DETERMINING THE ARCHITECTURAL ROLES OF EBF1 IN THE TOPOLOGICAL ORGANIZATION OF THE GENOME AND ITS FUNCTIONAL CHARACTERIZATION DURING B CELL FATE DETERMINATION

A Thesis

Submitted for the Degree of

Doctor of Philosophy

By

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Certificate

This is to certify that this thesis entitled **"DETERMINING THE ARCHITECTURAL ROLES OF EBF1 IN THE TOPOLOGICAL ORGANIZATION OF THE GENOME AND ITS FUNCTIONAL CHARACTERIZATION DURING B CELL FATE DETERMINATION"** submitted by **Priyanka Kriti Narayan** bearing registration number **14LAPH08** in partial fulfillment of the requirement for the award of Doctor of Philosophy in the Department of Animal Biology, School of Life Sciences, is a bonafide work carried out by him under my supervision and guidance.
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- **Priyanka Kriti Narayan** and Jagan Pongubala, **Functional characterization of EBF1 during B cell fate determination** Presented Poster at in the 14th THE CYTOMETRY SOCIETY ANNUAL CONFERENCE AND WORKSHOP,
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DECLARATION

I hereby declare that the results of the study incorporated in the thesis entitled **"DETERMINING THE ARCHITECTURAL ROLES OF EBF1 IN THE TOPOLOGICAL ORGANIZATION OF THE GENOME AND ITS FUNCTIONAL CHARACTERIZATION DURING B CELL FATE DETERMINATION"** submitted by me under the guidance and supervision of **Prof Jagan M.R Pongubala**, is original and independent research work. I also declare that it has not been submitted previously in part or in full to this University or any other University or Institution for the award of any degree or diploma.

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Table of Contents

ABBREVIATIONS

cDNA	Complementary DNA
qRT-PCR	Quantitative Reverse transcription- Polymerase Chain Reaction
SCF	Stem cell Factor
4-OHT	4-O-Hydroxy Tamoxifen
NEB	New England Biolabs
nt	Nucleotide
PBS	Phosphate Buffered Saline
µg	Microgram
µM	Micromolar
mL	Millilitre
MCSF	Macrophage Colony-Stimulating Factor
Kb	Kilobase
IL-7	Interleukin-7
IL-3	Interleukin-3
FBS	Fetal Bovine Serum
GMCSF	Granulocyte Macrophage Colony-Stimulating Factor
ER	Estrogen Receptor
EBF1	Early B cell Factor 1
dNTPs	Deoxyribonucleotide triphosphates
^0C	Degrees Celsius

DTT	Dithiothreitol
bp	Basepairs
3D	Three-dimensional
BAC	Bacterial artificial chromosome
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
GFP	Green fluorescent protein
Mb	Megabase
MPPs	Multipotent progenitors
Pax5	Paired box 5
Tris-HCl	Tris (hydroxymethyl) aminomethane hydrochloride
PC1	Principal component 1
min	Minutes
SSC	saline-sodium citrate

CHAPTER-1:

INTRODUCTION

1.1. A Brief Overview of the Haematopoietic System

Hematopoiesis generates a wide variety of cell types, each of which has a different function, are generated and constitute blood and the immune system throughout an organism's existence [1][2]. In the mammalian embryo, hematopoiesis starts in the yolk sac. Throughout the lifetime of an organism, hematopoietic stem cells (HSCs) are responsible for the creation and maintenance of numerous types of immune cells [3]. They have a tremendous capacity for self-renewal and are, by their own make-up, pluripotent. [4]. HSCs are often found in a specialized niche of bone marrow, which supports and provides the milieu for HSC self-renewal. The niche is a stem cell's milieu, which controls how often cells divide and how they differentiate. HSCs lose their capacity for self-renewal when they exit the niche and either begin to differentiate in response to cytokine signaling or die [5]. The concept of niche in other tissues has been developed from the research on the testes and ovarian stem cells of Drosophila [6][7]. The stem cell niche must alter as the location of hematopoiesis changes during vertebrate growth.

Adult mammals' bone marrow is home to a small population of cells known as hematopoietic stem cells (HSCs). They are at the top of a hierarchy of progenitors that gradually becomes limited to a number of or just one lineage [8]. Before birth, hematopoiesis occurs in multiple sites within the growing embryo. The para-aortic area of the embryo, the extraembryonic yolk sac (YS), the foetal liver, and the placenta are among these places. In the end, the process concludes in the bone marrow. In mice, the first occurrence of hematopoiesis in the Yolk Sac occurs as early as the eighth day of embryonic development, which corresponds to Carnegie Stage (CS) 8 in humans. During primitive hematopoiesis, along with rudimentary macrophages, the early erythroblasts contain large nuclei that express the embryonic globin gene [5].

During the development of blood stem cells in vertebrates, certain embryonic cells are allocated and specified in a variety of areas as the organism develops [9]. In the bone marrow, osteoblasts, Mesenchymal cells, reticular cells, osteoclasts, fat cells, endothelial cells, blood cells, and other cell types collaborate to promote the survival and function of HSCs. Such findings highlight the significance of niche-exposed soluble ligand-bound or membrane-bound cues for appropriate HSC behaviour.

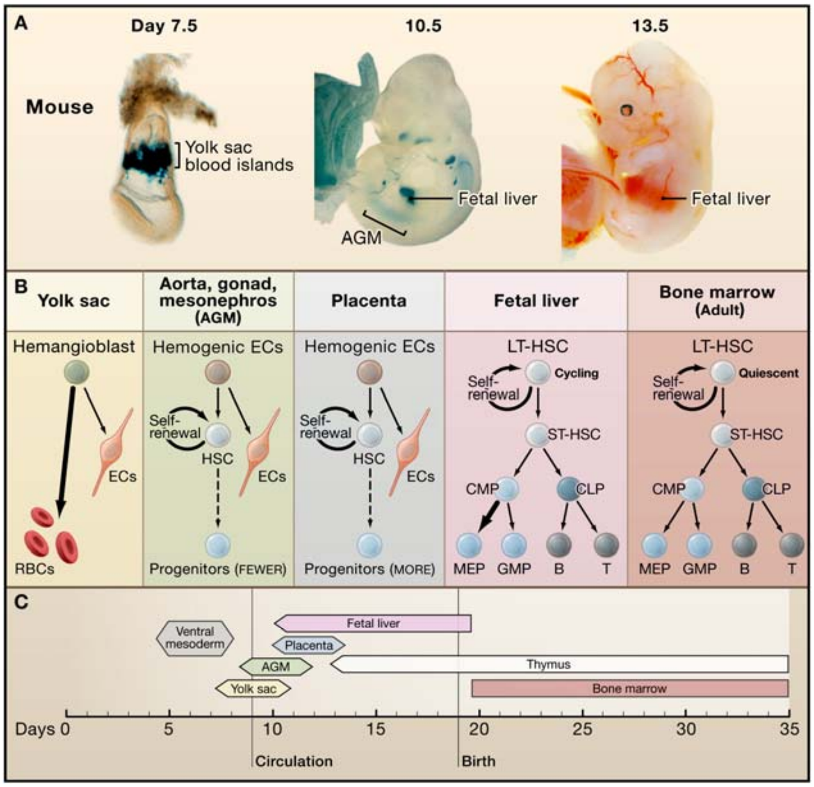


Fig 1.1. : Developmental Control of Mouse Hematopoiesis [7]

(A) Hematopoiesis begins in the yolk sac (YS) blood islands and progresses to the placenta, fetal liver, and aorta-gonad mesonephros (AGM) area (FL). YS blood islands are made visible by the lacZ labeling of transgenic embryos that express lacZ under the control of GATA-1. LacZ stains AGM and FL in Runx1-LacZ knockin mice.

(B) Hematopoiesis favors the generation of particular blood lineages in each location. Red blood cells (RBCs) and endothelial cells (ECs). Abbreviation: CLP, common lymphoid progenitor; ST-HSC, CMP, common myeloid progenitor; a short-term hematopoietic stem cell. MEP, megakaryocyte/erythroid progenitor; GMP, granulocyte/macrophage progenitor;

(C) Shifting hematopoiesis locations throughout developmental time frames.

1.2. Lineage restriction in HSCs

The present paradigm proposes that the beginning of the differentiation of Hematopoietic stem and progenitor cells (HSPC) is linked to the cessation of self-renewal and the creation of distinct multipotent, oligopotent, and eventually unipotent progenitor cell stages [10]. However, HSPCs show immense flexibility to produce multiple blood cell types which are made possible by a variety of mechanisms that regulate HSPC activity, including lineage restriction. Lineage restriction is a process by which HSPCs commit to a particular lineage and lose their ability to differentiate into other lineages. This commitment is irreversible and ensures that each cell type in the hematopoietic system performs its specialized function. Lineage restriction downstream of the HSPC continuum is crucial in maintaining blood cell homeostasis and preventing uncontrolled differentiation into unwanted lineages.

In addition, despite substantial research attempting to decipher the intricate networks driving stem cell lineage specification, the broad concepts underpinning regulatory mechanisms for lineage constraints remain obscure [11][12]. The decision-making process is comprised of numerous steps, including the formulation of a number of viable possibilities, the selection of one option from the range, and the determination of whether additional options are permanently closed or remain latent/clandestine. There is also the question of how a cell "remembers" and delivers its fate to its progeny. However, in order to comprehend the orchestration of gene expression in stem cell activity, it is necessary to develop a more accurate model of the network of regulatory components and the influence of external variables [13].

1.3. Cytokines Related to the Hematopoietic System

Cytokines are proteins that regulate the growth and function of numerous immunological and nonimmune cell types. They are of special interest to immunologists due to the importance they play in immunological regulation, the role they play in the pathogenesis of the disease, and the increasingly important function they play in the treatment [14]. An extrinsic mode of guidance for the lineage choice of multipotent progenitors is provided by the cytokines, which are soluble protein factors that are released by progenitor cells as well as adult cells. Cytokines serve a crucial role in the regulation of hematopoiesis because they influence a number of hematopoietic stem cell properties, particularly proliferation, maturation, survival, and function [15]. In vitro studies reveals that when combined with IL-11 or IL-3, the growth factors SCF, FLT-3L, and Tpo promote the long-term repopulation of LSK cells.. Cytokines, on the other hand, do more than only ensure the continued existence and multiplication of progenitors; they also serve as lineage educators. Depending on whether they are developed in the presence of G-CSF or M-CSF, granulocyte–macrophage progenitors (GMPs) will differentiate into either granulocytes or monocytes. Similarly, it has been discovered that myeloid-biased progenitors exhibit a poor response to IL-7, despite the fact that IL-7 signalling is known to be crucial for the early stages of development of T and B lymphocytes [16].

1.4. B cell lymphoid restriction

Lymphoid development commences with the creation of lymphocytes, which are white blood cells that contribute immensely to the body's immunological response. In the bone marrow, hematopoietic stem cells are what give rise to B and T lymphocytes, which are then categorised as such. T lymphocytes aid in the death of infected cells, whereas B lymphocytes grow into plasma cells that produce antibodies that guard the body against infection. Multiple variables, such as cytokines (signaling molecules) and transcription factors, influence maturation and differentiation.

The protein LMPP, which stands for "lymphoid maturation promoting factor," is a crucial element in early lymphoid development. Without LMPP, hematopoietic stem cells would develop into red blood cells or macrophages. Recent research has shown that, along with its role in early development, LMPP also regulates antibody synthesis by plasma cells later in life. Therefore, a deeper understanding of this protein's function could lead to novel ideas on how to enhance protection against infections.

The hematopoietic system is a crucial developmental model for unravelling the gene-regulatory networks underpinning the specification and commitment of the B-cell lineage. Lymphoid-primed multipotent progenitors, also known as LMPPs, develop into common lymphoid progenitors in the bone marrow (CLPs). Cellular immunity requires T cells, while humoral immunity relies on B cells. B cells express clonally different cell surface immunoglobulin (Ig) receptors that recognize the plethora of antigens, making them the primary arm of adaptive immunity. Lymphocyte development begins in the main lymphoid tissue (such as human fetal liver and fetal/adult bone marrow) and continues to secondary lymphoid tissue, where mature lymphocytes are produced. B-cell development in mammals occurs in a number of stages (lymph nodes and spleen). The plasma cells are the terminal differentiation point of B cells. The pluripotent hematopoietic stem cell (HSC) must undergo differentiation into more restricted progenitors in order for the process of blood cell formation to proceed continuously. These progenitors ultimately commit to one of a number of pathways and mature into cells of the designated lineage. Thus, when pluripotent progenitors commit to either the myeloid lineage or the lymphoid, they make either common myeloid progenitors(CMPs) or common lymphoid progenitors (CLPs) [17]. B, T, and NK cells are produced by the IL-7 receptor (IL-7R)+ CLP [18], whereas erythrocytes, megakaryocytes, granulocytes, and macrophages are produced by the IL-7R CMP (14).

1.5. Development of T cell

The thymus is the organ that is responsible for the creation of T cells. T cell development progresses through a series of discrete steps, the first of which occurs when hematopoietic stem cells in the highly specific milieu of the thymus decide to pursue a particular lineage. The pathway of development in the thymus is controlled by how the growing thymocytes interact with the local stromal cells and soluble cytokine growth factors [19]. A previous study determined that a constant migration of donor progenitors from the bone marrow (BM) to the thymus is required for the maintenance of long-term thymocyte differentiation. This is because the progenitor cells that reach the thymus have a limited lifespan under physiological conditions. On the other hand, progenitor cells have the potential to maintain long-term T-cell generation if the appropriate conditions are met [20]. When LMPPs enter the thymus, they are sensitive to the influence of Notch and DLL4 signaling, which causes them to preferentially develop in the direction of the T-lineage and become T cells. During the maturation of T cells, the expression of surface markers such as CD44, c-Kit, and CD25 can be utilized to characterise the double-negative (DN) stages. During the process of T cell formation, cells go through numerous stages of being double negative (DN) before transitioning into the condition of being double positive (DP).

T-cell differentiation, proliferation, and survival are all supported by the thymic epithelium, which accomplishes this by releasing a strong combination of growth factors and cell surface ligands. Among the many environmental signals provided by thymic stromal cells, the ligands for the Notch cell-surface receptors delta-like ligand 1 (DLL1) and DLL4 are particularly crucial. These ligands are responsible for triggering the growth of ETPs in the direction of the T-lineage. During the pro-T-cell phase, these ligands are essential for starting and keeping the T-cell developmental programme going. This is accomplished through the activation of early-T genes such as *Ptcra* and *Cd3e*, as well as important transcription factors such as *TCF-1* (which is encoded by *Tcf7*). This, in turn, is necessary for the continuation of T-cell development through the activation of critical factors such as *GATA3* and *BCL11B* [21]–[23]

T-cell progenitors go through gene rearrangements of T-cell receptors and construct TCR complexes; these processes are necessary for their continued existence and will determine their future activities. These cells can develop into several different lineages of T cells, notably γδT cells and αβT cells. The regulatory T cells (TReg cells,) natural killer T (NKT) cells, CD4+ T cells, and CD8+ T cells are all sub-lineages that originate from the αβ T cells; Nevertheless, once these cells have left the thymus and entered the periphery, their divergence into multiple sub-lineages has resulted in very divergent activities [24]. In order for this to take place, it is necessary for there to be co-expression of functioning T cell receptors (TCR), such as CD4 and CD8. In the end, there is only one type of receptor that is predominant, and it is this receptor that has the ability to either generate CD8+ T cells or CD4+ T cells. After leaving the thymus and migrating to the secondary lymphoid organs, naive CD4+ cells go through further differentiation to become one of several subpopulations of T helper (TH) cells. TH cells are responsible for assisting the immune system in fighting off infections and other diseases [25].

1.6. Molecular events during lymphoid restriction

The process of hematopoietic lineage differentiation is a complicated one that involves a wide range of changes in gene expression. This process of differentiation needs to be carefully controlled and regulated in order to develop a specific kind and number of mature cells. In order to successfully transition from one cell type to another, it is frequently necessary to alter the expression of hundreds or even thousands of genes. This is due to the fact that every cell type has its own unique, specialized gene expression program. It is still completely unknown how a repertoire of active and potentially active genes is maintained in a particular cell type and how it is reprogrammed during the process of switching from one cell type to another. This is only one of the numerous questions that have not been satisfactorily answered [26][27]. The regulation of transcriptional programs is accomplished by the coordinated actions of three primary mechanisms. One of the mechanisms relies on transcription factors, which are proteins that bind to particular sequence motifs and regulate particular groups of genes as a result [26][27] The two components that make up the second regulatory mechanism are called posttranslational changes of histones and the methylation of DNA. When combined, the processes of DNA methylation and post-translational modification of histones have the potential to produce an epigenetic memory. This memory can help a cell and its progeny keep their differentiated states across time [28]. HSCs are required to activate and silence specific gene expression programs in order to commit to a lineage, which is followed by differentiation into multipotent progenitors. [29]. The decision of which cell type to produce is known as fate choice, and it is controlled at the molecular level. Several key molecules have been identified that play a role in this process, including transcription factors, microRNAs, and chromatin-modifying enzymes.

1.7. Transcription factor taking part in B lineage restriction

Studies have genetically established that unique sets of transcription factors are necessary for the process of cell fate specification in both B and T cell lineages [30][17]. The B lymphocytic, as well as the erythrocytic lineages, have proven useful paradigms for investigating the regulatory networks that are responsible for cell fate specification. The transcription factor like Ets family proteins and PU.1, has shown a role in the lineage determination between myeloid and lymphoid cells. PU.1 is a crucial factor in the progression of lymphoid as well as myeloid lineages throughout embryonic development [31][32]. Multilineage cytokines (SCF, IL-6, and IL-3) promote the in vitro expansion of *PU.1-/-* hematopoietic progenitors; They cannot, however, respond to the lymphoid IL-7, myeloid GM-CSF, G-CSF, and M-CSF cytokines due to the inadequate expression of the relevant receptors. [33]. Restoring PU.1 expression with retrovirus allows PU.1-/- progenitors to differentiate into B macrophages and lymphocytes, as expected. Unexpectedly, lower PU.1 expression enhances B-cell development, while higher PU.1 concentrations inhibit B-cell development and encourage macrophage differentiation. This finding was made possible by the fact that macrophage differentiation was promoted by low PU.1 levels [34]. On a molecular scale, low PU.1 concentrations are known to initiate the IL-7R gene, in contrary to high PU.1 levels, which repress IL-7R expression [35]. As a result, graded expression of PU.1 determines whether early progenitors develop into myeloid or lymphoid cells, in part by determining their receptivity to IL-7.

B-lymphopoiesis in the bone marrow is dependent on two transcription factors: E2A and the early B-cell factor (EBF). If neither of these transcription factors is present, B-cell growth is halted prematurely. This happens before the immunoglobulin heavy-chain (IgH) gene undergoing the DH-JH rearrangement [36]. Many B-cell-specific genes, such as ***λ*5** and *VpreB, mb-1* and *B29,* and RAG1 and RAG2, are triggered by these regulators. This was revealed by inducing EBF and E2A expression in hematopoietic progenitor cells [37]. Additional key transcriptional regulators include the products of genes such as EBF1, Ikaros, E2A, Pu.1, and Pax-5 [38]. The EBF, E2A, and Pax-5 genes are specifically required at the moment of B lineage commitment. The Pu.1 and Ikaros genes, on the other hand, are involved in the establishment and development of multipotent progenitors. Previous Studies have shown that B cells stop developing in the bone marrow of Pax-5-deficient mice after the start of D-JH rearrangements but before the start of V-DJH rearrangements. This occurs after the beginning of D-JH rearrangements [39].

