since *RUNX3* gene expression was reduced upon NF-κB inactivation (Zhou et al. 2015). Investigation of the role of an NF-κB binding site in enhancer region 2 supported these observations, demonstrating that this site was required for the maintenance of basal activity of the enhancer, but its mutation did not impair EBNA2 activation. These data are consistent with a role for NF-κB in EBNA2-independent activation of the *RUNX3* super-enhancer. Since the EBV oncogene and CD40 mimic, Latent membrane protein 1, activates NF-κB in EBV-infected cells (Rowe et al. 1994), this may represent an additional route exploited by EBV to maintain high *RUNX3* levels in infected cells. Although NF-κB did not contribute to EBNA2-dependent activation of the *RUNX3* super-enhancer, deletion of an additional region of enhancer 2 next to the region containing identified RBP-J sites also led to a loss of EBNA2 activation implicating co-operative effects of

other as yet unidentified TFs, or the presence of additional variant RBP-J sites (Gunnell et al. 2016).

ChIP-sequencing analysis of other EBV TFs expressed in immortalised B cells (EBNA3A, 3B and 3C) also detected binding of EBNA3B and 3C to enhancer region 2 of the *RUNX3* super-enhancer (Gunnell et al. 2016; McClellan et al. 2012; McClellan et al. 2013). Microarray analysis of B cell lines infected with recombinant EBVs lacking all three EBNA3 proteins provided evidence for their role in activating *RUNX3* transcription (White et al. 2010) and this was confirmed at the protein level (Gunnell et al. 2016), a role that would not have been previously identified on the background of EBNA2 activation. Since these two factors also bind RBP-J on chromatin in a competitive manner with EBNA2 (Harth-Hertle et al. 2013; Wang et al. 2015a), it would appear that the activation of *RUNX3* by EBNA3 proteins may also be mediated through this TF and that EBNA2, 3B and 3C bind independently to the super-enhancer.

It therefore appears that at least two B cell TFs play key roles in *RUNX3* super-enhancer control in immortalised B-cells; NF-kB and RBP-J. Further experiments will be required to determine whether the *RUNX3* super-enhancer does indeed play a role in the regulation of *RUNX3* transcription in other cell-types as predicted and whether additional TFs are required to direct cell-type specificity in these contexts.

Cell-type specific feed-forward activation of *RUNX1* by EBV

Because *RUNX1* transcription is repressed in EBV-infected cells as a result of the induction of *RUNX3* by EBNA2, it was surprising that genome-wide binding analyses also identified binding sites for EBNA2 in a >100 kb region 139 to 250 kb upstream of

RUNX1 P1 (chr21:36,561,018-36,672,471, GRCh37/hg19)(Gunnell et al. 2016) (Figure 2). These binding sites coincide with regions assigned as super-enhancers in specific cell-types (e.g. Diffuse large B cell lymphoma cell-lines, a breast cancer line, mammary epithelial cells and skeletal muscle (dbSUPER, http://bioinfo.au.tsinghua.edu.cn/dbsuper/ (Khan and Zhang 2015)). EBNA2 binds at six main sites within this region in a cell-type specific manner, with binding detected in Mutu III BL cells and not in LCLs. The lack of activity of the *RUNX1* superenhancer in LCLs is supported by the lack of H3K27 acetylation in this region in the GM12878 LCL (Figure 2). Reporter assays examining EBNA2 responsiveness of the 6 main enhancer binding peaks identified enhancer region 4 as the main mediator of EBNA2 activation (Gunnell et al. 2016). As observed for *RUNX3*, EBNA2 activation of the *RUNX1* super-enhancer was dependent on the expression of RBP-J. The ability to access gene regulatory elements through association with RBP-J therefore appears be central in the control of *RUNX1* and *RUNX3* expression by EBV (Figure 3).