1.8. EBF1's function in the specification of B cell

For the mechanism of B lineage specification and commitment, including the activation of B-cell-specific gene expression and the inhibition of alternative lineage determinants, signaling cascades and transcriptional networks are necessary. It is crucial to activate gene expression that is specific to B cells in order to specify and commit to the B cell lineage. This is due to the fact that these processes entail the synthesis of genes that are specific only to B cells, in particular [40]. The specificity of the B cell lineage is determined by a circuit of transcription factors, including *E2A, Ebf1*, and *Foxo1*. This is accomplished by the activation of genes that code for a variety of molecules that are essential to the development of B cells as well as their activity. A system of transcription factors controls this process and keeps it running smoothly [41]. It is remarkable that the targeted deletion of most of *Ebf1* or *E12* -activated genes, such as IL7R, λ5, and Rag-1, causes B lymphopoiesis to malfunction later in development than in mice lacking *E2A* or *Ebf1*. This is because the B lymphopoiesis process stops earlier in mice that are deficient in E2A or Ebf1. This is due to the fact that animals lacking *E2A* or *Ebf1* reach this stage of the B lymphopoiesis process earlier than other mice [42]–[44]. Transduction of *Ebf1* in *PU.1-/-* cells promotes the development of defined pro-B cells by promoting IL-7-dependent proliferation, IgH recombination, and gene expression of early B lineage [45]. In EBF1-/- bone marrow, the production of B cells is inhibited prior to the expression of genes implicated in the B lineage and the initiation of IgH recombination. EBF1 has been found to play an important role in a variety of B cell development processes at the molecular level, including the control of Pax-5 and early B lineage gene expression, the maintenance of accessible Ig recombination signals, and the suppression of heterochromatin formation [46]–[48] *Ebf1* is a unique stage-specific DNA-binding protein expressed by adipocytes, olfactory neurons, and every cell that comes from the B lineage, with the exception of plasma cells that have reached the terminal differentiation stage of their development. *Ebf1* is involved in the regulation of the rob-1 gene, which is unique to pre-B and B lymphocytes. *Ebf1* has previously been identified as a protein that is unique to particular tissues and stages of development [38][49]. *Ebf1*, also referred to as the early B cell factor , can identify DNA through its N-terminal domain, which has an important zinc coordination motif. This protein can also form homodimers [49][50]. *Ebf1* is distinguished by the presence of a zinc coordination motif, in addition to several dimerization and transcriptional activation domains. There is evidence that *Ebf1* transcripts are present in B cell-derived cell lines at the early phase of development, but not in T cells or other hematopoietic cell lineages. The amounts of *Ebf1* transcripts in other non-lymphoid tissues are generally lower, with the exception of the spleen and adipose tissues, which both contain substantial numbers of *Ebf1* transcripts [50].

The modular domains that make up *Ebf1* are responsible for binding to DNA, dimerization, and activation of transcription. The amino-terminal half of *Ebf1* contains all of the essential sequences required for DNA binding, dimerization on optimum half-site sequences with adequate spacing, and transactivation. In order for *Ebf1* to bind DNA to natural sites, such as those located in the promoters of the mb-1 gene and numerous olfactory-specific genes, it is necessary for it to possess a separate dimerization domain that is composed of a-helical repeats. It is possible that the a-helical repeats serve to stabilize the links between *Ebf1*'s DNA-binding domains. This would enable the creation of homodimeric complexes even in the lack of DNA. A distinctive zinc-coordination motif is required in the DNA binding domain of EBF for DNA interaction. Additionally, it was shown that two different domains of EBF were responsible for mediating the activation of transcription. One of these domains coincides with the DNA binding domain. When *Ebf1* is transduced into EBF1-/- progenitors, all early B lineage genes, including those implicated in *CD19* and IgH recombination, are upregulated. Notably, in *EBF1-/-* progenitors, *Pax-5* failed to efficiently activate *CD19* or *mb-1* expression. This suggests that *Ebf1* is necessary for *Pax-5* to carry out its function of activating these two genes in order to achieve the desired results [45].

1.9. Spatiotemporal events during B cell development

Differentiation of hematopoietic stem cells (HSCs) is dependent on chromatin regulators, epigenetic modifiers, and several distal enhancers of promoters in order to transform pluripotency into multipotency and terminally differentiated lineage. Interactions between the promoter, insulator, and enhancer regions of chromatin are critical. In this way, enhancers can affect promoters that are not necessarily in close proximity to them. Therefore, it may not be possible for these two regions to interact in a linear form, which increases the likelihood of genome folding [51]. Gene promoters and distal enhancers drive lineage-specific gene expression throughout embryonic development. By guiding the recruitment of RNA polymerases and auxiliary components to transcription start sites, promoters initiate RNA synthesis. By binding chromatin remodelling complexes and sequence-specific transcription factors, enhancers control the expression of target genes at precise times and in specific tissues [52]–[54].

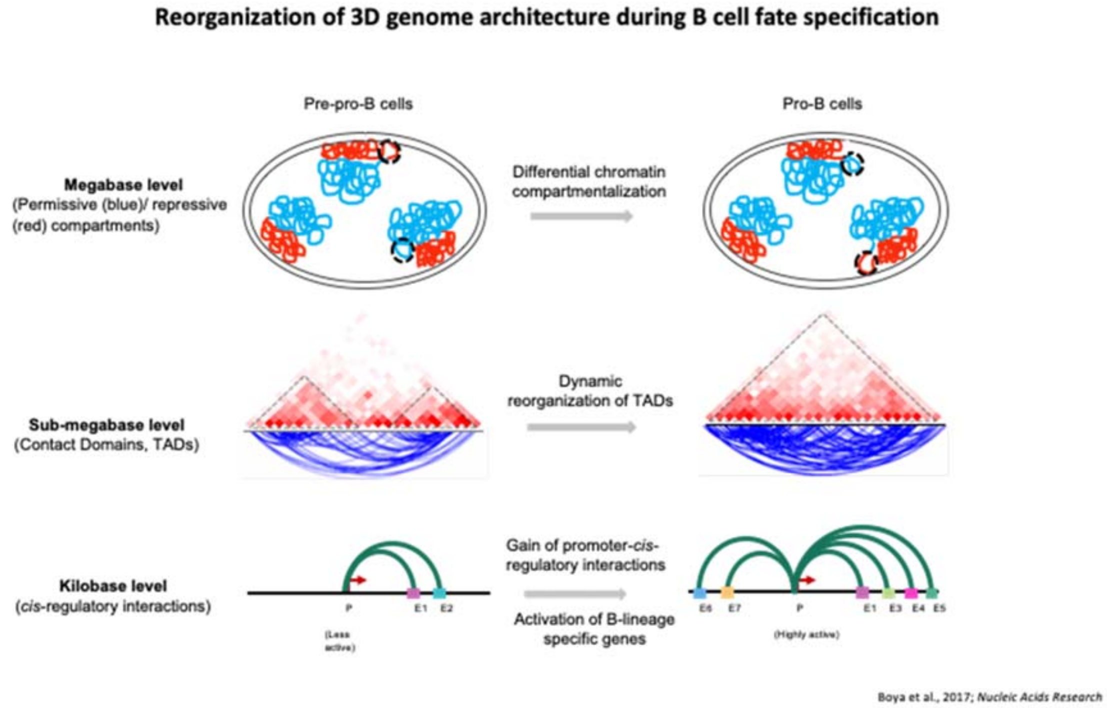
Previous studies have shown that the process of chromatin looping is necessary for the control of lineage-specific markers during development. Additionally, chromatin architecture, which is structured within the nuclear space and has a significant impact on genome function, is determined by chromatin looping[55]. Tracing the spatial architecture of chromatin fibres is a crucial first step in acquiring an understanding of the varying patterns of gene expression that precede developmental lineage change. In recent years, numerous methods that can deconstruct the three-dimensional architecture of chromatin looping have been revealed. For instance, fluorescence in situ hybridization, also known as FISH, is utilized on a large scale in order to determine the distance that exists between two genetic loci. Other methods have also been found that can deconstruct the three-dimensional architecture of chromatin looping [56][57]. In this instance, 200 kb probes derived from BAC or YAC have been utilized; nevertheless, fosmid is currently the most common type of probe, and it provides coverage of approximately 40 kb [55].

1.10. 3D Chromatin reorganization

It is crucial to understand how the genome organizes in three dimensions inside the nucleus in order to comprehend its function, as there is emerging evidence that implies the 3-D organization of the genome is strongly connected to the regulation of transcriptional programs [58]–[61]. Although the fundamentals of the organization of chromatin within the nucleus have been understood for over half a century, the comprehensive three-dimensional organization of chromatin and its association with cell-type-specific patterns of gene expression are not yet fully understood. During the course of the last century, advancements in microscopy techniques have made it possible to investigate chromosomal organization at ever-increasing levels of resolution and specificity [62]. Additional studies indicated that during interphase, chromosomes occupy a preferred position known as chromosomal territories, where large chromosomes are found close to the nuclear periphery and small chromosomes are found further within the nucleus [63][64]. This arrangement is further confounded by the discovery that gene-poor chromatin is concentrated near the nuclear periphery, whereas gene-dense chromatin is localized in the nucleus' core [65][66]. The spatial division of chromatin into active and inactive regions suggested that the location of genes within the nucleus influences their activity. Immunoglobulin heavy chain (IgH) loci are mainly located at the nuclear border in pro-T cells and multipotent progenitors, where they are transcriptionally silent, supporting the aforementioned notion. In contrast, these genomic loci are positioned away from the nuclear periphery in pro-B cells, where they undergo large-scale compaction and subsequent rearrangement [67]. The study of chromosome folding has been revolutionized over the course of the past decade as a result of the development of molecular approaches that are based on chromosome conformation capture (3C) technology. These approaches, when combined with methods of modeling and analyzing chromatin interaction data [68]–[70].

Recent Hi-C investigations have been utilized to a large extent for the purpose of researching the three-dimensional spatial structure of the genome within the nucleus. The discovery that the genome is divided into a compartment known as TAD (topologically associated domain), which demarcates functional epigenetic domains defined by unique chromatin marks, was one of the most significant aspects that Hi-C uncovered [71] Recent genome-wide investigations have made significant improvements to the comprehension of the genomic architecture underlying gene expression in higher eukaryotic organisms. Integrating investigations of transcription factor (TF) binding profiles, transcriptome, and epigenomes reveals the complex organisation of dispersed individual transcription units all across the genome. These analyses also show the causal links between chromatin state, regulatory DNA sequences, and transcriptional activity [52][54]. The interaction of regulatory elements, such as cell type-specific transcription factors, chromatin remodeling complexes, promoters, and enhancers that bind to these regulatory elements, is widely believed to be essential for accurate spatiotemporal gene expression. It is believed that these interactions control how genes are expressed [52].

The three-dimensional (3D) genome architecture of a cell is important for many cellular processes, including cell fate specification. The reorganization of the 3D genome during B cell fate specification is a complex process that is poorly understood. However, it is known that the 3D genome architecture has a role in the development of B cells and their ability to respond to antigens. In order to facilitate B cell development, the 3D genomic architecture is modified during B cell fate specification. This process is essential for the proper development of the immune system. Antibodies are created by B cells, which are a type of white blood cell. These antibodies are important for the body's response to infection and disease. The reorganization of the 3D genome during B cell fate specification allows for the development of different types of B cells.


Reorganization of 3D genome architecture during B cell fate specification

Boya et al., 2017; *Nucleic Acids Research*

Fig 1.10. Reorganization of 3D genome architecture during B cell fate specification[72]**.**

(A) chromatin is arranged into permissive and repressive compartments at the megabase level (A and B).

(B) At the sub-megabase level chromatin is organized as highly self-interacting domains known as TADS which are called topologically associated domains.

(C) At the Kilobase level chromatin is organized as a chromatin loop-like promoter enhancer interaction.

1.11. Epigenetic model for B cell development

Transcription factors, which bind to their target genes' DNA and regulate their expression, are the primary mediators of gene regulation. In recent years, mounting evidence has revealed that chromatin-based regulatory systems besides transcription factors may serve important roles in the development and maintenance of transcriptional programmes. These functions may be crucial, as they may aid in the initialization and upkeep of transcriptional programmes. This layer of control consists of DNA-bound histones with post-translational modifications, chromatin remodelling, and DNA methylation. The two most common forms of epigenetic alterations are known as DNA methylation and histone modification. As a result of the fact that

DNA methylation in vertebrates takes place almost solely within the context of CpG dinucleotides, the bulk of the CpG dinucleotides in the vertebrate genome has been modified to include methyl groups [73][74]. In the early mouse embryo and embryonic stem (ES) cells, non-CpG methylation occurs at a very low intensity; however, in somatic tissues, this intensity is drastically reduced [75][76].

More than one hundred distinct post-translational modifications, such as acetylation, methylation, phosphorylation, and ubiquitination, can occur on the core histones that are responsible for the formation of the nucleosome. These tend to take place in particular locations within the amino-terminal histone tails of the chromatin. In spite of the fact that a large majority of these alterations are still not well known, significant headway has been made in recent years regarding the comprehension of acetylation and methylation of lysine. In contrast, methylation of lysine can have a wide variety of consequences depending on which residue is changed, whereas acetylation of lysine is always linked to the accessibility of chromatin and the activity of transcription. This is in contrast to the case with lysine methylation, which always has the same impact. There is a correlation between transcribed chromatin and the methylation of histone H3 lysine 36 (H3K36) and H3 lysine 4 (H3K4). On the other hand, methylation of H3 lysine 27 (also known as H3K27), H3 lysine 9 (also known as H3K9), and H4 lysine 20 (also known as H4K20) often correspond with suppression. Different modifications of histones can have an effect on one another, and they may also have an effect on the methylation of DNA. Protein complexes that bind to modified histones or methylation cytosines may contribute to this interaction. [77][78].

A series of epigenetic regulatory events must take place before hematopoiesis and the early B cells development can begin. This is a prerequisite for both processes. The lineage of B cells is restricted as a result of epigenetic modifications, which are then followed by Ig V(D)J recombination and, ultimately, the formation of IgM-expressing immature B cells in the bone marrow (BM). The expression of the genes Ikaros and PU.1 is necessary for lymphoid lineage commitment. Despite the fact that the mechanism by which Ikaros and PU.1 change the chromatin state of their target genes is obscure[79]. PU.1 has been linked in a number of studies to a specific pattern of monomethylation on histone 3 at lysine 4, and previous research provides evidence of this link (H3K4me1)[80]. In addition to this, it has been demonstrated that DNA methylation is an essential component of the hematopoietic process. Trowbridge and colleagues discovered that the conditional deletion of DNA methyltransferases (DNMT)1 on the hematopoietic system reduces the potential of hematopoietic stem cells to self-renew. The absence of DNMT1 led to an increase in the rate of cell cycling as well as differentiation within the myeloid-restricted progenitor pool[81]. In a similar line, the data obtained by Brooke's group demonstrated that decreased DNMT1 expression encourages the development of myeloerythroid fate rather than lymphoid fate. This, in turn, causes a comparable enrichment of myeloerythroid transcription factors, which demonstrates the protective function that proper DNA methylation has in the differentiation of lymphocytes [82].

When HSCs are in the process of differentiating into CLP and pro-B cells, the early B-cell-specific *mb-1 (CD79a)* promoter becomes hypermethylated at CpG dinucleotides for the first time. As B cell growth proceeds, this methylation is followed by progressive demethylation of the methyl group. *Pax5* is able to assist in the transcriptional activation of mb-1 and the maintenance of stepwise B cell proliferation as a result of the discovery that the transcription factors *Ebf1* and *E2A* are responsible for the demethylation of the CpG sites on the mb-1 promoter [83]. The *mb-1* promoter undergoes epigenetic modifications as a result of *Ebf1*'s mediation, which results in an increase in the accessibility of the promoter DNA to transcription factors such as *Pax5*. It is interesting to note that ATP-dependent chromatin remodelling complexes, also known as CRCs, are also implicated in the regulation of mb-1 that is mediated by *EBF* and *Pax5* [84]. The expression of *E2A, EBF, RAG1,* and *RAG2* in nonlymphoid cells could induce chromatin reorganisation by producing an open chromatin conformation via recruitment of histone acetyltransferase-containing chromatin-remodeling complexes [48][85].

1.12. Switching nuclear locations at different stages of development

As opposed to a simple linear arrangement, the genome contains a more complex structure called Three Dimensional organization. It is important for it to fold into an intricate and cohesive pattern so that genetic elements can find each other with the required frequency. It is not yet known how higher-order topologies are organized using chromatin fibers; this will need to be investigated. Different folding patterns for chromosome topologies, including helical, radial, and mixed loop-helical folding of the genome, have been postulated. These folding arrangements can be coupled [86]–[88]. Imaging experiments conducted with electron microscopy have led researchers to the conclusion that chromosomes are made up of clusters of loops that are connected to one another by linkers [89]. Throughout the course of development, genes regularly alter their nuclear neighbourhoods [90]. Suppressed genes at the nuclear periphery or in heterochromatin territories can disengage and move into the nucleus, where they can become transcriptionally active at various times and stages depending on the cell type and developmental context [90]–[92]. Multiple instances of B and T cell development demonstrate that transcriptional activity and genomic structure are functionally linked. For instance, the relocation of these loci to a confined nuclear core is intricately connected to the rearrangement of lineage-specific antigen receptors [93][94].

Throughout the duration of the cell's development, the great majority of genes in B cells stay in the same compartments, but a considerable number of genes (ten percent) switched from compartment A to compartment B and vice versa, exhibiting necessary modifications in transcript levels, according to a comparative Hi-C analysis. This was discovered despite the fact that the majority of genes continue to reside in the same compartments throughout the cell’s developmental progression [72][87]. Several genes undergo a process of repositioning that enables them to shift from compartment B to compartment A when the pro-B cell stage progresses from the pre-pro-B stage. Notable examples of this process include*, Satb2, Ebf1, Tead1, Tlr4*, and *Pou2af1.* There was a dramatic increase in the number of connections between the promoter and the enhancer after genes were moved from compartment B to compartment A, leading to higher levels of gene expression. Alternatively, during the pro-B cell stage, genes such as *cKit, Satb1*, and *Cd34* in addition to important determinants of alternate cell fates such as *Zbtb16, Vav3, Gata3, Sox6,* and *Klf4* migrate to the B compartment [87][72]. Recent research has shown that broad alterations in the nuclear architecture of plasma cells are responsible for orchestrating the fate of plasma cells. The Ebf1 loci is suppressed in developing plasma cells by moving it from the euchromatic to the peri-centromeric heterochromatin area. This movement takes place during the process of plasma cell development. Concomitantly, a different group of components, namely *Prdm1, Ell2,* and *Atf4* obtains the euchromatin state [95]. Therefore, a recurring concept that directs the formation of adaptive immune cells is the selective activation or silencing of genes throughout the shift from multipotent progenitors to differentiated B and T cells during the course of development [96].

1.13. Igh relocalization

The bone marrow is the source of hematopoietic stem cells, from which B lymphocytes are developed and the process of their development involves a series of steps that take place in sequential order. To comprehend the processes behind normal B-cell generation, it is crucial to identify and then characterize the various B-cell precursor (BCP) subgroups. At this point in time, there is a widespread consensus among researchers that the expression of the *PAX5* transcription factor is the event that kickstarts the commitment to the B-cell lineage. This is caused by the production of genes that are unique to B cells, such as *CD79a* (or Iga), and *CD19*, as well as the suppression of genes that are not suitable for the B lineage. Pro-B cells are defined as cells that express *CD79a* but do not have *CD19*. This is because *CD79a* expression is one of the early indications of commitment to the B-cell lineage. After this comes the stage of pre-B cells, which, because they display CD19, are considered to be mature B cells [97]. At this point, the recombination activating genes begin the process of V-D-J recombination at the immunoglobulin (Ig) heavy chain (IGH) locus (RAG1 and RAG2) [40].

In order for a mature B lymphocyte to generate a functional immunoglobulin, one of its two immunoglobulins heavy chain (IGH) genes must be rearranged during the V(D)J recombination process that occurs in bone marrow B-cells[98]. The finding that immunoglobulin genes go through somatic DNA rearrangement was a huge breakthrough in the science of immunology, inspiring ongoing research to identify and define the mechanisms that mediate and control this process. The variable (V), diversity (D), and joining (J) gene regions of the immunoglobulin (Ig) genes are rearranged in an orderly form during development of in the bone marrow (BM) to generate the basic Ig repertoire. Ig heavy chain gene (IGH) rearrangement comes before Ig light chain gene (ILC), and DH to JH joining comes before VH to DJH joining [99]. All of these gene segments are encircled by recombination signal sequences (RSSs) that are constant and act as recognition sites for the RAG1 and RAG2 V(D)J recombinase proteins. This RSSs are found all around the gene segments. Research into the molecular mechanism of the reaction was greatly sparked by the discovery of the recombination-activating genes RAG1 and RAG2, as well as the subsequent validation of their direct role in triggering V(D)J recombination by creating site-specific DNA double-strand breaks (DSBs) [100]. The V(D)J recombination process can be theoretically separated into two distinct stages: the cleavage phase and the joining phase. To accomplish this, researchers have performed in-depth biochemical analyses of the RAG proteins, characterised V(D)J recombination intermediates, and identified components involved in repairing these intermediates. These studies have made it possible for this to be accomplished. The fact that the cleavage phase occurs first is one of the factors that contributes to the differentiation between these two stages. After the cleavage step comes to the joining phase of the processed [101]

Objectives of the study

Development of B cells has been shown to be under the control of various TFs like *PU.1, Ebf1, E2A, Pax5*, and *IRF4*, as mutation of any one of the TFs results in blockade of B cell development at a specific stage. Previous studies have demonstrated that Ebf1 is the primary determinant for the development of B cells. To understand the architectural role of *Ebf1* in B cell fate specification we considered *Ebf1-/-* and *Rag2-/-* system which is pre-pro B and Pro B cell to identify genome-wide chromatin reorganization using HiC, which was previously done in the lab. Our HiC study showed that chromatin is hierarchically organized into TADs and chromatin loops, in agreement with earlier research.

- At the megabase level, chromatin is divided into permissive and repressive compartments (A and B).
- At the sub-megabase level chromatin is organized as highly self-interacting domains known as TADS which are called topologically associated domains.
- At the Kilobase level chromatin is organized as a chromatin loop-like promoter enhancer interaction.

Our studies have shown that chromatin undergoes global reorganization and differential compartmentalization to dictate B lineage-specific gene expression patterns. To gain a thorough understanding of the link between *Ebf1* and differential chromatin compartmentalization during B cell fate specification, we have framed two objectives:

Objective 1: Probing the role of *Ebf1* in differential chromatin compartmentalization during B cell fate commitment.

Objective 2: Functional characterization of *Ebf1* during B cell developmental progression.

CHAPTER-2:
MATERIALS AND METHODS

2.1. Materials

List of consumables, kits, and molecular biology reagents utilised in this work.

2.1.1. Reagents and Chemicals

Table 2.1. List of Chemicals, enzymes, and reagents utilized in this research.

Chemicals, Enzymes, and Molecular biology reagents	Source
T4 DNA ligase	New England biolabs
Sodium Hydroxide pellets	Sigma-Aldrich
Taq polymerase	New England Biolabs
Tris base	Sigma-Aldrich
T4 Polynucleotide kinase	New England Biolabs
Trizol/Trizol-LS	Invitrogen
Phosphatase, Calf Intestinal	New England Biolabs
Power Sybr Green Mastermix	Applied Biosystems
Random Hexamers	Invitrogen
Restriction Enzymes	New England biolabs
RNAsin plus RNase inhibitor	Promega
Sodium Chloride	Sigma-Aldirch
Phusion Polymerase	New England Biolabs
Magnesium Chloride	Sigma
Bacterial Agar	Himedia
Nuclease-free water	Ambion
DNA polymerase I, Klenow Fragment	New England Biolabs
Hydrochloric acid	Sigma-Aldrich
Isopropanol	Sigma-Aldrich
Luria Bertani Broth	Himedia
Superscript III Reverse Transcriptase	Invitrogen
DH5α E.coli strain	Invitrogen
Dithiothreitol	Promega
Ethanol, Molecular Biology grade	Hayman
EDTA	Sigma-Aldrich

Deoxyadenosine triphosphate (dATP)	Invitrogen
Deoxycytidine triphosphate (dCTP)	Invitrogen
Deoxyguanosine triphosphate (dGTP)	Invitrogen
Deoxythymidine triphosphate (dTTP)	Invitrogen
Ampicillin	Sigma-Aldrich
Agarose	SeaKem

2.1.2. Disposable material and Kits

Table 2.2. List of disposable materials and kits utilised in this research.

Disposables / Kits	**Source**
Cell strainer, 70µm	BD Biosciences
CellStar Cell culture plates (24-well)	Corning Inc.
CellStar Tissue culture dishes (60 mm)	Corning Inc.
CellStar Tissue culture flasks (T75, T25)	Corning Inc.
Cryovials, 1.5mL	Corning Inc.
Falcon Tubes (15 ml, 50 ml)	BD Falcon, Tarsons, Nunc
Filter tips	Rainin, Mettler Toledo
Glove	Genaxy
Nucleospin Gel Extraction and DNA purification Kit	Macherey Nagel
Pipette tips	Tarsons
Plasmid purification Midiprep/Maxiprep kit	Macherey Nagel
Reaction Tubes (1.5 ml, 2 ml)	Tarsons
Cuvette	Bio-Rad
FISH Tag™ DNA kit	invitrogen

2.1.3. Cell culture media and reagents

Table 2.3. List of the cell culture media and other reagents utilised in this study.

Cell culture media and reagents	Source
4-O-Hydroxy Tamoxifen (4-OHT)	Sigma-Aldrich
DMEM	Gibco
Fetal Bovine Serum (FBS)	Gibco
Flt3L	R&D Systems
IL-7	R&D Systems
SCF	R&D Systems
Fugen6	Promega
HEPES	Sigma-Aldrich
L-glutamine	Gibco
MEM alpha	Gibco
Opti-MEM	Gibco
Penicilllin Streptomycin Solution	Gibco
β-Mercaptoethanol	Gibco
Polybrene	Sigma-Aldrich
RPMI	Gibco
Syringe filter	Merck

2.1.4. Technical Equipment

Table 2.4. List of Important technical equipment used in this study.

Technical Equipment	Source
Bacterial Incubator and Shaker	Thermo Scientitifc
Biosafety Cabinet	Thermo Scientitifc
Cell culture Incubator	Thermo Scientitifc
Refrigerated Centrifuge Sorval Legend Xtr	Thermo Scientitifc
FACSAriaIII	BD Biosciences
LSRFortessa (SORP)	BD Biosciences
GeneAmp PCR System 9700	Applied Biosystems
Nanodrop ND 1000	Thermo Fisher

Thermomixer	Eppendorf
StepOne Plus Real-time PCR	Applied Biosystems
Confocal Microscopy	Leica TCS SP8 STED
Gene Pulser	**Bio-Rad**

2.1.5. List of primers

Table 2.5. List of qRT-PCR primers used for quantifying mRNA transcripts.

Primer name	Sequence
GAPDH_FP	GGTGAAGGTCGGTGTGAACG
GAPDH_FP	CTCGCTCCTGGAAGATGGTG
SATB2_FP	TGAGCGGGATGTGATCTATG
SATB2_RP	GTTTCCACAAACACGGAGGT
SATB1_FP	GTTTCCACAAACACGGAGGT
SATB1_RP	TCCTCTTCCTTTCGGAGGAT
CD24A_FP	TTCCCCAAATCCAAGTAACG
CD24A_RP	AACCTGTGCCCAATTTCAAGTG
CD79b_FP	TCAGAAGAGGGACGCATTGT
CD79b_RP	GGATGATGAGGAGGGTCTGG
Pou2AF1_FP	GACATGTACGTGCAGCCTGT
Pou2AF1_RP	CGGGTGTAGCAGTGCTTCTT
ZFP521_FP	AGCGACATCACAGAGCACA
ZFP521_RP	TCTCCGAAATCACACCCTTC
BST1_FP	CTTGCTGACAGTGCTTCTGG
BST1_RP	TGGCTGTGCAGTTTTTGTTC
GFRA2_FP	GCCTTCTGCCTCTTCTTCTTT
GFRA2_RP	GGAGCTGCAGTTGGATTCAG

Table 2.6. Primers for constructing EBF deletion mutants:

Primer name	Sequence
Δ35-50	F1 - CGC CTC GAG CC ATG GAG CCG CTG
	R1 - A GTG AGC CCG GGC GCC CTG CAT CCA CGT CC

	F2 - G CAG GGC GCC CGG GCT CAC TTT GAG AAG CAG
	R2 - CGC GAA TTC TCA CAT GGG AGG GAC AAT CAT G
Δ51-107	F1- CGC CTC GAG CC ATG GAG CCG CTG
	R1 - G GTA GTG GAT GGC CAG ACC CAC CCC GCT CTG
	F2 - G GGT CTG GCC ATC CAC TAC CGG CTC CAG CTC
	R2 - CGC GAA TTC TCA CAT GGG AGG GAC AAT CAT G
Δ108-156	F1- CGC CTC GAG CC ATG GAG CCG CTG
	R1 – T GAT CTC GTG CCC ATT ATT GGT CTT TTC GCT G
	F2 – C AAT AAT GGG CAC GAG ATC ATG TGC AGC CG
	R2 - CGC GAA TTC TCA CAT GGG AGG GAC AAT CAT G
Δ157-175	F1 - CGC CTC GAG CC ATG GAG CCG CTG
	R1 – C TGA GGG AGT TGT GAG CAA TAC TCG GCA CAT TTC
	F2 – A TTG CTC ACA ACT CCC TCA GAT CCA GTG
	R2 - CGC GAA TTC TCA CAT GGG AGG GAC AAT CAT G
Δ176-251	F1 - CGC CTC GAG CC ATG GAG CCG CTG
	R1 – A GGG CGT ACC CTC ATT TCG GTT GCC ACA GC
	F2 – C CGA AAT GAG GGT ACG CCC TCT TAT CTG GAA C
	R2 - CGC GAA TTC TCA CAT GGG AGG GAC AAT CAT G
Δ252-352	F1 - CGC CTC GAG CC ATG GAG CCG CTG
	R1 – A GCC GTA GTC TTC CGA GGG GTC AAG CCT CCG AG
	F2 – C CCC TCG GAA GAC TAC GGC TTC CAG AGG TTA C
	R2 - CGC GAA TTC TCA CAT GGG AGG GAC AAT CAT G
Δ353-412	F1 - CGC CTC GAG CC ATG GAG CCG CTG
	R1 – G AGG GAC ACT GAT GGT GGG TTC ATT GAG TGC TG
	F2 – A CCC ACC ATC AGT GTC CCT CGG AAC CAC AAC
	R2 - CGC GAA TTC TCA CAT GGG AGG GAC AAT CAT G
CΔ428-591	F 1- CGC CTC GAG CC ATG GAG CCG CTG
	R1- T CAA GCA GCG CGAAGTGTTAGCAAGGGCTGGG

	F2- T AAC ACT TCG TCA AGC AGC GTG TCA CCA CAT
	R2- CGC GAA TTC TCA CAT GGG AGG GAC AAT CAT G

Table 2.7. Primers utilised in reporter assays.

Primer name	Sequence
Satb2_E1_FP	CGGCGCTAGCCCCCTTCCGTCTTCCACTTC
Satb2_E1_RP	CGGCAGATCTTTTCCCTCCAGTCCAATCCC
Satb2_E2_FP	CGGCCTCGAGCTTCTCGGTAGCCACTCCTG
Satb2_E2_RP	CGGCCTCGAGCGGGGAGGAAGTTCACTAGG
Satb2_E3_FP	CGGCGCTAGCGATGCTCCATGAACTGCTCC
Satb2_E3_RP	CGGCAGATCTTCATTCTGGTTCTCTGGCGT
RV primer FP	CTAGCAAAATAGGCTGTCCCC
Pou2af1_E1_FP	CGGCGCTAGCAGAAGCCTGAGTGTCTCTGT
Pou2af1_E1_RP	CGGCAGATCTAACCACCATACAGCACAGGA
Pou2af1_E2_FP	CGGCGCTAGCGCACGCCCAGTCACATTAAA
Pou2af1_E2_RP	CGGCAGATCTAACCAACCATTGCCACAGAA
Pou2af1_E3_FP	CGGCGCTAGCCCCTGGCTATGAGGTCCTCT
Pou2af1_E3_RP	CGGCAGATCTACCCTGTGCACACTAGTTCT
Cd79b_E1_FP	CGCGCTAGCGACCCACGTTGTCATAGTTGAC
Cd79b _E1_RP	CGCCTCGAGGACCTCTGCCCACTAATAATCTG
Cd79b _E2_FP	GCCGCTAGCCCTGTTCCGCAGTTCCTTTG
Cd79b -E2-RP	CGCGCTAGCCATATACCCACTGTATTAGTCG
Cd79b -E3-FP	GCCGCTAGCGATAGACAGACTTCATGAGGTTC
Cd79b -E3-RP	CGCCTCGAGCCAGTGTGGGGAAATAACCAG
Cd24a-E1-FP	CGCGCTAGCGGAGGTGTCTCAGCTCTTTCT
Cd24a -E1-RP	CGCCTCGAGCTTGTCTACAACCTTCAACAGC
Cd24a -E2-FP	CGCGCTAGCCATTTTAGGTAGTGGTGTGAG
Cd24a -E2-RP	GCGCTCGAGGAAGTGAGGGAACCTTACAG
Cd24a -E3-FP	CGCGCTAGCATGGTCCCACAGTTGTTACTAC
Cd24a -E3- RP	GCGCTCGAGCCTAAGAAACCACCAGCATCT
Cd24a-E4-FP	CGCGCTAGCGCATTGTGGTGCCGTGAATTG

Cd24a-E4-RP	CGCCTCGAGCTGAAGAGGTGTGATAGCTGAG
Cd24a-E5-FP	GCGGCTAGCGAATTATTGGTGAACCCACAC
Cd24a_E5_RP	GCGCTCGAGCTGGAGGGAAGCCATTAGGAT
Cd24a_E7_FP	CGCAGATCTGCCTAGCTGTTCATGCACTAATG
Cd24a_E7_RP	CGCAGATCTCATTTCCTCGAGGTTGACACTG
GFP_FP	CGCAAGCTTATGGTGAGCAAGGGCGAGGAGC
GFP_RP	CGCATCGATTTACTTGTACAGCTCGTCCATG
ER-FP	GCCGAATTCCGAAATGAAATGGGTGCTTCAG
ER_RP	CGCGAATTCTCAGATCGTGTTGGGGAAGCCC

2.1.6. BAC Clones used in the study

BAC Clone Name	**Length/Size**	**Chromosome Number**	**Spanning the Gene**
RP23-471J18	190 kb	17	SATB1
RP23-268C12	192 kb	1	SATB2

2.2. Methods

2.2.1. Vectors and constructs

In all of the cloning studies, Phusion polymerase was utilized to amplify the sequences encoding cDNAs for the genes or markers being studied. Either the primers had been treated with T4-polynucleotide kinase or they had restriction sites built into them so that sticky end cloning could be performed with them. Both of these methods were used (for blunt end cloning). After the vectors had been digested with the appropriate restriction enzymes, the DNA polymerase I, Klenow fragment was applied to them in cases where it was required to blunt the vectors. After that, calf intestinal phosphatase, also known as CIP, was utilized in order to eliminate the 5'-phosphates (whenever required). After the gel had been removed from or purified using Nucleospin Gel extraction and DNA purification columns, the amplified insert and vector were joined together using T4 DNA ligase. This step was followed by the ligation step. The ligation combinations resulted in the production of a competent strain of bacteria known as DH5. The procedure known as "Colony PCR" was carried out on the colonies that were obtained using Taq DNA polymerase. Sanger sequencing and restriction digestion were used to validate the accuracy of the clones. With the assistance of the Plasmid Maxiprep/Midiprep columns, all of the constructs that were to be transfected were produced on a massive scale and in an ultrapure form.

The full-length mouse EBF1 cDNA was sub-cloned from vectors containing these sequences and then joined to the C-terminal domain of Estrogen Receptor to create MigR1-EBF1.ER constructs (ER). This fusion was then inserted into the retroviral vector MigR1-IRES-hCD2 or MigR1-IRES- GFP.

2.2.2. Cell culture

Pre-pro-B cells (*Ebf1-/-* progenitors) were cultured on the OP9 cells (stromal cell) in Opti-MEM supplemented with fetal calf serum (4% v/v), streptomycin (10 µg/ml), penicillin (10 U/ml) β mercaptoethanol (50 µM), as well as IL-7 (5 ng/ml), SCF (10 ng/ml) , and Flt3L(10 ng/ml). With the exception of adding only 5 ng/ml of IL-7 to the culture media, *Rag2-/-* cells, also known as Pro-B cells, were grown in a conditions that were essentially identical to those that had previously been described. The RT-PCR experiment was carried out with both pre-pro-B cells and pro-B cells.

The 38B9 cells grew in RPMI 1640 complete medium with 10% (v/v), FBS added to it along with Penicillin-Streptomycin (10 U/mL), β -mercaptoethanol (50 µM), and 2mM L-glutamine. After the cells had reached confluence, they were split by tapping and then they were plated in fresh complete media. Every other day, the cells were passaged.

OP9 or OP9-DL1 (stromal cells) were grown in MEM alpha complete media with FBS 10% (v/v), Penicillin and Streptomycin (10 U/mL), β- mercaptoethanol (50µM), and 2mM L-glutamine. After the cells attained confluency, they were passaged with 0.25 percent trypsin and then plated in a fresh complete medium. Every other day, the cells were subjected to passage.

Both 293T and Plat-E cells were grown in DMEM complete media that had 10% (v/v) foetal bovine serum, 10 U/mL penicillin-streptomycin, 50 µM β -mercaptoethanol, and 12.5 mM HEPES. After the cells attained confluence, they were split with 0.25 percent trypsin and then plated in a fresh complete medium. On alternate days, the cells were passed.

2.2.3. Viral transductions

Retroviral constructs were transfected into Plat-E and 293T cells. As previously stated, the retroviral constructs were transfected into Plat-E or 293T cells along with packaging vectors carrying the viral gag-pol and envelope proteins (Pongubala et al., 2008). A swinging bucket rotor was used to spin-infect Ebf1-/- progenitors in 24-well cell culture plates for 2.5 hours at 25°C in the presence of polybrene (final concentration of 10 ug/mL) after 48 hours of transfection. Fresh media enriched with cytokines was added to the spin-infected cells before plating them on OP9/OP9-DL1 stroma. After 48 hours, transduced cells were either kept on OP9/OP9DL1 stromal cells at the appropriate conditions or used for differentiation experiments after FACS sorting according to the selection marker(s).