Although EBNA2 has the ability to target *RUNX1* and activate its transcription, it is unclear why EBNA2 would target *RUNX1* for activation, when its upregulation of *RUNX3* serves to repress *RUNX1* transcription (Spender et al. 2005). It is possible that the cell-type specificity of *RUNX1* targeting is important here. Experiments in LCLs highlight the importance of *RUNX1* repression for LCL growth, but EBNA2 activation of the *RUNX1* super-enhancer was observed in BL cells and not in LCLs. In BL cells, the characteristic *IG-MYC* translocation leads to growth deregulation through *MYC* overexpression. In this background, the repressive effects of *RUNX1* on growth may be overridden by the effects of *MYC*. It is also possible that the expression of some *RUNX1* might provide some advantage for the survival or growth of BL cells.

EBNA3B and 3C also bind *RUNX1* super-enhancer regions in the same cell-type specific manner as EBNA2, indicating multiple points of control by EBV (Gunnell et al. 2016) (Figure 3). However, rather than contributing to *RUNX* upregulation as observed at *RUNX3*, these proteins appear to attenuate *RUNX1* expression. These dual roles of the EBNA3s are entirely consistent with their known roles as activators and repressors of transcription and their association with both co-activators and co-repressors (Knight et al. 2003; Cotter and Robertson 2000; Subramanian et al. 2002; Paschos et al. 2012) (Table 1).

RUNX1 regulation by intronic super-enhancer elements in haematopoietic cells

RUNX1 expression is controlled by enhancer elements in mouse and human cells, but unlike the intergenic upstream enhancers targeted by EBV, these enhancers are located in the region between the *RUNX1* P1 and P2 promoters. Comparative genomics combined with DNase hypersensitivity site (DHS) analysis identified a 194 bp enhancer in the mouse genome located 23.5 kb downstream of the *RUNX1* P1 transcript ATG (Nottingham et al. 2007). This +23 enhancer drives early haemopoietic-specific gene expression in mouse embryos in a pattern consistent with that of endogenous *RUNX1*. *In vitro* analysis of conserved TF binding sites in the enhancer identified conserved RUNX, Gata2 and Ets sites as crucial for its activity and RUNX1, Gata2 and the Ets TFs Fli-1, Elf1 and PU.1 bound the enhancer *in vivo* (Nottingham et al. 2007). Binding of the Gata-associated SCL, Lmo2 and Ldb1 complex was also detected by ChIP. Transgenic enhancer-reporter embryos confirmed that mutation of the Gata and Ets motifs severely disrupted enhancer activity in the embryo, although mutation of the RUNX motif had little effect (Nottingham et al. 2007). Unlike in zebrafish, where

RUNX1 P1 contains sites that control tissue-specific expression, the mouse P1 and P2 promoters alone lack tissue-specific activity, but tissue-specific expression of either promoter can be controlled by +23 kb enhancer (Bee et al. 2009).

In human cells, P1 and P2 display some cell-type specific activity and an equivalent 500 bp +23 kb enhancer (RE1) in intron 1 was found to regulate *RUNX1* expression in a haemopoietic cell-specific manner (Markova et al. 2011). The RE1 element was able to activate transcription from *RUNX1* P1 and P2 in reporter assays in Jurkat T leukaemic cells, from P2 in K562 erythroleukaemic cells, but failed to activate either promoter in HEK293 epithelial cells. A further 400 bp element identified in *RUNX1* intron 5.2 based on the presence of lymphoid-specific DHSs (RE2) did not activate *RUNX1* expression in reporter assays in any cell background. Chromosome conformation analysis detected haemopoietic cell-specific interactions between both P1 and P2 and RE1 (Markova et al. 2011) and showed that the promoters also interact with one another, forming a chromatin hub resembling that observed at *RUNX3* (Gunnell et al. 2016). RE2 also interacted with both P1 and P2, but separately, and this was not cell-type specific (Markova et al. 2011). Given the lack of detectable enhancer activity for RE2, this region may play an architectural role in the organisation of the locus, although a role as a matrix attachment region was ruled out (Markova et al. 2012).