2.2.4. Flow Cytometry Analysis

Single-cell preparations for cell analysis or sorting were prepared in MACS Buffer (consisting of 1X PBS with a pH of 7.4, 0.5% BSA, and 2 mM EDTA). Anti-mouse CD19 and anti-mouse CD25 antibodies were used to stain the cells, as well as any combination of these antibodies that was necessary. Examining or sorting the stained cells required the use of either a BD LSRFortessa (SORP) or a BD FACSAria III (BD Biosciences) instrument. Using the application FlowJO, an analysis of the data was performed (TreeStar Inc).

2.2.5. *In Vitro* differentiation assays

Ebf1 and *Pax*5 were expressed in *Ebf1-/-* progenitor using retroviral transduction in order to differentiate *Ebf1-/-* progenitors towards B-lineage. Prior to any further experimental study, FACS-sorted transduced cells were plated on OP9 stroma and grown in the presence of Flt3L (10ng/mL), IL-7 (5ng/mL), and SCF (10ng/mL) cytokines. In order to stimulate differentiation toward the T-lineage before to conducting experimental analyses, On OP9-DL1 stroma, Ebf1-/- progenitors were grown for seven days with 10ng/mL of Flt3L, 10ng/mL of SCF, and 5ng/mL of IL-7. All cultures were given fresh medium every three days, and samples were collected on day 7 of cultivation and examined using flow cytometry (Pongubala et al., 2008).

2.2.6. Isolation of total RNAs

Total RNA was used to quantify mRNA transcripts. By using a pipette, cells were suspended in Trizol and homogenized. According to one mL of Trizol, 0.2 mL of Chloroform was added, followed by a vigorous shake and a five-minute incubation period at room temperature. To separate the aqueous and organic phases, the samples were centrifuged at 4°C for 15–20 minutes. The interphase and lower organic layer were left behind, while the upper aqueous phase was transferred to a new tube. After adding an equivalent volume of isopropanol to the mixture and centrifuging it at 4 degrees Celsius for half an hour, the RNA was precipitated. Subsequently, 75% ethanol was used to wash the pellet before it was quickly dried and suspended in nuclease-free water.

2.2.7. Quantitative RT- PCR

Studies using quantitative RT-PCR were carried out in order to investigate the degree to which the candidate gene in question was expressed. Trizol(Sigma) was used as the extraction method for RNA. The spectroscopic examination was performed using a Nanodrop spectrophotometer in order to check the integrity of the RNA (Nanodrop Technologies). After that, the RNA was transcribed in the opposite direction to produce cDNA. qPCR (Applied Biosystems, USA) was performed on this cDNA using primers that were unique to the genes that were being investigated.

The ΔΔCt approach, which was discussed earlier, was utilized in the conducting of the analyses. In a nutshell, the average Ct values from triplicate samples were determined for each gene in control and treatment samples. After that, the Ct values were computed by normalizing them against the Ct values of a control gene, as shown in the formula [ΔCt = Ct(sample) – Ct(standard)]. After that, these Ct values were put to use in the comparison between the control group and the treated group, as well as in the calculation of the fold change [ΔΔCT = ΔCt(treatment) – ΔCt(sample), fold change = 2^-(ΔΔCT)].

2.2.8. Statistical analysis

Microsoft Excel(2016) and GraphPad Prism9 were used to conduct statistical analyses.

2.2.9. 3D FISH

2.2.9.1. Nick Translation

The 190 kb and 192 kb bacterial artificial chromosome (BAC) probes RP23-471J18 and RP23-268C12 respectively against specific genes were obtained from Children's Hospital Oakland Research Institute's BACPAC Resource Center (BPRC). The BAC clones were cultured in LB medium with 12.5µg/ml Chloramphenicol {Sigma}. BAC extraction was carried out using the MN NucleoBond Xtra BAC. Purified BAC DNA was subjected to Nick translation using standard protocol to make the probes. Nick translation of BAC DNA was then performed by FISH Tag™ DNA Kit with an amine-modified nucleotide, which connected the amine-modified BAC DNA to an amine-modified fluorophore. DNase I is utilized during nick translation to generate single-strand breaks. By exchanging the existing nucleotides for new ones at the 3' ends of these "nicks," DNA Polymerase I "translates" the nick and enables the insertion of tagged nucleotides. Aminoallyl-dUTP was chosen because to its high incorporation efficiency with DNA Polymerase I and future possibilities for chemical bonding with amine-reactive dyes or haptens. It is essential to achieve a balance between the nicking done by DNase I and the translation done by DNA polymerase I

Note: Regardless of the efficiency of the approach outlined here, DNAse I from various batches or companies may need to be titrated.

A working solution of DNase I was prepared (1:200) on ice according to the instructions below.

Component	Volume
NFW	89.5
Nick Translation Buffer(10X)	10ul
Let the mixture sit on ice for 10 minutes, and then add:	
DNase I (Component O)	0.5UL
Final Volume	100UL

2.2.9.2. Slide preparation

Cells were put on microscopic slides coated with poly-L-lysine for 30 minutes in an incubator at 37°C. 10 minutes of fixation with paraformaldehyde at a final concentration of 4% followed by permeabilization, denaturation, two-day probe hybridization, washing counterstaining, and confocal imaging. Throughout these processes, the cells' three-dimensional shape was preserved.

2.2.9.3. Hybridisation & Detection

Co-denaturation of tissue sections and probe was performed at 80°C for 2 minutes exactly. At least 20ng of DNA probe/ slide was hybridized at a time. The hybridization of the slides was place in a humidified dark chamber at 37°C for 1-2 days.

2.2.9.4. Post-hybridisation Washes

Following the completion of the hybridization procedure, the slides were immersed in 2x SSC for an extended period of time until the coverslips became pliable enough to be removed. The slides were then washed for 15 minutes at 45 degrees Celsius in 50% formamide/2x SSC, for 15 minutes at 63 degrees Celsius in 0.2x SSC, then it was washed for 5 minutes at 45 degrees Celsius in 2x SSC. The sample was then washed in 2x SSC at room temperature for 5 minutes, followed by 5 minutes in PBS at room temperature after which it was stained with 1:2 DAPI with gold antifade in 2X SSC.

2.2.9.5. Microscopy and Processing

Images were acquired by a 100x with a 2X optical zoom and a 0.12m Z step between optical slices of a Leica TCS SP8 STED confocal microscope using the LASX software provided by Leica. Loci were given a score based on whether or not they were connected to the periphery of the nucleus. The stacks were processed for analysis and distances were measured for manual validation using ImageJ software. Manual validation was performed on 100-150 nuclei per cell type. The unpaired t-test was employed to analyse intralocus distances statistically.

2.2.10. Electroporation

The electroporation method was based on Transfection by Electroporation of RAW264.7 Macrophages by Stephan T. Smale (2010).

38B9 cells were cultured in a T-75 flask containing a growth medium. When cells were 50–80 percent confluent, passage occurred. The cells were counted using a hemocytometer. Electroporation was performed in a cuvette with a 0.4-cm electrode gap. 10 ug of pGL3 vector and 0.5 g of renilla vector were utilized with 6x10^6 cells in 200uL of culture media. Each cuvette was subjected to 250V using a Bio-Rad Gene Pulser X cell and a 950-uF capacitor.

CHAPTER-3:

RESULTS

Objective I

3.1. Probing the role of EBF-1 in differential chromatin compartmentalization during B cell fate commitment.

To examine the architectural role of EBF1 in B cell fate specification, previously, our lab generated genome-wide chromatin interactome data using Hi-C in *Ebf1-/-* and *Rag2-/-* cells, which represent pre-pro-B and pro-B cell stages [46]. Our Hi-C analysis confirmed previous findings that chromatin is hierarchically organized into compartments, TADs, and chromatin loops. At the level of megabases, chromatin is structured into permissive and repressive compartments (A and B). At the sub-megabase level, chromatin is organized as topologically associated domains (TADs), which are highly self-interacting regions. At the kilobase level, chromatin is arranged as a chromatin loop-like promoter-enhancer interactions [72]. Confirming earlier research, it was demonstrated that chromatin undergoes global remodeling and differential chromatin compartmentalization to determine gene expression patterns specific to the B lineage [72].

IgH relocalization and recombination are defining characteristics of B lineage specification. In pre-pro-B cells, the IgH locus is positioned near the nuclear periphery, whereas in pro-B cells, it shifts away from the nuclear periphery **Fig 3.1A,** and undergoes proximal and distal VDJ recombination. However, it has already been demonstrated that EBF1 is the primary determinant of B cell fate; nevertheless, EBF1 was postulated to have a vital role in the relocalization of IgH during the differentiation of MPP into pro-B cells. In addition, the nuclear localization of IgH was investigated in the absence and presence of EBF1 to assess its role in chromatin rearrangement during B cell fate determination.

To comprehend the function of EBF1 in Igh relocalization, two model systems were employed: *Ebf1-/-* cells (representing MPP, pre-pro-B cell stage) and EBF1 transduced in *Ebf1-/-* cells (representing pro-B cell stage) as mentioned in **Fig3.1.B.** Next, 3D FISH was done on the IgH locus. For 3D gene loci, FISH BAC DNA was isolated for the specific gene **Fig3.1.C**. and a probe was prepared by Nick Translation **Fig3.1.D.** 3D FISH shows that in *Ebf1-/-* cells IgH alleles were located at the nuclear periphery. Interestingly, we observed that upon ectopic expression of *Ebf1* in *Ebf1-/-* cells, IgH alleles were relocated toward the center of the nucleus. EL4 is a transformed T cell serving as a negative control, while pro-B is a positive control for the expression of IgH alleles. The restoration of EBF1 expression in *Ebf1-/-* cell progenitors triggers the rearrangement of IgH loci **Fig3.1.E**, according to these investigations. These investigations reveal that EBF1 is crucial for chromatin compartmentalization during B cell fate specification. The graph shown in **Fig3.1.F**, represents the percentage of nuclear localization of IgH loci, approximately 100 nuclei from each cell type were scored for subnuclear localization, and the percentage of resolved IgH pairs is represented. C-center, P-periphery. These findings demonstrate that restoration of EBF1 expression in *Ebf1-/-* cell progenitors initiate the rearrangement of IgH loci. Further, these findings support the hypothesis that EBF1 may play an essential role in chromatin compartmentalization during specification of B cell fate.

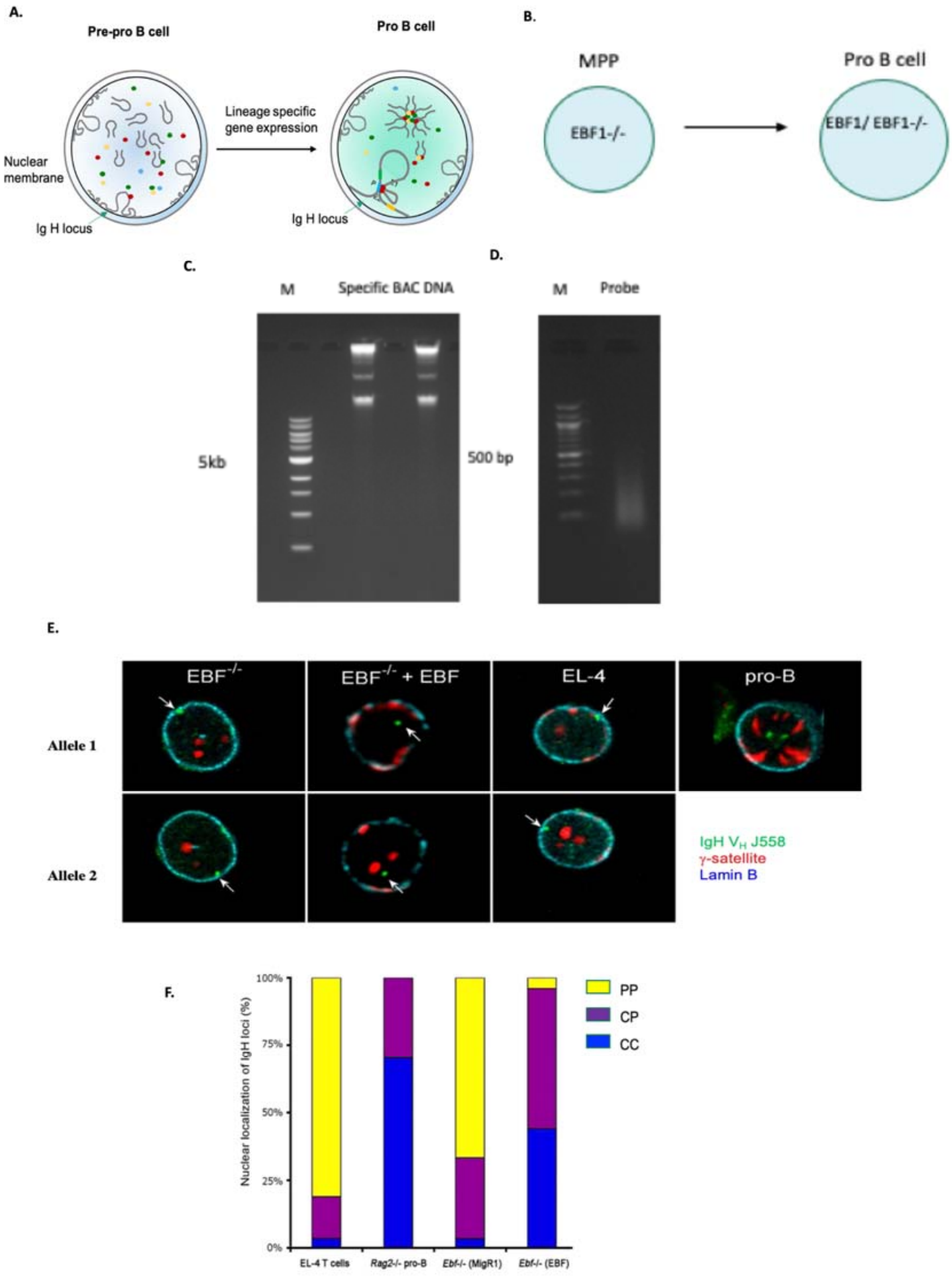


Fig3.1. Restoring EBF1 expression in Ebf1-/- progenitors promotes IgH locus rearrangement.

(A) Diagrammatic illustration of IgH relocalization in pre-pro-B cell and pro-B cell.

(B) Diagrammatic representation of two model systems *Ebf1-/-* cell(which represent MPP) and EBF1 transduced in *Ebf1-/-* cell represent pro B cell used in this study.

(C) Electrophoresis of specific BAC DNA after isolation.

(D) Probe preparation by Nick translation for 3D DNA FISH.

(E) High-scale compaction of the IgH locus is detected by 3D DNA FISH when EBF1 is restored to the Ebf1-/- progenitor. On interphase nuclei, two-color FISH was done using the VhJ558 (green) and gamma satellite (Red) probe sets. The nuclear lamina was detected by staining for anti-Lamin B1 Blue. EL4, Rag2-/- Ebf1-/- (migR1) and *Ebf1-/-* (EBF1) cells were processed.

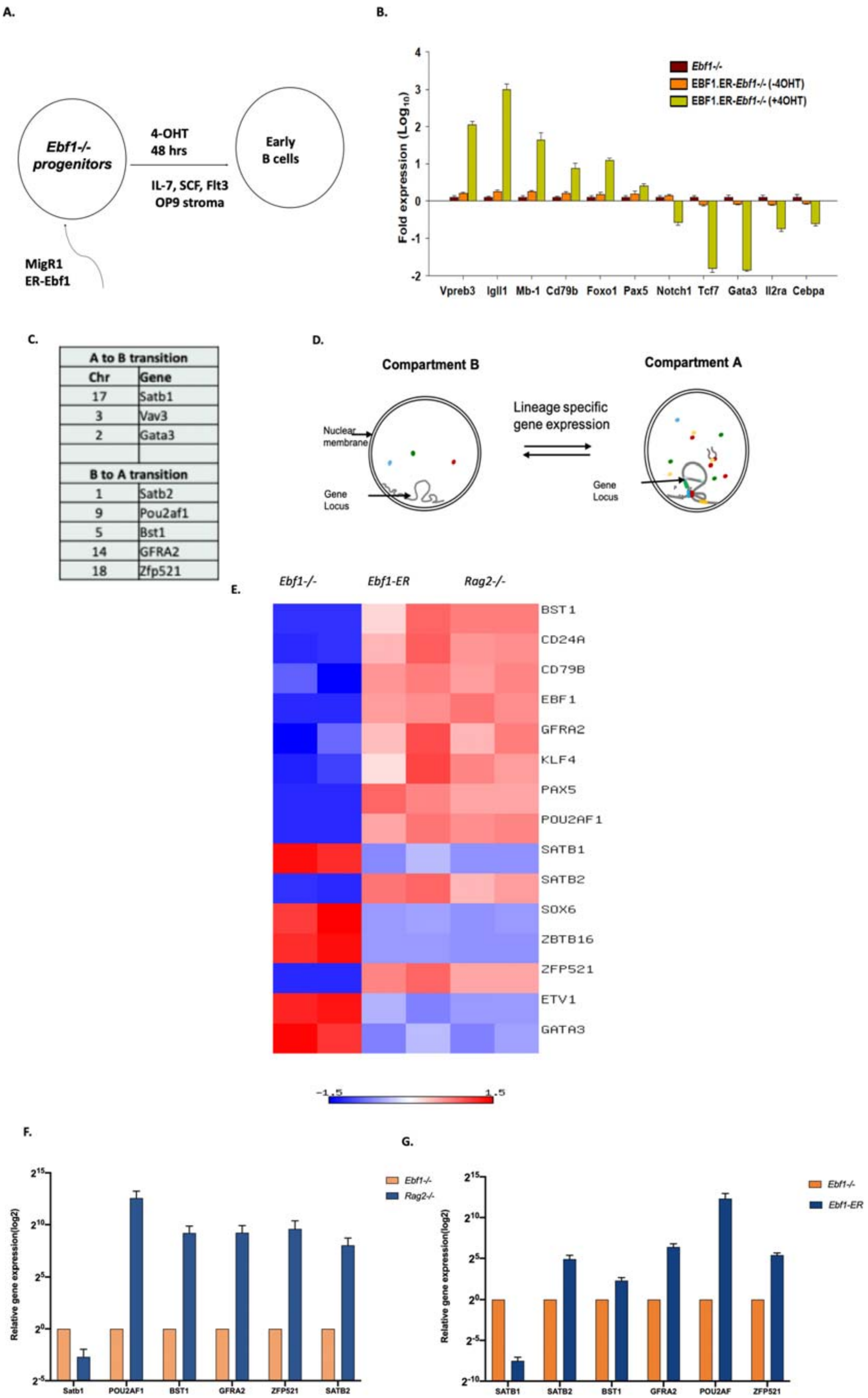
(F) Graph representing the percentage of nuclear localization of IgH loci. Approximately 100 nuclei from each cell type were scored for subnuclear localization and percentage of resolved IgH pairs is represented. C-center, P-periphery.

3.2. A list of differentially switching genes during the progression from pre-pro b to pro-B cell stage.

Recent research indicates that chromatin is further organized into Topologically Associating Domains, which are regions of varied sizes that are dense and highly self-interacting (TADs). In numerous cell types, the stability and uniformity of these chromatin domains have been observed. We used Principal Component Analysis (PCA) at 1 Mb resolution to better understand how the two distinct cell types differ in their chromatin interaction patterns. This allowed us to examine the differences between the two types of cells. These studies, which were carried out previously in the lab, demonstrated that chromatin can be partitioned into either the A or the B compartments, which are distinguished from one another, respectively, by a high number of interactions or a dearth of interactions. It was shown that the A compartments include a higher number of genes than the B compartments, and these genes have a CpG island content that is four times higher than what is seen in the B compartments. Compared to the B compartments, the A compartments showed much greater transcript levels and were enriched for active histone modifications (H3K4me1, H3K4me3, and H3K9/14ac) [72]. This suggests that the compartmentalization of chromatin parallels the activity of genes in both types of cells.

MPPs must express EBF1 in order to transition into the pro-B cell stage. Previous studies have demonstrated that *Ebf1-/-* progenitors can differentiate into committed CD19+ pro-B cells by having the transcription factor EBF1 restored [102]. We used a hematopoietic system, which serves as a great developmental model for B cells, to achieve our goal of achieving better-coordinated differentiation towards the B-lineage and capturing the first alterations that occur during this process. Our earlier studies showed that ectopic production of EBF1 could enable *Ebf1-/-* progenitors to develop into B-lineage cells by using an inducible retroviral construct that expresses full-length mouse EBF1 connected to the C-terminal hormone binding domain of Estrogen Receptor (MigR1-EBF1.ER) **Fig3.2.A**. In order to analyse the induction of EBF1 target genes, 4-O-Hydroxy-Tamoxifen (4-OHT) was used to treat *Ebf1-/-* cells for two days in the presence of Flt3L, IL-7, and SCF. As expected, RT-PCR examination of early B-lineage specific genes (*Pax5, Cd79b, Mb-1, Vpreb3, Foxo1, Igll1*) revealed increased expression levels after Ebf1 induction. Furthermore, genes like *Gata3, Tcf7, Il2ra* (T lineage-specific), and *Cebpa*(Myeloid lineage-specific) were repressed in 4-OHT-treated EBF1.ER-Ebf1-/- cells **Fig3.2.B.** This analysis shows that we were able to generate an efficient model system to study B lineage developmental program.

q PCR analysis has corroborated the observation of changes in gene expression using RNA-seq data **Fig3.2.E.** for differentially switched genes. Moreover, RT-PCR analysis showed that Rag2 -/- cells, which represent pro-B cells, strongly expressed well-known early B-lineage genes like *Pou2AF1, Satb2, Gfra2,* and *Bst1,* while *Satb1*, which switches from an active compartment to an inactive compartment in pre-pro-B cells to pro-B cells, was down-regulated **Fig3.2.F**. RT-PCR research revealed the same strong expression of genes such as *Pou2af1*, *Satb2*, *Gfra2*, and *Bst1* in EBF1-ER-*Ebf1-/-* cells **Fig3.2.G.** treated with tamoxifen, reveal the involvement of EBF1 in gene switching from an active compartment to an inactive compartment. In addition, alternate lineage genes, including *Satb1* (T lineage-specific), exhibited suppression, which was shown to be downregulated by EBF1 expression in EBFER and *Rag2-/-* cells. Gene expression analysis of differentially switched genes revealed that genes switching from the compartment B to the compartment A are transcriptionally active in EBFER and *Rag2-/-* cells and repressed in *Ebf1-/-* cells, and vice versa.



A to B transition	
Chr	Gene
17	Satb1
3	Vav3
2	Gata3
B to A transition	
1	Satb2
9	Pou2af1
5	Bst1
14	GFRA2
18	Zfp521

Fig3.2: Gene expression analysis of differentially switching genes in *Ebf1-/-* vs *Rag2-/-* cells

(A) Diagrammatic illustration of *EBF1-/-* and EBF1-ER system.

(B) RT-PCR research showed an increase in early B-lineage gene expression (*Igll1, Vpreb3, Foxo1, Mb-1,Pax5,* and *Cd79b)*. Furthermore, 4-OHT-treated EBF1.ER-Ebf1-/- cells displayed repression of genes from various lineages, including as *Gata3, Tcf7,* and *Il2ra* and *Cebpa*, which have previously been shown to be downregulated following EBF1 expression (at 1uM final concentration).

(C) List of differentially switching genes from Permissive compartment(A) to Repressive compartment (top panel) and Repressive compartment to Permissive compartment.

(D) Diagrammatic illustration of Differential gene switching from compartment A to compartment B and vice versa

(E) RNA seq data for differentially switching genes in *Ebf1-/-*, EBF1.ER-*Ebf1-/-* and *Rag2-/-*

(F) Transcript levels of differentially switched genes *Satb1,Pou2af1,Bst1,Gfra2, Zfp521,Satb2* were analysed using RT-PCR in *Ebf1-/-*, vs *Rag2-/-*.

(G) Transcript levels of differentially switched genes *Satb1,Pou2af1,Bst1,Gfra2, Zfp521,Satb2* were analysed using RT-PCR in *Ebf1-/-*, vs EBF1.ER-*Ebf1-/-*.

3.3. 3D DNA FISH of differentially switching loci within their chromosome territory.

As described above, PCA analysis reveals that *Satb1* resides in an active compartment in pre-pro-B cell cells, while *Satb2* resides in an inactive compartment in pre-pro-B cells. To rigorously test this, we have employed 3D gene loci FISH in pre-pro-B, and pro-B cells. In *Ebf1-/-* cells, *Satb1* tagged with ALEXA FLUOR 488, colored in green, is positioned in the middle of the nucleus, whereas *Satb2*, colored in red, is located close to the nuclear periphery **Fig3.3**. In EBF1-ER cells, however, *Satb2*, tagged with Alexa Fluor 594, colored in red, positioned towards the nucleus center, while *Satb1*, tagged with Alexa Fluor 488 and colored in green, is localized towards the nuclear periphery **Fig3.3**. Box plots representing the distance distribution of the loci from the periphery of 150 nuclei are shown in **Fig3.3**.

Satb1, but not *Stab2,* is active in the absence of EBF1 since *EBF1-/-* cells recapitulate MPPs and can differentiate into both B and T cells. On the other hand, *Satb2* becomes active in the presence of *Ebf1* when *Ebf1* is ectopically expressed in *Ebf1-/-,* however, *Satb1* switches from the active to the inactive compartment. Thus, our results confirm the conclusion of a recent study that EBF1 affects *Satb2* and **Satb1** either directly or indirectly. Furthermore, it is demonstrated that EBF1 is important for the differential compartmentalization associated with the determination of the B lineage.

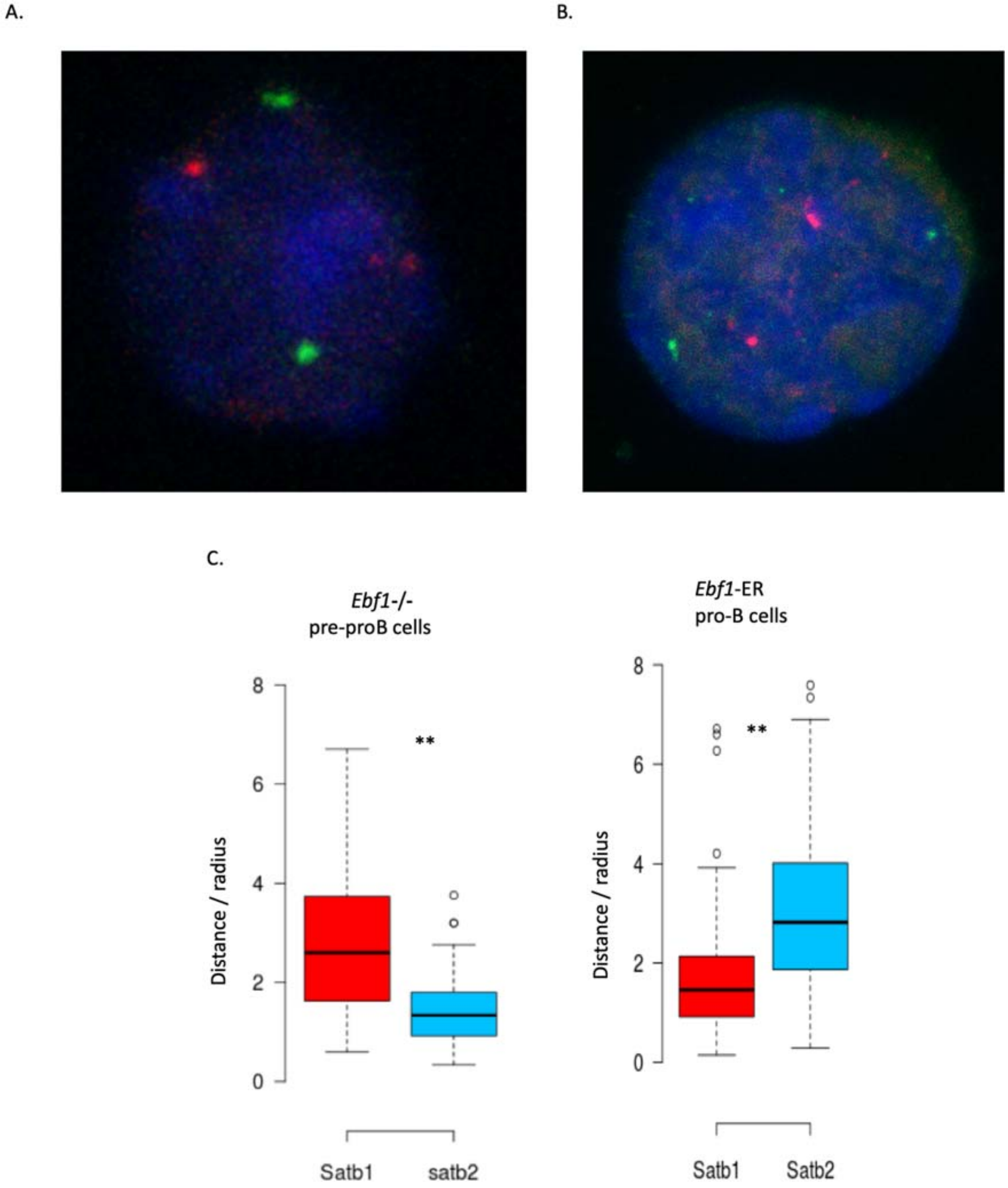


Fig3.3. 3D DNA FISH of differentially switching loci within their chromosome territory

- **(A)** *Ebf1*-/- cells probed with *SATB1* tagged with Alexafluor 488 (green) and *SATB2* tagged with Alexafluor 594 (red).

(B) *EBF1*-ER cells probed with SATB1 tagged with Alexafluor 488 (green) and *SATB2* tagged with Alexafluor 594 (red)

(C) Box plots representing the distance distribution of the loci from periphery of the nucleus in 150 nuclei.

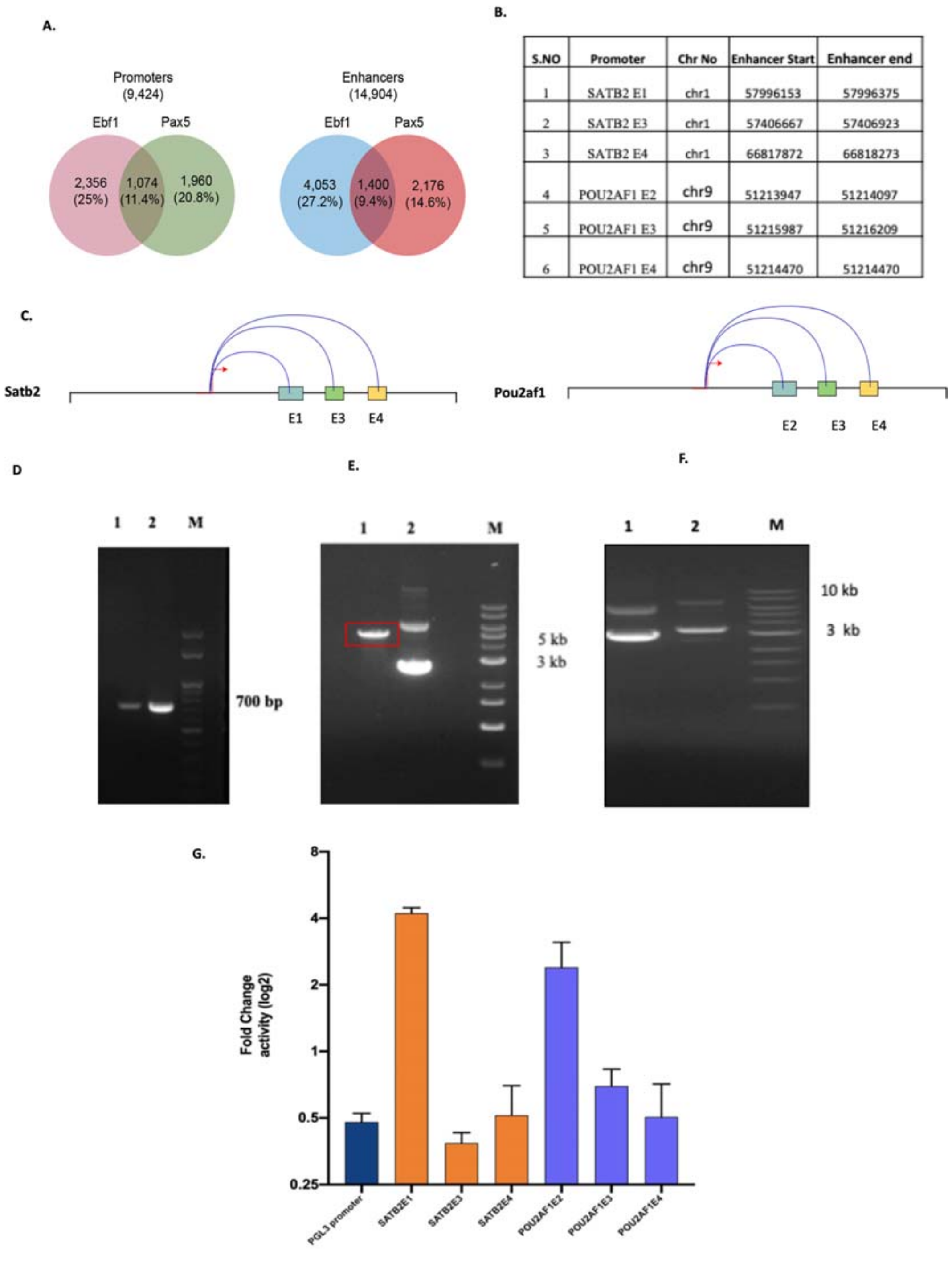
3.4. **Differential chromatin compartmentalization is regulated in part by Ebf1 binding to cis-regulatory elements.**

Based on the differential chromatin compartmentalization of *Satb1* and *Satb2* in developing B cells we hypothesized that EBF1 regulates chromatin compartmentalization by binding to promoter–cis-regulatory regions of differentially switched genes. To test this hypothesis, we have chosen the potential genes *Satb2* and *Pou2af1*.

After proving the involvement of EBF1 in differential chromatin compartmentalization, we hypothesise that the stimulation of B lineage-specific genes may be regulated at many tiers, along with the binding of EBF1 and *Pax5* to their respective target promoter-cis-regulatory interacting regions. We used the data we obtained from ChIP-seq on pro-B cells, which included interactions between promoters and cis-regulatory elements, to test this hypothesis. Interestingly, we discovered that in pro-B cells, EBF1 binds to 5390 (57.2%) promoters and 7629 (51.2%) cis-regulatory elements(enhancers) engaged in long-range interactions as characterized by in situ Hi-C, either alone or in combination with Pax5 **Fig3.4.A**.

De novo motif analysis (TFs) was performed for putative *cis*-regulatory elements for the genes whose chromatin state was determined by PCA. To test this hypothesis, we have chosen the potential genes *Satb2* and *Pou2af1*. Three potential enhancers were chosen for each gene **Fig3.4.B**, and all six enhancers **Fig3.4.C.** were cloned into the PGL3 Promoter vector **Fig3.4.D**. Following the transfection of 38B9 cells that had been transformed into pro-B cells, reporter assay experiments were conducted. In the instance of *Satb2*, E1 demonstrated the highest activity among the three, but *Pou2af1* exhibited higher E2 activity **Fig3.4.G**.

These studies suggest that EBF1 regulates chromatin compartmentalization by binding to promoter and enhancer elements of differentially switched genes. These studies imply that EBF1 participates in the development of cis-regulatory interactomes that are exclusive to the B lineage. These studies can be extended further by knocking out these enhancers and checking the effect on chromatin rearrangement in B cell development.



S.NO	Promoter	Chr No	Enhancer Start	Enhancer end
1	SATB2 E1	chr1	57996153	57996375
2	SATB2 E3	chr1	57406667	57406923
3	SATB2 E4	chr1	66817872	66818273
4	POU2AF1 E2	chr9	51213947	51214097
5	POU2AF1 E3	chr9	51215987	51216209
6	POU2AF1 E4	chr9	51214470	51214470

Fig3.4: EBF1 binds in parts to the cis-regulatory regions to control the B lineage-specific gene expression pattern.

(A) De novo motif analysis (TFs) was performed for putative cis-regulatory elements for the genes whose chromatin state was determined by PCA.

(B) List of putative enhancers and their location for genes *Satb2 and Pou2af1*

(C) Diagrammatic illustration of Satb2 and Pou2af1 enhancers

(D) Amplification of SATB2 E3 enhancers

(E) Restriction digestion of PGL3PV with Nhe-I and Bgl-II

(F) Clone confirmation of SATB2 E3 enhancers

(G) Luciferase assay of putative enhancers for Satb2 and Pou2af1 in 38B9 cells.

Objective 2:

3.5. Functional characterization of EBF-1 during B cell developmental progression

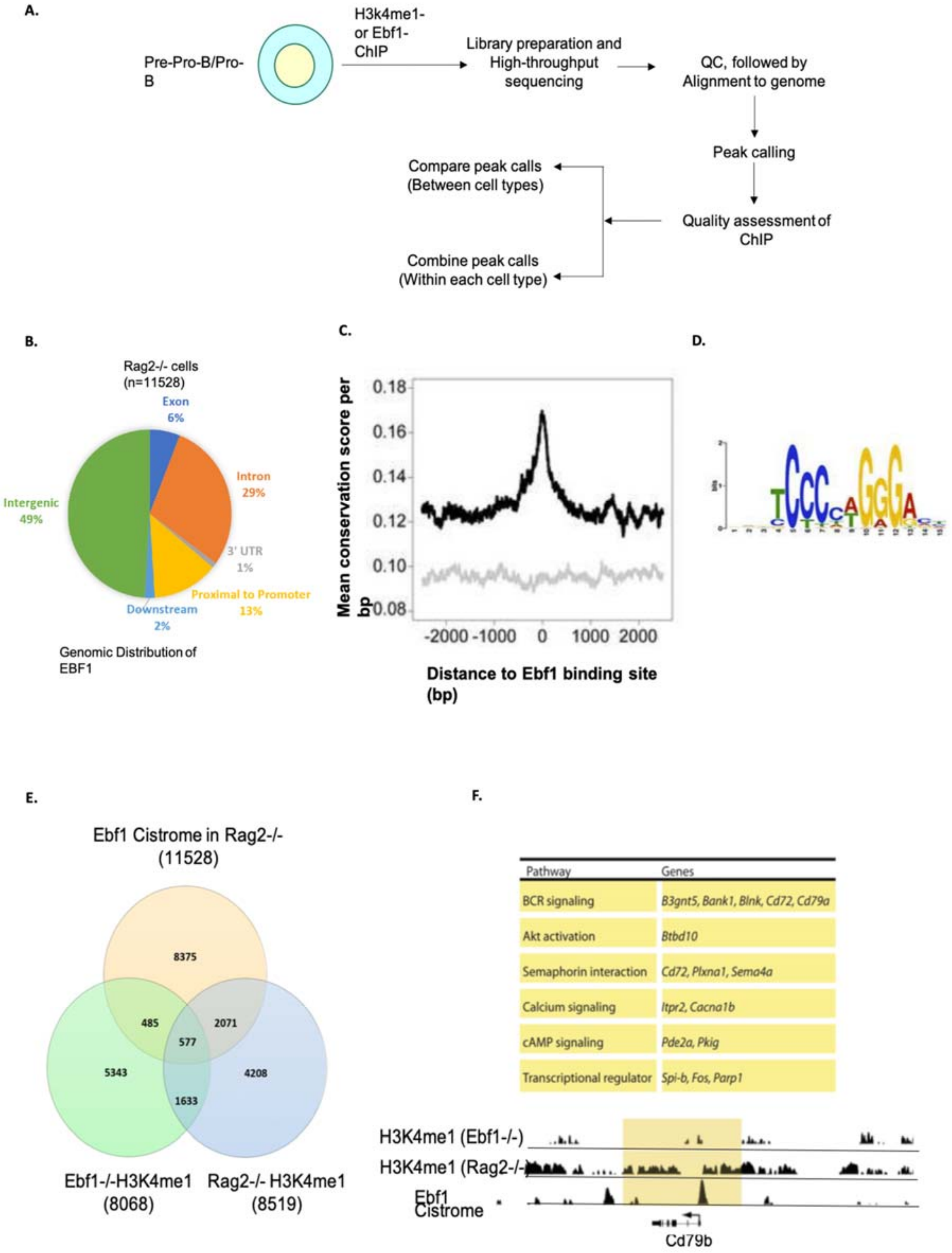
After demonstrating the involvement of EBF1 in chromatin remodeling, we sought to determine the epigenetic modifications of chromatin following the expression of EBF1 during development of B Cell ChIP-seq **Fig3.5.A.** was done on pre-pro-B cells and pro-B cells for EBF1 and H3K4me1 to find out how EBF1 controls the development of B cells at the molecular level.