Recent analysis confirmed the importance of the region between P1 and P2 in the regulation of *RUNX1* expression in Jurkat cells (Kwiatkowski et al. 2014). T-cell acute lymphoblastic leukaemia (T-ALL) cell-lines, including Jurkat, were found to be highly sensitive to treatment with a selective covalent inhibitor of the TFIIH kinase subunit CDK7 (Kwiatkowski et al. 2014). Genome-wide analysis in Jurkat cells identified

RUNX1 as one of a subset of genes that were selectively downregulated on inhibitor treatment. A *RUNX1* super-enhancer covering the entire 173 kb region between P1 and P2, containing the previously described RE1 element, was identified through examination of H3K27 acetylation profiles. The association of CDK7 with this entire super-enhancer region and the susceptibility of super-enhancers to inhibition likely explains the enhanced suppression of *RUNX1* transcription in these cells. T-ALL cell-line sensitivity to low-dose drug treatment is also probably enhanced as a result of the loss of expression of *TAL1* and *GATA3,* two other key components of the auto and cross-regulatory *RUNX1* transcriptional network (Kwiatkowski et al. 2014).

It is therefore clear that cell-type and developmental stage specific expression of *RUNX1* can be controlled by at least two extremely large super-enhancer regions, one located upstream of the gene locus that is exploited by EBV TFs in B cells and one located in an intronic region between the P1 and P2 promoters. Given the prediction that the upstream region has H3K27 acetylation levels consistent with superenhancer function in other cell-types, it is likely that this region has further cell-type specific and potentially disease specific roles in controlling *RUNX1* expression.

Notch control of *RUNX* gene expression

The mechanism of RUNX super-enhancer control by EBV in immortalised B cells requires association of viral TFs with RBP-J. This reinforces the known interplay between the Notch and *RUNX* pathways in many developmental systems. For example, haematopoiesis in *Drosophila* is associated with Notch signalling-dependent induction of *RUNX1* (Lozenge) in specific precursor cells (Lebestky et al. 2003). *RUNX1* was also identified as a direct target gene induced by activated Notch1 signalling in murine

mesodermal cells, but not embryonic stem cells (Meier-Stiegen et al. 2010) and in mouse NIH3T3 cells Notch1 upregulates *RUNX1* transcription (Nakagawa et al. 2006) and Notch1-RBP-J null mice have impaired haematopoietic potential and reduced *RUNX1* expression in specific compartments (Nakagawa et al. 2006). Notch signalling also controls *RUNX1* expression and haematopoietic stem cell development in Zebrafish (Burns et al. 2005). Although most studies have concentrated on *RUNX1*, *RUNX3* has also been shown to be a direct target of Notch in murine endothelial cells (Fu et al. 2011).

The role of RUNX proteins as transcriptional regulators in EBV-immortalised B cells

In addition to targeting *RUNX* genes via their super-enhancers to control *RUNX* transcription in EBV-infected cells, EBV TFs may also regulate cellular genes in association with RUNX proteins. Evidence for a role for RUNX proteins in EBV-directed gene control in B cells is based on the presence of RUNX motifs and/or RUNX3 binding at human genome binding sites for EBNA2 and EBNA3 proteins. Motif analysis of EBNA2 binding sites in the IB4 LCL found that 43% had significant enrichment for a RUNX motif (Zhao et al. 2011). Cluster analysis of cell TF binding or the presence of TF motifs at EBNA2 sites was used to determine whether specific sets of TFs co-bound to EBNA2 binding sites. These analyses identified a cluster of EBNA2 binding sites with RUNX motifs that were also bound by EBF and RBP-J. A separate cluster of EBNA2 sites was also identified that was bound by RBP-J in association with RUNX motifs, but in the absence of EBF. Interestingly, although the RUNX-EBF-RBP-J co-bound sites overlapped with significant binding sites of the

histone acetyl transferase p300 (37%) and had high H3K4 monomethylation levels, the RBP-J-RUNX co-bound sites showed the least overlap with p300 (5%) binding and had low H3K4 monomethylation levels (Zhao et al. 2011). The most highly expressed RUNX protein in these LCLs is RUNX3, but the analyses did not distinguish between RUNX1 and RUNX3 binding. These observations are interesting, however, in the context of a previous report where RUNX3 was found to suppress Notch activation of RBP-J dependent transcription through its association with the Notch intracellular domain (NIC) (Gao et al. 2010). Given that EBNA2 mimics NIC in its association with RBP-J, it is possible that RUNX3 might associate with EBNA2 to repress transcription at RBP-J bound sites and that the presence of EBF counteracts this repression. An EBF site in the viral LMP1 promoter has been shown to play a in role in EBNA2 activation of the promoter, although the involvement of RUNX3 in LMP1 transcription control is unknown (Zhao et al. 2011).