To begin, an EBF1 ChIP-Seq analysis was performed on Rag2-/- pro-B cells to investigate the landscape of EBF1 binding in order to obtain insight into the molecular basis upon which EBF1 orchestrates the development of B cells. From these studies, we identified 11,508 regions that were bound by EBF1. Examination of the genomic distribution of EBF1 binding sites revealed that EBF1 targets a diverse set of sequences, many of which reside in close proximity to or within genes, including promoters (13%) and introns (29%) **Fig3.5.B**. In addition, we examined the degree of conservation of DNA sequences +/-2.5 kb from EBF1 target sites. Conservation measurements across 30 vertebrate genomes were created using phastCons and downloaded from the UCSC genome browser and compared to control sequences that were generated from random sites in the genome. We observed that DNA sequences spanning *Ebf1* binding sites in *Rag2-/-* pro-B cells were statistically more conserved than sequences from genomic locations that were randomly chosen. We note that the degree of conservation was even more pronounced for sequences that were +/-250bp of Ebf1 target sites relative to randomly selected DNA regions.

To determine whether EBF1binding is associated with modifications in the chromatin structure at enhancer elements during B cell commitment, we performed ChIP-seq for H3K4me1, an epigenetic mark associated with enhancer elements, in *Ebf1-/-* progenitors and *Rag2-/-* pro-B cells expressing EBF1. We observed that although the overall number of regions enriched for H3K4me1 was similar between *Ebf1-/-* progenitors (n=8068) and *Rag2-/-* pro-B cells (n=8519), enhancer landscape slightly enhanced in pro-B cells and that nearly 20-30% of these regions overlapped in the two cell types. When we examined the status of H3K4me1 modified chromatin spanning EBF1 binding sites in *Rag2-/-* pro-B cells, we observed regions that were marked with H3K4me1 prior to EBF1 expression and that this state was maintained in committed pro B cells (577). In addition, we observed an increase in regions enriched for

H3K4me1 (2071) as cells transitioned from a multipotential progenitor state to a committed state that requires EBF1 expression.

These findings demonstrate that EBF1 expression is associated with the acquisition of the H3K4me1 mark at specific regions in the genome and suggest that EBF1 may direct the deposition of H3K4me1 at these loci. We note that EBF1 expression and binding were also associated with the loss of this active histone mark at 485 genomic regions and that EBF1 may antagonize such a chromatin modification in the context of repressing alternate lineage genes.



Pathway	Genes
BCR signaling	*B3gnt5, Bank1, Blnk, Cd72, Cd79a*
Akt activation	*Btbd10*
Semaphorin interaction	*Cd72, Plxna1, Sema4a*
Calcium signaling	*Itpr2, Cacna1b*
cAMP signaling	*Pde2a, Pkig*
Transcriptional regulator	*Spi-b, Fos, Parp1*

Fig3.5 Genome-wide Ebf1 target sites.

(A) Experimental strategy for Chip seq for Ebf1 and H3K4me1 in Pre pro B cell and Pro B cells.

(B) Ebf1 binding landscape in Rag2-/- pro B cells using ChIPseq.

(C) & (D) Degree of conservation of DNA sequences +/-2.5kb from Ebf1 target sites. (E) ChIP-seq for H3K4me1, an epigenetic mark associated with enhancer elements in Ebf1-/- progenitors and Rag2-/- pro B cell expressing EBF1. (F) Status of H3K4me1 modified chromatin spanning EBF1 binding sites in Rag2-/- pro B cells.

3.6. By mediating cis-regulatory interactions, EBF1 regulates b lineage-specific gene expression patterns.

Using Hi-C data, the genome-wide promoter *cis*-regulatory interactions in pre-pro-B and pro-B cells were determined. Analysis of these interactions revealed that promoter *cis*-regulatory interactome undergoes extensive rewiring during B-cell fate commitment and is connected with a B lineage-specific gene expression pattern. For example, *Cd24a* and *Cd79b*, which are highly expressed in pro-B cells, exhibit a greater number of *cis*-interactions in pro-B cells than in pre-pro-B cells. Similarly, the quantitative RT-PCR study **Fig3.6.C.** demonstrated higher amounts of *Cd79b* and *Cd24a* transcripts in pro-B cells than in pre-pro B cells. To examine the enhancer activity, six and three potential enhancers for the *Cd24a* and *Cd79b* genes were selected respectively. We cloned these enhancers into the PGL3 promoter vector and performed reporter assays. In the case of *Cd24a*, only E1 and E2 out of six enhancers displayed increased activity, whereas, in *Cd79b*, the activity of all three enhancers increased **Fig3.6.D**. These results suggest that EBF1 is involved in the development of the cis-regulatory interactome specific to the B lineage. These investigations reveal that EBF1 regulates chromatin compartmentalization and promoter *cis*-regulatory interactions to determine the B cell fate of MPPs.

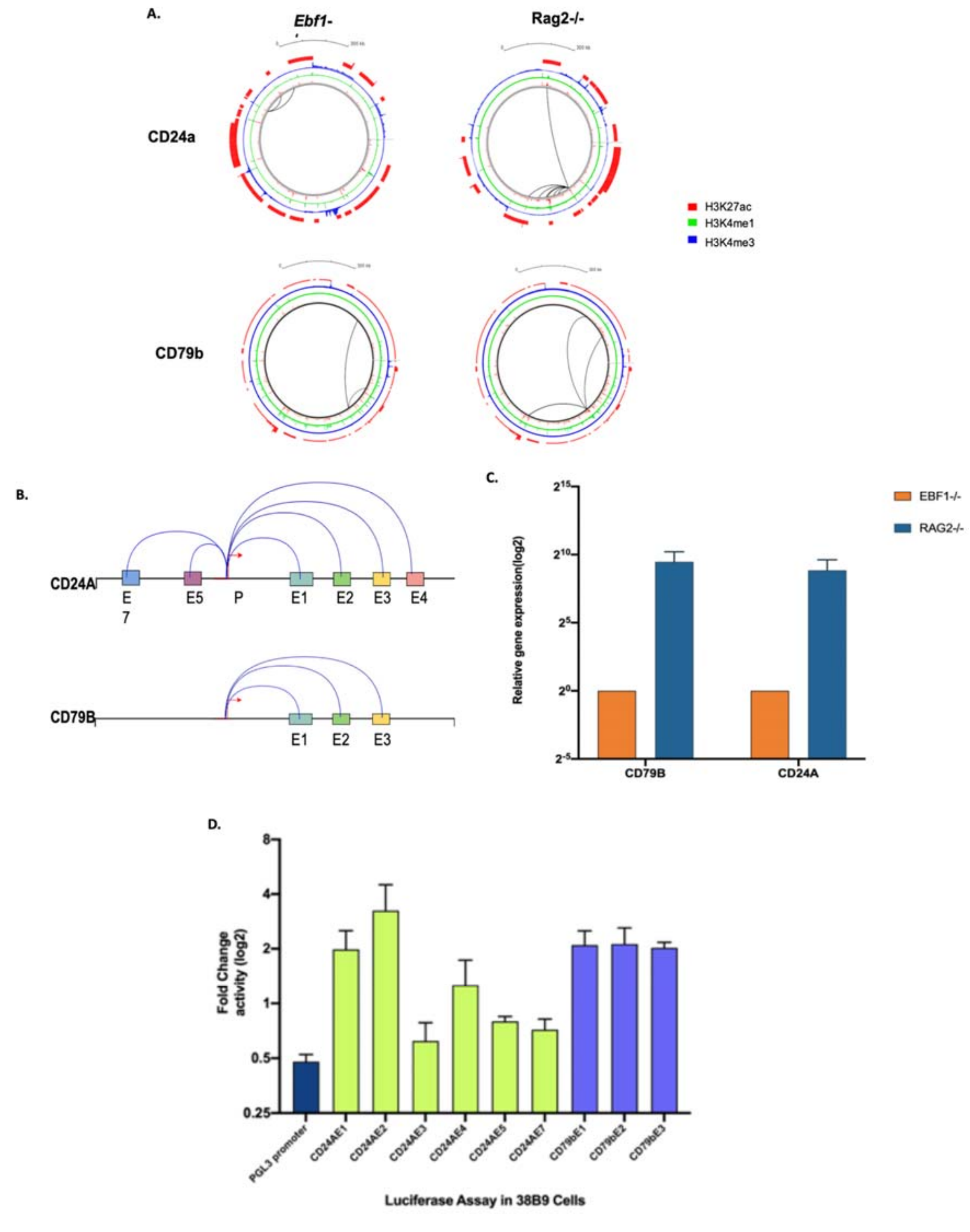


Fig3.6: By regulating Cis-regulatory interactions, EBF1 controls the gene expression pattern peculiar to the B lineage.

(A) Genome-wide promoter cis-regulatory interactions in pre-pro B and pro-B cells.

(B) Diagrammatic representation of enhancers for *cd79b* and *cd24a*.

(C) RT-PCR analysis of transcript levels of *CD79b* and *CD24a in Ebf1-/- and Rag2-/-*.

(D) Luciferase assay of putative enhancers for *cd79b* and *cd24a* in 38B9 cells.

3.7. EBF1 promotes B lineage development even under T lineage conditions

Gain-of-function studies were conducted to thoroughly test the role of EBF1 in driving B cell fate decisions at the expense of alternative lineage genes. MPPs transduced with EBF1 and PAX5 and cultured in B and T cell conditions for seven days; the developmental choices of these cells were then evaluated using FACS **Fig3.7.A**.

CD19 is a B cell marker, whereas CD25 is a T cell-specific marker, as seen in MPP transduced with EBF1 and Pax5 and grown under B cell conditions is displayed in the upper panel. CD19-positive cells were observed when EBF1 induced the development of MPPs into B cells, as predicted. Pax5, the second determinant of B cell fate, similarly differentiates these MPPs into CD19-positive B cells, but to a lesser degree than EBF1. Interestingly, under T cell conditions, (OP9 DL1) EBF1 was able to drive MPPs into CD19-positive B cells at the expense of T lineage development. **Fig3.7.B.**

In accordance with the FACS analysis, a gene expression study of these cells revealed that CD19-positive B cells that were developed under T cell conditions expressed early B lineage-specific genes **Fig3.7.C**. Furthermore, VDJ recombination events were also monitored. It is widely established that B lineage specification is associated with successful VDJ recombination at the IgH locus. Despite being cultured under T lineage conditions, Ebf1-transduced MPPs that differentiated into CD19-positive B cells displayed effective VDJ recombination. These results demonstrated that Ebf1 is the primary determinant of B cell fate commitment by suppressing alternative lineage choice **Fig3.7.D**.

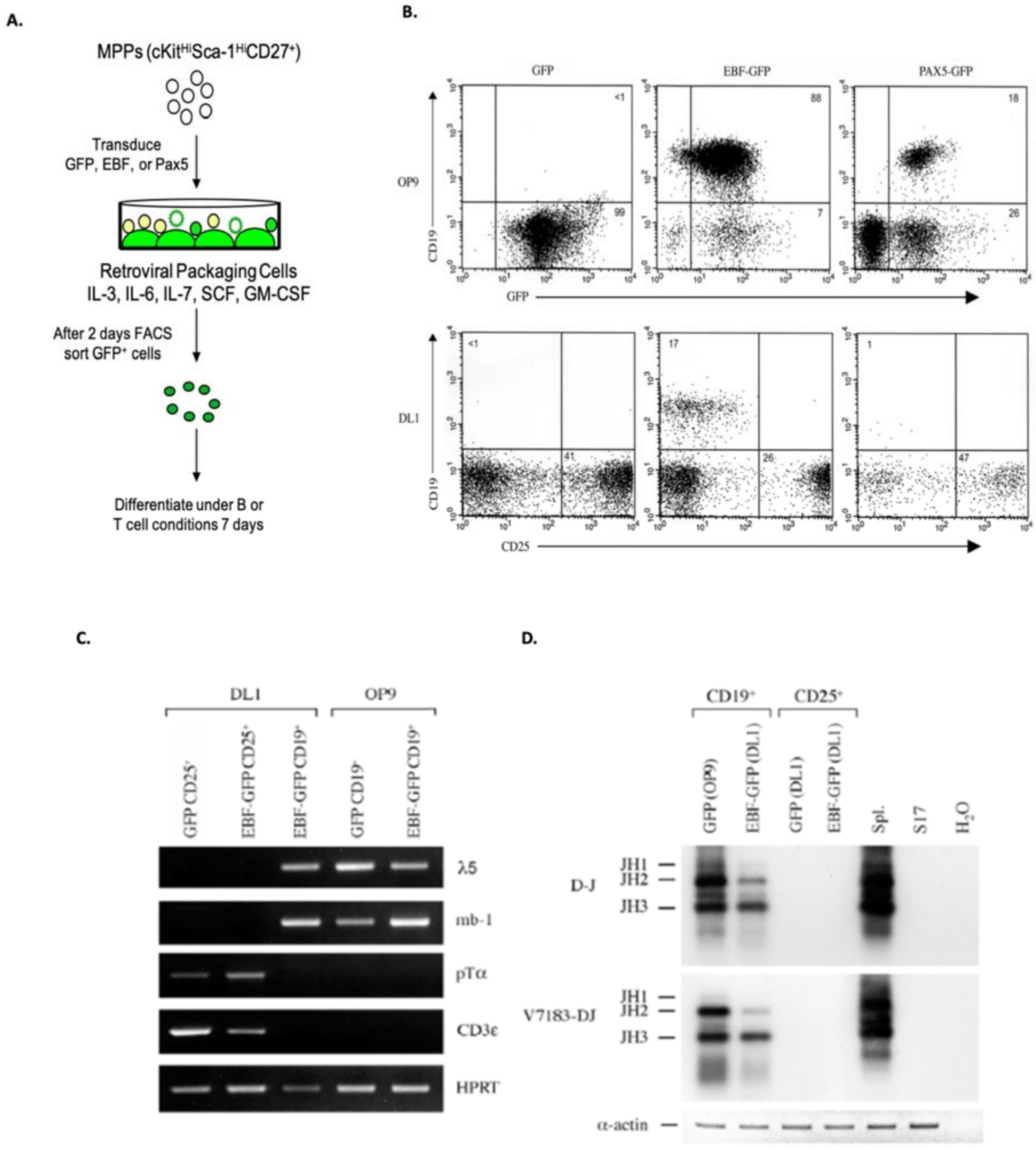


Fig3.7: EBF1 promotes B lineage development even under T lineage conditions

- **(A)** Experimental strategy for transduction and gain of function study.
- **(B)** FACS analysis for developmental choices B lineage development MPP transduced with EBF1and Pax5 and cultured under B cell condition and T cell condition.

(C) Gene expression analysis of B lineage development MPP transduced with EBF1 and Pax5 and cultured under B cell condition and T cell condition.

(D) Southern blot analysis of VDJ recombination at the IgH locus of MPP transduced with EBF1and cultured under B cell condition and T cell condition.

3.8. Construction of EBF1 deletion mutants by overlap extension PCR

We were interested in further analyzing the significance of the EBF1 domains in B cell development and relocalization, given the importance of Ebf1 in B lineage determination and repression of alternate lineage genes. Early B-cell factor (Ebf1) is a transcription factor with a highly conserved N-terminal DNA-binding domain, a Helix-loop-Helix domain, a dimerization domain, and a less conserved C-terminal transcriptional activation domain (TAD) **Fig3.8.A**. The DNA binding domain is distinguished by its unique zinc-binding motif. Although the biological and molecular functions of EBF1 during B cell development have been widely explored, it remains unclear how each domain of EBF1 regulates B lineage-specific gene expression. Given its postulated significance as a critical determinant of the fate of B cells, various EBF1 deletion mutants were created by overlap extension PCR, with the exception of the C-terminal deletion mutant **Fig3.8.B** and cloned into the MigR1 vector **Fig3.8.C** and a functional study was conducted.