The potential involvement of RUNX proteins in EBV-mediated gene control also extends to the EBNA3 proteins. ChIP-sequencing analysis of epitope tagged EBNA3C found that similar to EBNA2, 44% of EBNA3C sites were enriched for RUNX motifs (Jiang et al. 2014). Availability of RUNX3 ChIP-seq data for this later study also allowed analysis of binding overlap, which revealed that an even higher proportion (64%) of EBNA3C sites in the human genome were also bound by RUNX3 (Jiang et al. 2014). EBNA3C sites that were co-bound by RUNX3 were found in 5 different clusters with different sets of cell TFs, some of which did not contain RBP-J, indicating that other TFs play a role in EBNA3C binding to DNA. Interestingly one EBNA3C-RUNX3 co-bound site cluster was associated with chromatin marks found at strong promoters, but lacked RBP-J binding or any enrichment for RUNX motifs, consistent

with some RUNX binding in the absence of a consensus site. Despite the 'active' chromatin profile these sites were also co-bound by the Sin3A co-repressor, highlighting a potential role for RUNX3 in gene repression, in this case in the 'shutting down' of active promoter sites. Three other EBNA3C-RUNX3 clusters had mostly strong enhancer chromatin marks and RUNX motifs and included either PU.1, RBP-J and EBNA2, or BATF and IRF4. EBNA3C binding was enhanced in the presence of RUNX3 and RUNX3 has higher binding signals at sites bound by EBNA3C indicating some mutual enhancement or stabilisation of binding.

A high degree of overlap with RUNX3 binding was also detected in LCLs at binding sites for epitope tagged EBNA3A (63%) or EBNA3B (83%) (Schmidt et al. 2015; Wang et al. 2015a). For EBNA 3A, 7 clusters of cell TF co-association were identified with 5 of them showing enrichment for RUNX3 binding and the presence of RUNX motifs (Schmidt et al. 2015). All of these 5 site clusters displayed chromatin marks associated with either weak or strong enhancers. Interestingly, in contrast to EBNA3C and its cell TF clusters, there were no detected associations of RUNX3 with EBNA3A in TF clusters containing co-repressors, indicating that RUNX3 association with different EBV TFs may be distinct and have different roles in different contexts. However, analysis of these datasets is hindered by the extensive overlap between the binding sites of EBNA2 and the EBNA3s (McClellan et al. 2013; Wang et al. 2015a), making it difficult to assign specificity to any interactions or co-operation. Interestingly, analysis of unique EBNA2, EBNA3A, 3B and 3C binding sites revealed that RUNX motifs are only enriched at sites bound by EBNA2 alone or EBNA3C alone and not for EBNA3A and EBNA3B, indicating that there may be specificity in the potential association between EBV TFs and RUNX3 (Wang et al. 2015a).

Although there is no evidence to date of any direct or indirect interactions between EBV TFs and RUNX proteins, taken together these genome-wide binding studies strongly implicate RUNX3 in EBV-directed cellular gene regulation, through direct or indirect interactions at gene regulatory elements. This is consistent with the immortalisation of B cells by EBV TFs through the hijacking of cell TFs that drive the B-cell transcription program.

It is noteworthy that there is significant RUNX3 binding at the *RUNX3* super-enhancer and multiple RUNX motifs are present, including in enhancer 2, the most EBNA2 responsive region (Figure 4). In fact there is a RUNX consensus site within the region of enhancer 2 that seems to play a co-operative role in EBNA2 activation (Gunnell et al. 2016), so it will be interesting to determine the effects of mutating the RUNX binding site in this region on EBNA2 responsiveness of the superenhancer. RUNX3 also bound to the *RUNX3* P1 promoter (Figure 4). This is interesting given the known role of RUNX3 binding to two conserved RUNX sites in the *RUNX1* P1 promoter in the repression of *RUNX1* transcription in B cells (Spender et al. 2005) and the auto-repression of *RUNX2* via the same sites in rat (Drissi et al. 2000) . The tandem *RUNX1* P1 RUNX sites are completely conserved in the human *RUNX3* P1 promoter so it is possible that binding of RUNX3 to these sites may auto-repress its own P1 promoter. Consistent with this, *RUNX3* transcripts are derived from P2, not P1 in EBV immortalised B cells (Spender et al. 2005). Further analysis is required to determine the role played by RUNX3 in the regulation of its own enhancer and promoters, and indeed how RUNX3 binding to RUNX1 P1 may influence *RUNX1* enhancer activity.

Future directions and outstanding questions

Studies of *RUNX* gene control in B cells immortalised by the tumour-associated Epstein-Barr virus have led to the identification of super-enhancers that play a key role in controlling the cell-type specific expression of both *RUNX3* and *RUNX1*. Further investigation is now needed to address the potential role of these super-enhancers in the spatial and temporal control of *RUNX* gene expression in the haemopoietic and other developmental systems and their contribution to *RUNX* gene deregulation in other disease states. The known susceptibility of super-enhancers to perturbation and their key role in lineage-specific growth regulation has already made them attractive targets for new transcription inhibitory molecules that have been shown to block T-ALL, small cell lung carcinoma, neuroblastoma and triple negative breast cancer cell growth (Chipumuro et al. 2014; Christensen et al. 2014; Kwiatkowski et al. 2014; Wang et al. 2015b). The perturbation of these EBV-driven super-enhancers may offer new therapeutic possibilities for the treatment of EBV-associated malignancies, given the key role played by RUNX proteins in the regulation of EBV-infected cell growth.

In spite of all this progress, there are still many questions regarding the role of *RUNX3* and *RUNX1* in B-cell gene regulation and growth control. One important priority is to determine in detail which genes are targeted by *RUNX3* and *RUNX1* in B cells and how combination with other cell TFs determines the dual roles of these proteins in gene activation and repression. It is also notable that the *RUNX1* super-enhancer is only active and bound by EBV TFs in Burkitt's lymphoma cells and not in the LCLs examined to date. Repression by the higher levels of RUNX3 in LCLs may be one possible explanation (Gunnell et al. 2016), but there may well be other effects of the

high levels of MYC in Burkitt's lymphoma cells on the super-enhancer, and MYC binding sites that are bound by MYC/MAX are present in this region. Bringing together the rapidly increasing ChIP-sequencing information with functional studies of promoter/enhancer elements and further functional TF interactions is likely to give an even more comprehensive understanding of the roles of *RUNX* genes in B cell immortalisation in the near future.

Figure captions

Table 18.1 EBV latent proteins and their roles and expression in EBV immortalised B cells. Epstein-Barr nuclear antigens (EBNAs) are found in the nucleus and Latent membrane proteins (LMP) are found in the cell membrane. Genes essential for transformation of peripheral resting B cells are indicated (+). EBNA-LP (leader protein) plays an accessory role (+/-). Burkitt's lymphoma tumour cells display a latency I gene expression pattern but drift in culture to the latency III pattern seen in initially transformed cells and LCLs. For more information on latent gene function see (Kempkes and Robertson 2015; Cen and Longnecker 2015)

Fig. 18.1 Binding of EBV transcription factors at human *RUNX3* on chromosome 1. ChIP-sequencing reads for EBNA2 and EBNA3 proteins (3A, 3B and 3C) in Mutu III BL cells and the GM12878 LCL and H3K27ac signals in GM12878 from ENCODE. *RUNX3* runs right to left in the human genome. The arrow indicates the promoter active in B cells. Numbering indicates the major EBNA2 binding sites in the 18 kb upstream super-enhancer. The super-enhancer is bound by EBNA2 and active in both cell lines