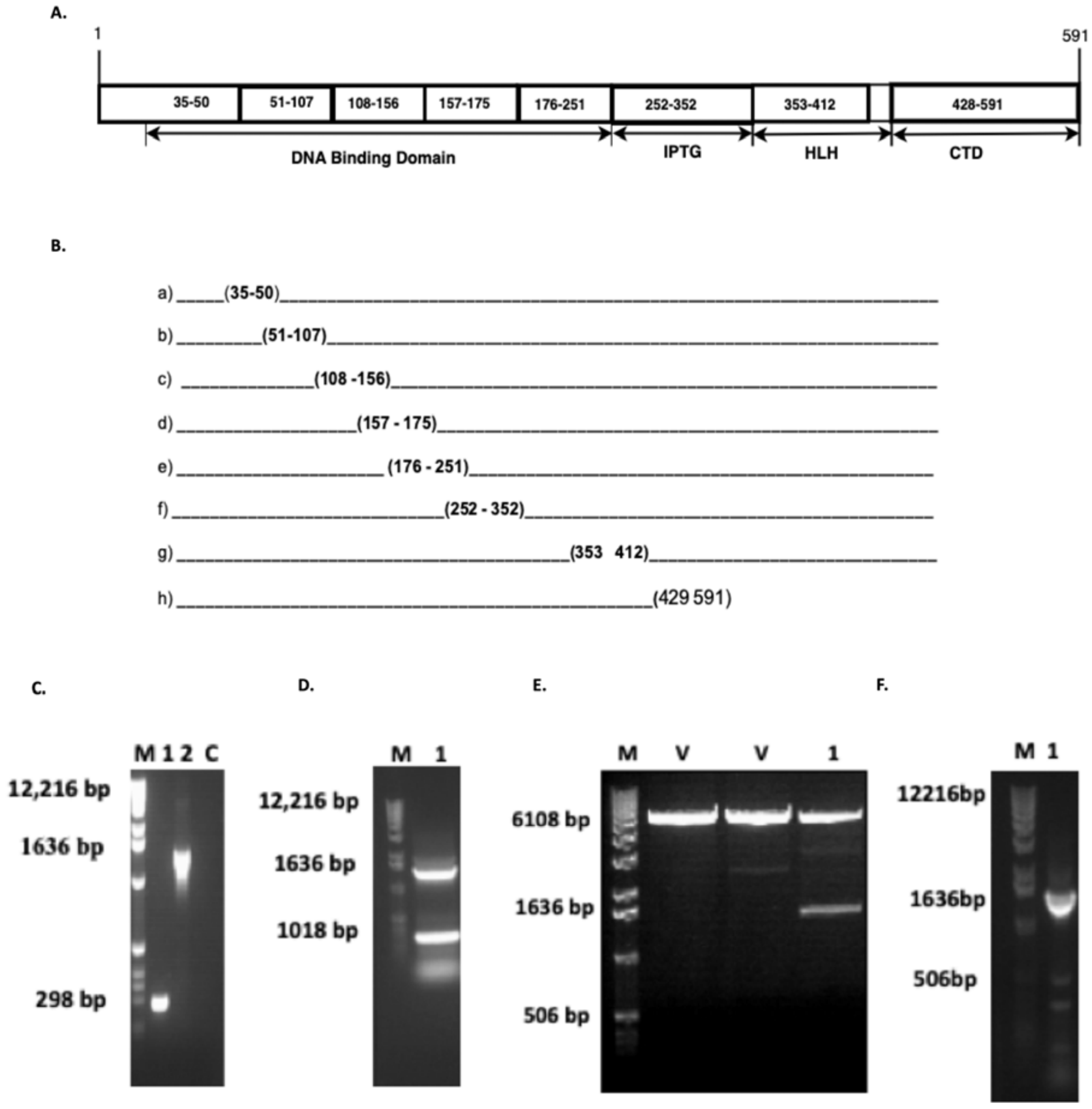


Fig3.8: A graphic illustration of the generation of several deletion mutations in early B cell factor1.

(A) Structure of early B factor (EBF1)

(B) Diagrammatic representation of generation various EBF1 Deletion Mutant by overlap extension PCR except for C terminal deletion mutant.

(C) Amplification of Δ108-156

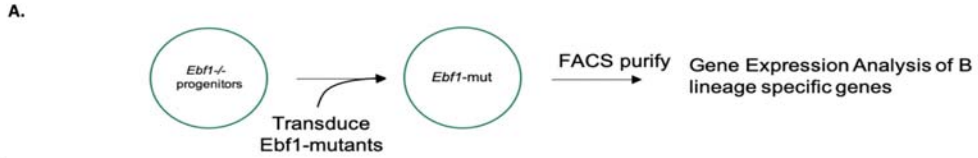
(D) EBF1 deletion mutant (Δ108-156) by overlap extension PCR

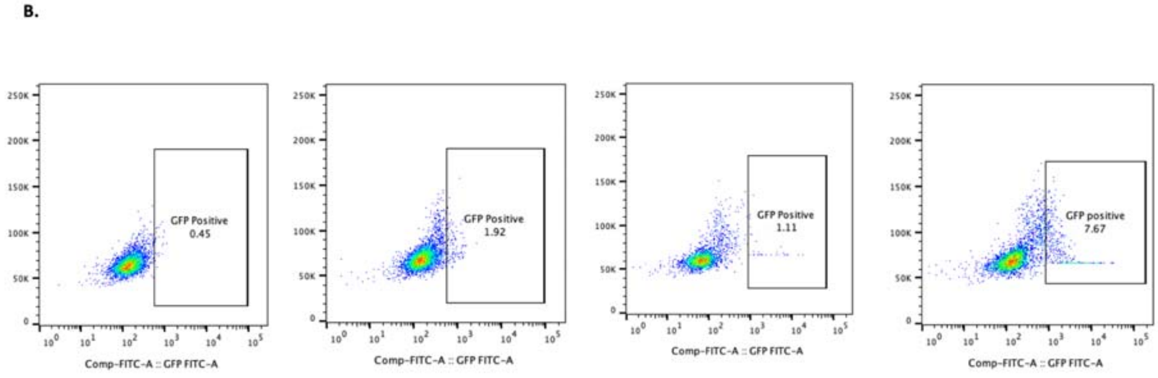
(E) Confirmation of clone (Δ108-156) by Rx Xho1/EcoR1.

(F)

3.9. Functional analysis of EBF1 deletion mutant into Ebf1-/- progenitors

After transducing *Ebf1-/-* cells with retroviral EBF1 mutant constructs **Fig3.9.A.**, GFP-expressing EBF1 mutant/ *Ebf1-/-* cells were sorted by FACS after 48 hours **Fig3.9.B**. Mammalian progenitor cells are notoriously difficult to transfect, and this is the first time electroporation has been standardized in these cells.



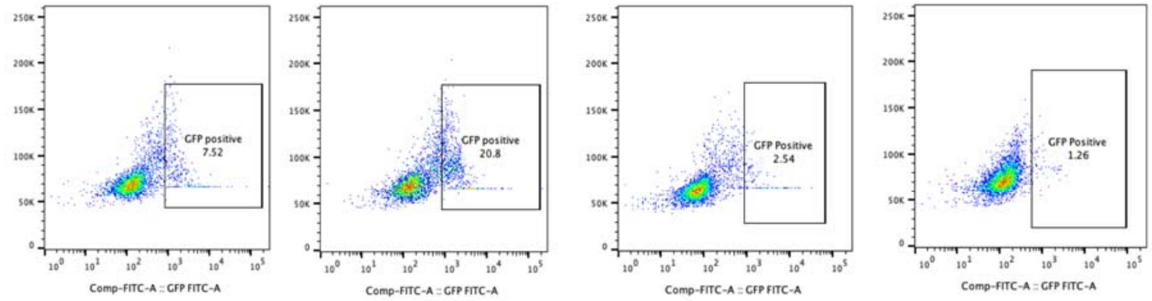


EBF1-/- unstained

Δ 35-50 in EBF1-/-

Δ51-107 in EBF1-/-

Δ108-156 in EBF1-/-

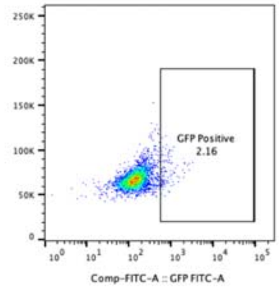


Δ157-175 in EBF1-/-

Δ 176-251 in EBF1-/-

Δ 251 in EBF1-/-

Δ353-412 in EBF1-/-



Δ428 in EBF1-/-

Fig3.9: Functional analysis of EBF1 deletion mutant into *Ebf1*-/- progenitors

(A) Diagrammatic representation of retroviral transduction of Ebf1 in *Ebf1-/-*
(B) FACS sorting of EBF1 deletion mutant after 48 hours of retroviral transduction.

3.10. Expression analysis of B cell markers in EBF1 deletion mutants.

To explore the role of EBF1 domains in the initiation of B cell differentiation, After being transduced with several EBF1mutants, EBF1-deficient progenitors were examined using real-time PCR to assess the expression of a group of B cell-specific genes.

During these experiments, *Ebf1-/-* cells were used as a negative control, and 38B9 representing pro-B cells, were used as a positive control. The deletion mutants were transduced/electroporated in *Ebf1-/-* cells and cultured. According to the hypothesis, if a specific domain is required for the functional activity of EBF1, it should not activate its target gene during differentiation and instead mimic the gene expression pattern of an *Ebf1-/-* cell.

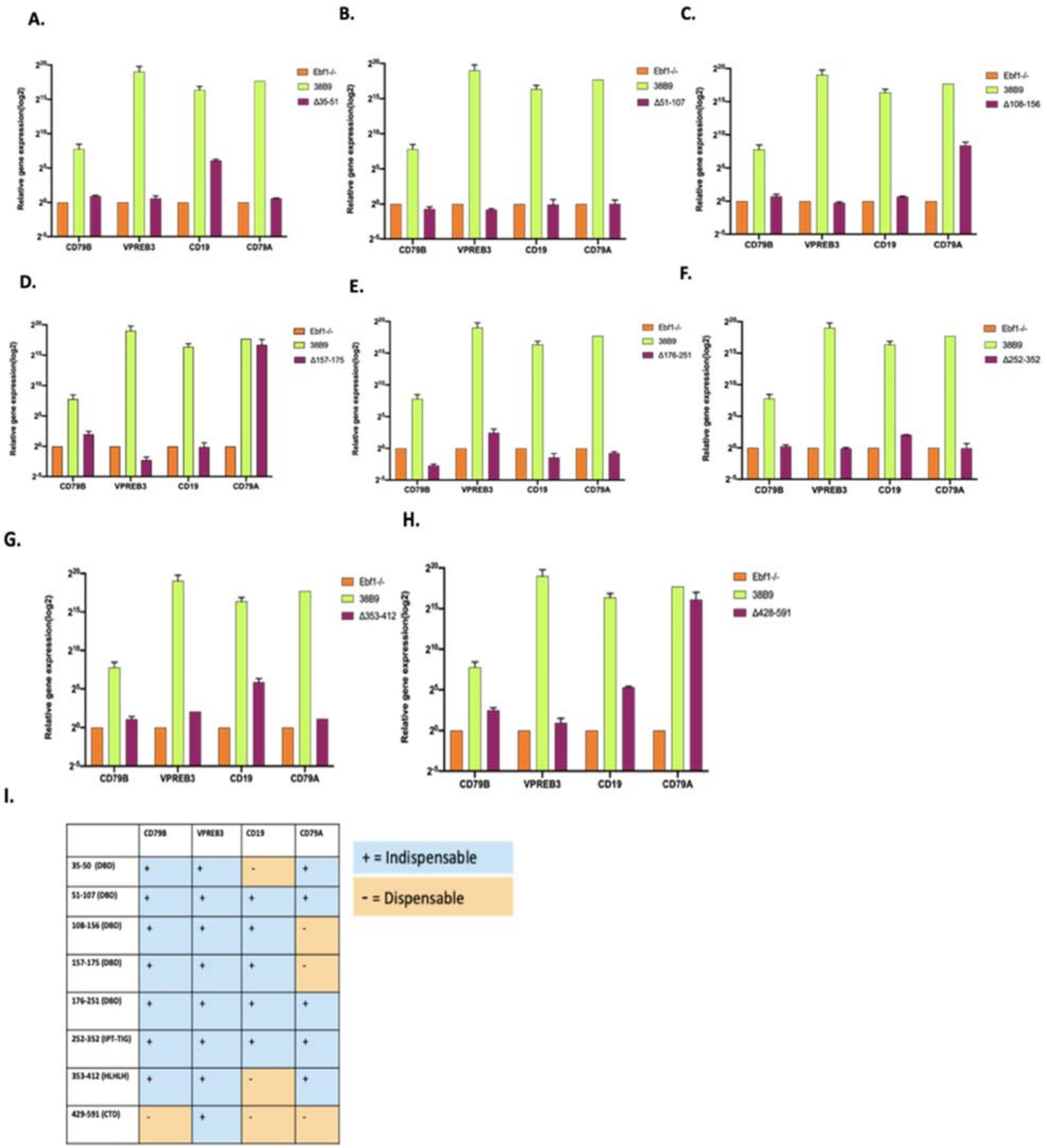
Although it is known that DBD is required for the binding of the EBF1 target gene, it is obscure which crucial areas of DBD are necessary for EBF1 binding and how they contribute to the B cell-specific gene expression pattern. To investigate this, five EBF1 deletion mutants spanning the DBD were generated and their function in target gene expression was investigated.

Except for *Cd19*, all genes in the del mutant 35-50 depend on the 35-51 region of DBD **Fig3.10.A**. Interestingly, del 51-107 did not activate any of the study's target genes **Fig3.10.B**. It demonstrates that the 51-107 DBD area is crucial for the activation of these target genes. Deletion of 51-107 is expected to prevent the binding of EBF1 to its target genes, which explains why no expression is observed. The 51-107 DBD region is necessary for the binding of EBF1 to its target genes[49]. Unlike the del mutant 51-107, the del mutants 108-156 and 157-175 exhibited a similar defect in expression impairment of EBF1 target genes CD79A. **Fig3.10.C&D**. CD79A is unaffected in del mutants 108-156 and 157-175.

All genes are downregulated in del mutant 176-251 and del mutant 252-352 **Fig3.10.E&F**, suggesting that this domain may play a crucial role in B cell development. It demonstrates that the del 176-251 DBD region is necessary for the activation of these target genes. It has been believed that deletion of 176-251 eliminates the binding of EBF1 to its target genes, which explains the absence of expression. Consequently, this demonstrates conclusively that these two areas of DBD are essential for the functional activity of EBF1 to activate target genes.

Again, it implies that the 252-352 region is crucial for EBF1 target gene binding. Like the TIGIPT domain, it enhances the binding of EBF1 target genes. Previously, it has been demonstrated that TIGIPT, coupled with DBD, is essential for DNA binding. However, they have only demonstrated this for one gene, mb1/cd79a[50]. To investigate why lambda five is not affected, an EBF1 ChIP for lambda5 must be performed in this deletion mutant.

Except for CD19, all B cell-specific genes are down-regulated in del 353-412, demonstrating its dependence on the 353-412 area **Fig3.10.G**. In the del mutant 428-591, all EBF1 target genes are predicted to be downregulated **Fig3.10.H**. Surprisingly, the majority of target genes were shown to be partly reliant despite the absence of a transactivation domain. Recent research conducted by Grossdechel, characterized the *in vivo* function of a C-terminal deletion mutant[103]. Similar EBF1 target genes that are independent of CTD have been identified



	CD79B	VPREB3	CD19	CD79A
35-50 (DBD)	+	+	-	+
51-107 (DBD)	+	+	+	+
108-156 (DBD)	+	+	+	-
157-175 (DBD)	+	+	+	-
176-251 (DBD)	+	+	+	+
252-352 (IPT-TIG)	+	+	+	+
353-412 (HLHLH)	+	+	-	+
429-591 (CTD)	-	+	-	-

+ = Indispensable

- = Dispensable

Fig3.10:Expression analysis of B cell markers in EBF1 deletion mutants

(A) RT-PCR analysis of transcript levels of B cell markers in Δ35-50.

(B) RT-PCR analysis of transcript levels of B cell markers in Δ51-107.

(C) RT-PCR analysis of transcript levels of B cell markers in Δ108-156.

(D) RT-PCR analysis of transcript levels of B cell markers in Δ157-175.

(E) RT-PCR analysis of transcript levels of B cell markers in Δ176-251.

(F) RT-PCR analysis of transcript levels of B cell markers in Δ251-352.

(G) RT-PCR analysis of transcript levels of B cell markers in Δ353-412.

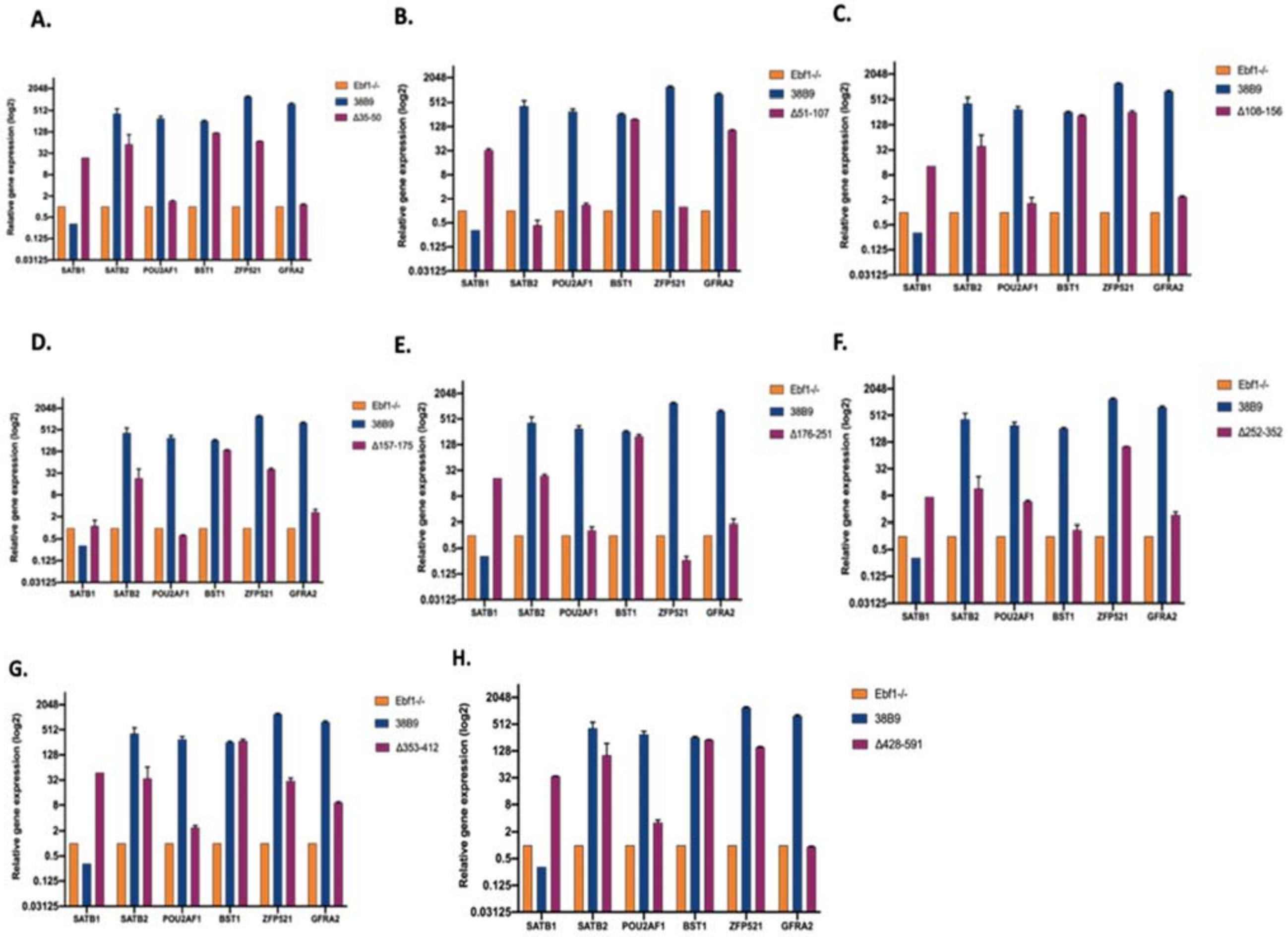
(H) RT-PCR analysis of transcript levels of B cell markers in Δ428-591.

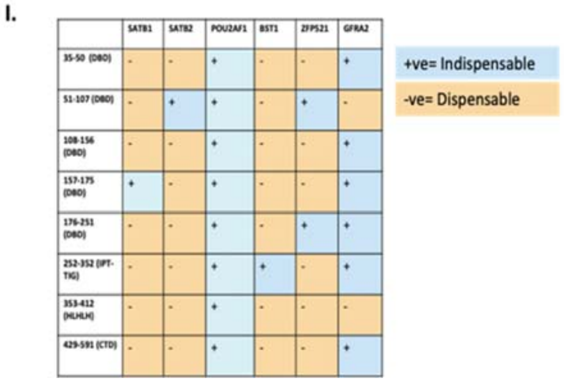
(I) Summary table of expression analysis of B cell markers in EBF1 deletion mutants.