Fig. 18.2 Binding of EBV transcription factors at human *RUNX1* on chromosome 21. The P1 and P2 promoters and the RUNX1a, RUNX1b and RUNX1c isoforms are shown. Labelling as in Fig. 1. Numbering indicates the major EBNA2 binding sites in the 111 kb upstream super-enhancer, which is bound by EBNA2 and active in Mutu III BL cells but not GM12878 cells

Fig. 18.3 Model of EBV control of the *RUNX* transcriptional network in different B cell backgrounds. EBNA2, 3B and 3C bind to the *RUNX3* super-enhancer (SE) in

EBV-infected B cells to activate *RUNX3* transcription. RUNX3 then binds to the *RUNX1* P1 promoter to repress *RUNX1* transcription with its binding partner CBFβ. **a** In EBV-infected BL cell-lines expressing the latency III pattern of gene expression (Table 1), RUNX1 is also regulated positively through EBNA2 binding to a *RUNX1* SE. EBNA3B and 3C attenuate this EBNA2 activation, but higher RUNX1 expression is maintained. **b** In EBV-infected LCLs the *RUNX1* SE is inactive and not bound by EBNA2, 3B or 3C and *RUNX1* is repressed by RUNX3/CBFβ

Fig. 18.4 RUNX3 binding at *RUNX1* and *RUNX3*. **a** Binding of RUNX3 at human *RUNX3* on chromosome 1. ChIP-sequencing reads for RUNX3 in GM12878 cells from ENCODE are shown alongside ChIP-sequencing reads for EBNA2 in GM12878. Labelling as in Fig. 1. **b** Binding of RUNX3 at human *RUNX1* on chromosome 21. ChIP-sequencing reads for RUNX3 in GM12878 cells from ENCODE are shown alongside ChIP-sequencing reads for EBNA2 in Mutu III BL cells. Labelling as in Fig. 2

Table 18.1

EBV latent protein	Essential for immortalisation	latency I expression	latency III expression	Function
EBNA1	+	+	+	Viral genome maintenance and segregation, latent replication, transcription activation.
EBNA2	+	-	+	Transcriptional activation of all viral latent genes and many cell genes. Binds RBP-J and PU.1.
EBNA3A	+	-	+	Transcriptional activation and repression. Competes with EBNA2 to bind RBP-J. Polycomb repressor complex-mediated cell gene silencing.
EBNA3B	-	-	+	Transcriptional activation and repression. Competes with EBNA2 to bind RBP-J. Tumour suppressor.
EBNA3C	+	-	+	Transcriptional activation and repression. Competes with EBNA2 to bind RBP-J. Polycomb repressor complex-mediated cell gene silencing. Deregulates the cell-cycle.
EBNA-LP	+/-	-	+	Coactivates genes with EBNA2.
LMP1	+	-	+	Transforming oncogene. Constitutively active CD40-like receptor. Activates NF-κB, JNK and p38 signalling pathways. Enhances cell survival and growth.
LMP2A	-	-	+	BCR mimic and decoy. Activates PI3-K/AKT. Regulates autophagy and blocks viral reactivation.
LMP2B	-	-	+	Blocks viral reactivation. Impairs activation of LMP2A.

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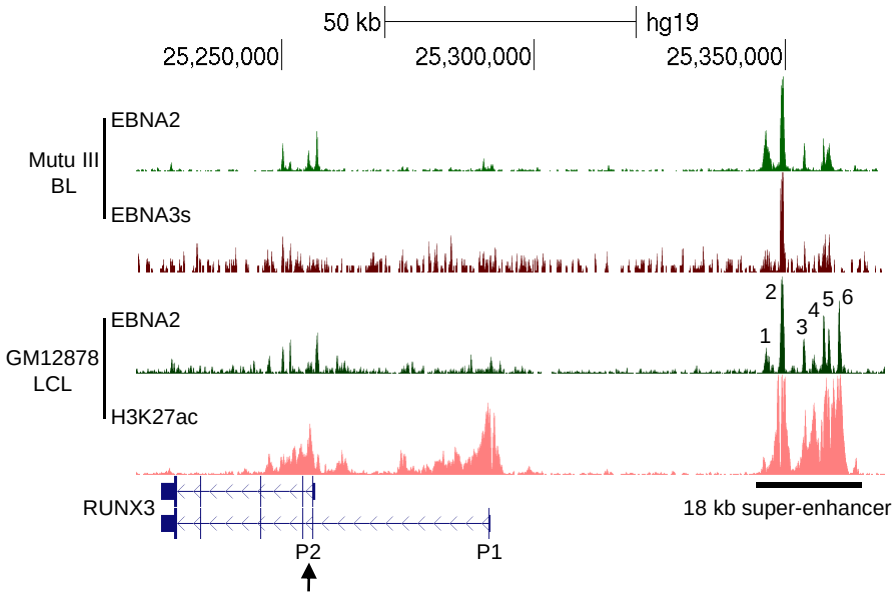
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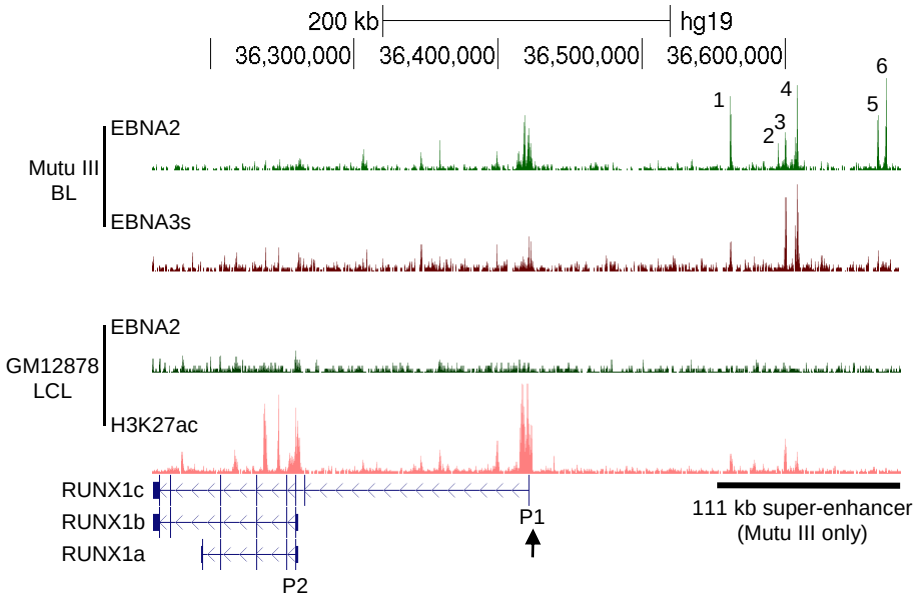
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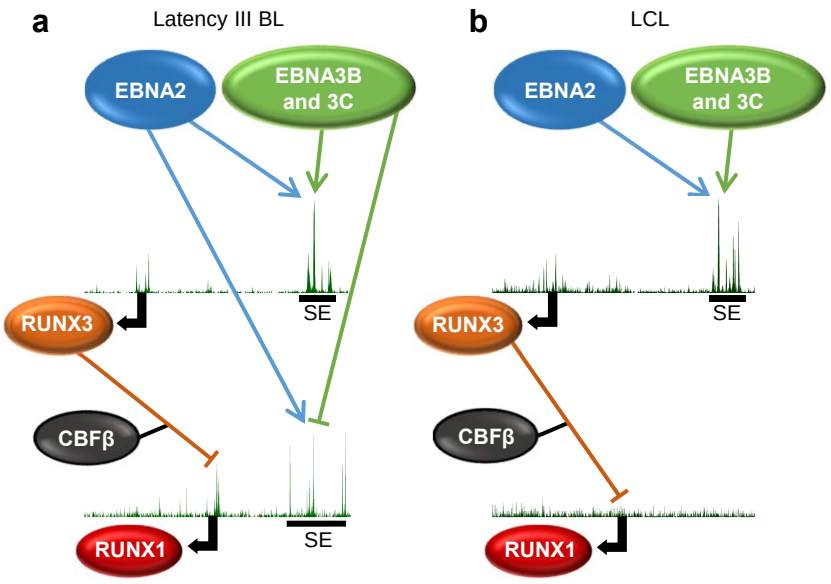
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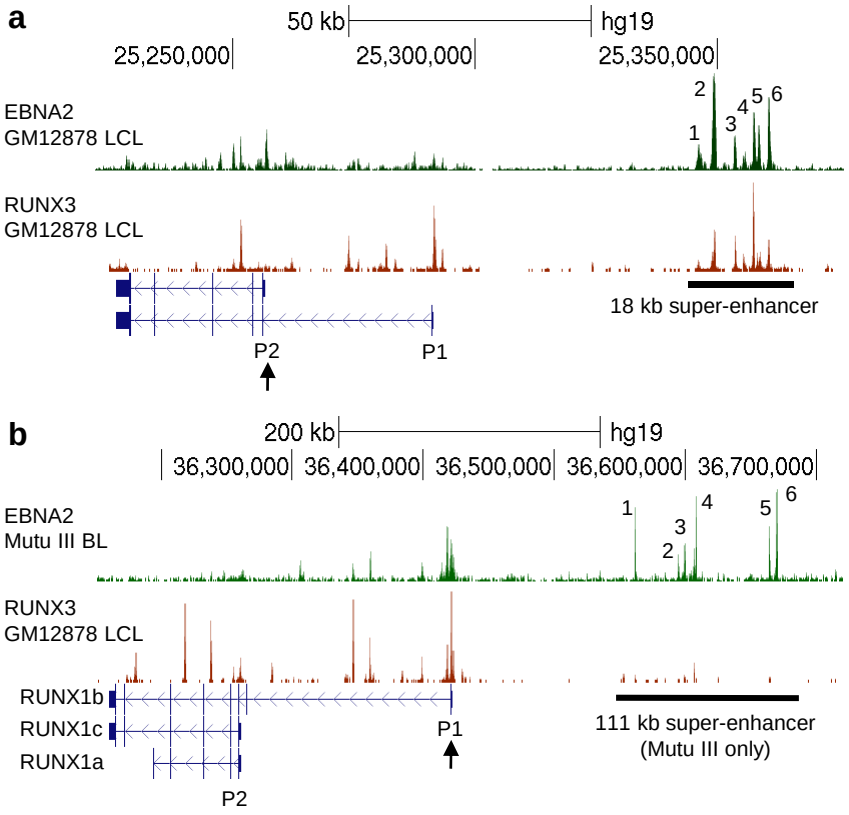
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50 kb
hg19
25,250,000
25,300,000
25,350,000
Mutu III
BL
EBNA2
EBNA3s
GM12878
LCL
EBNA2
H3K27ac
1
2
3
4
5
6
RUNX3
P2
P1
18 kb super-enhancer


200 kb
hg19
36,300,000
36,400,000
36,500,000
36,600,000
1
2
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EBNA2
Mutu III
BL
EBNA3s
EBNA2
GM12878
LCL
H3K27ac
RUNX1c
RUNX1b
RUNX1a
P1
P2
111 kb super-enhancer
(Mutu III only)


a
Latency III BL
EBNA2
EBNA3B
and 3C
SE
RUNX3
CBFβ
SE
RUNX1
b
LCL
EBNA2
EBNA3B
and 3C
SE
RUNX3
CBFβ
RUNX1


a
50 kb
hg19
25,250,000
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EBNA2
GM12878 LCL
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RUNX3
GM12878 LCL
18 kb super-enhancer
P2
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200 kb
hg19
36,300,000
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EBNA2
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RUNX3
GM12878 LCL
RUNX1b
RUNX1c
RUNX1a
P1
P2
111 kb super-enhancer
(Mutu III only)