3.11. Expression analysis of switched genes in EBF1 deletion mutants

All other domains of EBF1 are essential for the expression of early B lineage genes in cells transduced with various deletion mutants, with the exception of the C-terminal domain, which exhibited a partial deficiency. Quantitative RT-PCR analysis of differentially switched genes has also been performed to determine the function of each domain of EBF1 in target gene regulation. As indicated before, *Ebf1-/-* cells are MPP and serve as a negative control, while 38B9 cells are pro-B cells and serve as a positive control. Except for *Pou2af1* and *Gfra2*, the expression of other genes in the deletion mutant 35-50 is independent of the 35-50 region of DBD **Fig3.11.A**. Previously, it was demonstrated that 51-107 is essential for the expression of early B lineage genes; however, in the case of differentially switching genes, it was discovered that 51-107 is not necessary for the expression of all B lineage genes **Fig3.11.B**. This could be because EBF1 does not bind and regulate the expression of these genes directly. For example, Pou2af1, a well-established EBF1 target, is downregulated in all deletion mutants. This data suggest that the expression of *Pou2af1* and *Gfra2* is dependent on the 35-50 DNA binding domain. Like early B lineage genes, the del mutants 108-156 and 157-175 showed a partial expression deficiency in differentially switching genes **Fig3.11.C&D**.

All EBF1 deletion mutants partially affect the differentially switched genes, so here we assume that EBF1 is not directly binding and regulating the expression of these genes except for Pou2AF1, a well-known EBF1 target, which is impacted in the majority of EBF1 deletion mutants. We conclude that EBF1 is not directly binding and regulating the expression of these genes.





	SATB1	SATB2	POU2AF1	BST1	ZFP521	GFRA2
35-50 (DBD)	-	-	+	-	-	+
51-107 (DBD)	-	+	+	-	+	-
108-156 (DBD)	-	-	+	-	-	+
157-175 (DBD)	+	-	+	-	-	+
176-251 (DBD)	-	-	+	-	+	+
252-352 (IPT-TIG)	-	-	+	+	-	+
353-412 (HLHLH)	-	-	+	-	-	-
429-591 (CTD)	-	-	+	-	-	+

+ve= Indispensable

-ve= Dispensable

Fig3.11: Expression analysis of switched genes in EBF1 deletion mutants

(A) RT-PCR analysis of transcript levels of switched genes in Δ35-50

(B) RT-PCR analysis of transcript levels of switched genes in Δ51-107

(C) RT-PCR analysis of transcript levels of switched genes in Δ108-156

(D) RT-PCR analysis of transcript levels of switched genes in Δ157-175

(E) RT-PCR analysis of transcript levels of switched genes in Δ176-251

(F) RT-PCR analysis of transcript levels of switched genes in Δ252-352

(G) RT-PCR analysis of transcript levels of switched genes in Δ428-591

(H) Summary table of expression analysis of switched genes in EBF1 deletion mutant.

CHAPTER-4:

DISCUSSION

In order to determine the specific cell destiny of multipotent progenitors, it is necessary to exercise precise and coordinated control over gene expression[58][59][104] [60]. Previous study reveals that a number of transcription factors (TFs), including PU.1, EBF1, E2A, PAX5, and IRF4, regulate the development of B cells. This is because a mutation in any one of these TFs causes a stop in the progression of B cell growth at a certain stage. Our lab has shown that the transcription factor EBF1 is the most important factor in the formation of B cells. Because EBF1 is able to restore the growth of B cells even when PU1, E2A, and IL7R are absent. Loss of function experiments have revealed that *Ebf1-/-* cells, despite being cultured in lymphoid conditions, are unable to express B lineage-specific genes like λ5, VpreB, and mb-1. Instead, these cells express genes that are specific to the myeloid and T cell lineages. B cell development is inhibited in the absence of EBF1 at the stage of a lymphoid progenitor cell that expresses B220, Flt3, and IL-7R but does not express CD19. EBF1-/- cells have been shown to develop into T cells when grown on OP9-DL1 stroma and into myeloid lineages when cultured with myeloid cytokines such as GMCSF and M-CSF. This study adds to the existing body of evidence demonstrating the importance of EBF1 in determining cell fate by showing that it is also required for the restriction of alternate cell fates.

Previously in our laboratory, we performed a genome-wide expression investigation of EBF1 by ectopically expressing EBF1 in *Ebf1-/-* cells. The purpose of this study was to identify the underlying mechanisms through which EBF1 promotes B-cell growth. Based on these findings, we were able to identify a subset of genes whose expression EBF1 regulates through positive or negative feedback loops. There were 662 genes in total, and 363 of those genes were active while 299 were repressed. According to the findings of these analyses, EBF1 activates the genes that are essential for B cell specification, while alternate lineage genes are suppressed.

To explore the architectural significance of EBF1 in B cell fate specification, we used HiC to find genome-wide chromatin rearrangement in EBF1-/- and Rag2-/- pre-pro B and Pro B cells. In line with previous studies, our HiC investigation indicated that chromatin is hierarchically structured into TADs and chromatin loops. [105] [71][59][70][106]. Chromatin is compartmentalized into permissive and repressive regions at the mega-base level. (A and B). At sub, megabase level chromatin is organized as highly self-interacting domains known as TADS which are called topologically associated domains. At the Kilobase level chromatin is organized as a chromatin loop-like promoter enhancer interaction. Our studies have shown that chromatin undergoes global reorganization and differential compartmentalization to dictate gene expression specific to B lineage patterns.

However, a comprehensive knowledge of how the multidimensional chromatin architecture regulates transcriptional activity specific to B cell is not yet available. To get a complete knowledge of the interplay between EBF1 and differential chromatin compartmentalization during B cell fate specification, first, we sought to investigate whether there is evidence that EBF1-dependent chromatin compartmentalization occurs during B cell fate commitment.

Both relocalization and recombination of IgH are considered to be the hallmarks of the B lineage specification. In pre-pro-B cells, the IgH locus is found in the nuclear periphery, but in pro-B cells, it moves away from the nuclear periphery and goes through proximal and distal VDJ recombination. However, it has already been proven that EBF1 is the primary determinant of B cell fate selection. Nevertheless, we proposed that EBF1 might be playing an essential role in the relocalization of IgH during the differentiation of MPP to pro-B cells. The nuclear localization of IgH has been examined in the lack and presence of EBF1 to determine whether it plays a role in determining B cell fate determination. To comprehend the role of EBF1 in Igh relocalization, two model systems were utilized: Ebf1-/- cells (representing the MPP, pre-pro-B cell stage) and Ebf1-/- cells transduced with EBF1 (representing pro-B cell stage) Moreover, 3D FISH was performed on the IgH locus. In Ebf1-/- cells, IgH alleles were found to be localized near the nuclear periphery, as demonstrated by 3D FISH. Interestingly, that when we ectopic expression of EBF1 in Ebf1-/- cells, the IgH alleles moved into the center of the nucleus. These findings suggest that the rearrangement of IgH loci is initiated following induction of EBF1 expression. In addition, these data lend credence to the speculation that EBF1 may play a significant part in the compartmentalization of chromatin throughout the process of determining the fate of B cells.

chromatin is non-randomly arranged into permissive (A) and repressive (B) compartments, according to our in situ Hi-C research, which is consistent with that of previous investigations [59][70]. We discovered a unique set of genes that move between the A and B compartments during the developmental progression from the pre-pro-B to pro-B cell stage. During the shift from pre-pro-B to pro-B, genes necessary for B cell development, such as Satb2, Pou2af1, Tlr4, Bst1, and Gfra2, move from the repressive compartment to the permissive compartment. Zbtb16, Gata3, Satb1, and Klf4 are among the crucial genes that move to the B compartment in pro-B cells, where they are suppressed. These genes are also involved in alternate lineage differentiation processes. Then, to investigate if the differential chromatin compartmentalization is associated with the B-lineage-specific gene expression pattern, we analyzed the number of transcript levels in pre-pro-B cells and pro-B cells as determined by RNA-Seq. And we discovered that A compartments had significantly greater transcript levels than B compartments. The gene expression analysis of differentially switched genes revealed that genes switching from the B compartment to the A compartment are transcriptionally active in pro B cells and repressed in pre-pro B cells, and vice versa. The observation of changes in gene expression utilizing RNA-seq data for differentially switched genes has been supported by q PCR analysis. Moreover, RT-PCR analysis revealed higher expression of well-known early B-lineage genes such as Pou2AF1, Satb2, Gfra2, and Bst1 that switch to A compartment in pro B cells. whereas Satb1, which switches from an active compartment to an inactive compartment in pre-pro-B cells to pro-B cells, exhibited down-regulation.

To thoroughly investigate the role of EBF1 in differential gene switching, we chose satb1 and satb2 as potential genes and utilized 3D gene loci FISH in pre-pro-B cell and pro-B cell populations. Our 3D gene loci FISH data suggest upon ectopic expression of EBF1 allows satb1 to move from the nuclear center to periphery and it becomes transcriptionally inactive in pro-B cells. On the other hand, Satb2 relocates from the nuclear periphery to the center of the nucleus and becomes transcriptionally active in pro-B cells. These results demonstrate that EBF1 is essential for the differential chromatin compartmentalization associated with the determination of the B lineage.

After confirming the involvement of EBF1 in differential chromatin compartmentalization, we hypothesise that Ebf1 and Pax5 binding to their target promoter-cis-regulatory interaction regions is just one of many levels of regulation involved in the activation of genes that are particular to the B lineage. In order to verify this, we integrated the promoter-cis-regulatory interaction data obtained by Hi-C with the data obtained by EBF1/PAX5 ChIP-seq in pro-B cells. Importantly, we discovered that in pro-B cells, Ebf1 binds alone or in conjunction with Pax5 to 57.2% of promoters and 51.2% of enhancers involved in long-range interactions. De novo motif analysis (TFs) was performed on probable cis-regulatory regions for the genes for which PCA indicated the chromatin state. We have chosen the candidate genes Satb2 and Pou2af1 to test this theory. Each gene was assigned three possible enhancers, and all six enhancers were cloned into the PGL3 Promoter vector. After transfection of 38B9 cells changed into pro-B cells, reporter assay tests were undertaken. In the case of Satb2, E1 demonstrated the highest activity of the three, whereas Pou2af1 displayed the highest E2 activity. These findings suggest that EBF1 modulates chromatin compartmentalization via binding to promoter and enhancer regions of differentially expressed genes.

After proving EBF1's role in chromatin remodeling, we analyze the functional importance of EBF1 during B-cell development. ChIP-seq was performed on pre-pro-B cells and pro-B cells for EBF1 and H3K4me1 to provide light on the molecular mechanism by which EBF1 orchestrates B cell development. We discovered 11,508 EBF1 binding sites. EBF1 targets a varied variety of sequences, the majority of which exist in close proximity to or within genes, including promoters (13%) and introns (28%). The phastCons program was used to generate conservation measurements across 30 vertebrate genomes and we found that DNA sequences including EBF1binding sites in Rag2-/- pro-B cells were statistically more conserved than sequences from random genomic positions.

To examine if Ebf1 binding is linked with chromatin structural alterations at enhancer sites during B cell commitment, we performed ChIP-seq for H3K4me1, an epigenetic mark associated with enhancer regions, in Ebf1-/- progenitors and Rag2-/- pro-B cells expressing Ebf1. Although the total number of H3K4me1-enriched regions was comparable across Ebf1-/- progenitors (n=8068) and Rag2-/- pro-B cells (n=8519), the enhancer landscape was slightly expanded in pro-B cells, and 20-30% of these regions overlapped between the two cell types. Examining the status of H3K4me1 modified chromatin spanning EBF1 binding sites in Rag2-/- pro-B cells, we discovered areas that were marked with H3K4me1 prior to EBF1 expression and that this condition was maintained in committed pro B cells (577). Moreover, we found an increase in H3K4me1-enriched areas (2071) when cells moved from a multipotent progenitor state to a committed state that needs EBF1 expression. These findings indicate that EBF1 expression is related with the acquisition of the H3K4me1 mark at certain genomic loci and imply that EBF1 may be responsible for the deposition of H3K4me1 at these loci. We observe that EBF1 expression and binding were similarly associated with the loss of this active histone mark at 485 genomic locations and that Ebf1 may inhibit this chromatin modification in the context of suppressing alternate lineage genes.

The genome-wide promoter cis-regulatory interactions of pre-pro-B and pro-B cells were determined using Hi-C data. These analyses revealed that the promoter cis-regulatory interactome undergoes extensive rewiring during B-cell fate determination and is related to a gene expression pattern specific to B lineage. Cd24a and Cd79b, which are strongly expressed in pro-B cells, have more cis-interactions in pro-B cells than in pre-pro-B cells. Similarly, the quantitative RT-PCR analysis revealed that pro-B cells had more Cd79b and Cd24a transcripts than pre-pro B cells. To evaluate enhancer activity, six and three putative enhancers were chosen for the Cd24a and Cd79b genes, respectively. These enhancers were cloned into the PGL3 Promoter vector, and reporter assay experiments were conducted. In Cd24a, only E1 and E2 out of six enhancers demonstrated increased activity, whereas, in Cd79b, all three enhancers displayed higher activity. These results implicate EBF1in the foundation of the lineage-specific cis-regulatory interactome for the B lineage. EBF1 could potentially regulate chromatin compartmentalization and promoter cis-regulatory interactions to determine the B cell fate of MPPs.

Using gain-of-function experiments, the role of EBF1 in directing B cell fate decisions at the expense of alternative lineage genes was exhaustively examined. The developmental options of MPPs transduced with EBF1 and PAX5 and grown in B and T cell settings for seven days were examined using FACS. As predicted, CD19-positive cells were found when EBF1 stimulated the maturation of MPPs into B cells. PAX5, the second B cell fate determinant, converts these MPPs into CD19-positive B cells to a lesser extent than EBF1. Interestingly, EBF1 was able to push MPPs into CD19-positive B cells at the expense of T lineage selection when cultured under T cell condition. In agreement with the FACS analysis, gene expression analysis of these cells revealed that CD19-positive B cells generated under T cell conditions expressed early B lineage-specific genes. EBF1-transduced MPPs that differentiated into CD19-positive B cells exhibited efficient VDJ recombination despite having been grown under conditions favorable to the T lineage. These findings indicate that EBF1 is the major determinant of B cell fate commitment by inhibiting alternative lineage choice.

Given the role of EBF1 in B lineage determination and repression of alternate lineage genes, we were interested in further studying the significance of the EBF1 domains in B cell development and relocalization. Although the biological and molecular functions of Ebf1 during B cell development have been extensively studied, it is still unknown how each domain of EBF1 regulates the expression of B lineage-specific genes. This work revealed that, with the exception of the C-terminal domain, all other EBF1 domains are required for the activation of early B lineage genes. In addition, we discovered that the expression of the majority of differentially switched genes is partially influenced by the loss of EBF1 in several mutant.

CHAPTER-5:

SUMMARY

To determine multipotent progenitors' cell fate, precise and coordinated gene expression is needed[58][59][71][60]. B cell development is regulated by several transcription factors (TFs), including PU.1, EBF1, E2A, PAX5, and IRF4. Mutations in any of these TFs terminate B cell development at a specific point. Our lab found that EBF1 primary determinant in B cell development. EBF1 can develop B cells without PU1, E2A, or IL7R. Ebf1-/- cells grown in lymphoid conditions cannot express b lineage-specific genes including VpreB, mb-1, and λ5. Instead, these cells express myeloid and T cell-specific genes. In the absence of EBF1, B cells stop developing at the stage of a lymphoid progenitor cell that expresses Flt3, IL-7R, and B220, but not CD19. Previous research has demonstrated that EBF1-/- cells can develop into T cells when cultured on OP9-DL1, and they can differentiate into a myeloid lineages when cultured under myeloid cytokines like GMCSF and M-CSF. This study found that EBF limits alternative cell fates and specifies B cell fates.

To understand EBF1's architectural involvement in B cell fate specification, we used HiC to find genome-wide chromatin rearrangements in EBF1-/- and Rag2-/- pre-pro B and Pro B cells. Our HiC study showed that chromatin is hierarchically organized into TADs and chromatin loops, as previously reported [71][70][59]. At the mega-base level, chromatin is structured into permissive and repressive compartments (A and B). At the sub-megabase level, chromatin is structured into topologically associated domains (TADS). At the kilobase level, chromatin forms a promoter enhancer loop. We found that global chromatin reorganization and differential compartmentalization control gene expression specific to B lineage. However, how the multilayer architecture of chromatin controls B cell-specific transcriptional activity is still unknown. To fully comprehend EBF1 and differential chromatin compartmentalization during B cell fate specification. First, we investigated EBF1-dependent chromatin compartmentalization during B cell fate commitment.

B lineage specification involves IgH relocalization and recombination. In pro-B cells, the IgH locus shifts away from the nuclear periphery and undergoes proximal and distal VDJ recombination. However, it has been established that EBF1 is the major determinant of B cell fate choice. EBF1 may be necessary for IgH relocalization during MPP to pro-B cell differentiation. In the absence and presence of EBF1, IgH nuclear localization has been studied to evaluate its role in B cell fate determination. Two model systems were used to understand EBF1's role in Igh relocalization: Ebf1-/- cells (pre-pro-B cell stage) and Ebf1-/- cells transduced with EBF1 (representing pro-B cell stage) 3D FISH was performed on the IgH locus. 3D FISH showed IgH alleles towards the nuclear periphery in Ebf1-/- cells. The IgH alleles migrated into the nucleus when we ectopically expressed EBF1 in Ebf1-/- cells. These findings show that IgH locus rearrangement begins when EBF1 expression is restored in Ebf1-/- cell progenitors. These findings further support the notion that EBF1 may help compartmentalization of chromatin during B cell fate determination.

Previous in vitro in situ Hi-C experiments we performed showed that chromatin is non-randomly structured into A and B compartments [59][70]. Additionally, we identified a discrete set of groups of genes that shifted from the A to the B compartment during the transition from pre-pro-B to pro-B cells. *Tead1, Satb2, Tlr4, Pou2af1, Bst1, Gfra2,* and other B cell development genes switch from the B compartment to the A compartment during the transition from pre-pro-B cell stage to pro-B cell stage. *Zbtb16, Klf4, Gata3, Satb1,* and other genes involved in alternate lineage differentiation, move to the compartment B in pro-B cells. To test if differential chromatin compartmentalization is linked to the B-lineage-specific gene expression pattern, we measured transcript levels in pre-pro-B and pro-B cells using RNA-Seq and we found that A compartment exhibited much higher transcript levels than B compartments. The gene expression analysis revealed that Genes switching from the B compartment to the A compartment is transcriptionally active in pro-B cells and repressed in pre-pro B cells, and vice versa. RT-PCR also showed robust expression of early B-lineage genes such as *Pou2AF1, Satb2, Gfra2*, and *Bst1* that transition to A compartment in pro B cells. whereas *Satb1,* which changes from an active compartment to an inactive compartment in pre-pro-B cells to pro-B cells, was down-regulated.

We selected satb1 and satb2 as prospective genes and used 3D gene loci FISH in pre-pro-B and pro-B cell populations to study EBF1's role in differential gene switching. Our 3D gene locus FISH findings reveal that when EBF1 is ectopically expressed in pro-B cells, satb1 travels from the nuclear core to the periphery and becomes transcriptionally silent. Satb2 moves from the nuclear periphery to the nucleus center and becomes transcriptionally active in pro-B cells. These findings show that EBF1 is necessary for differential chromatin compartmentalization during B lineage determination.

After demonstrating EBF1's role in differential chromatin compartmentalization, we hypothesized that Ebf1 and Pax5 binding to target promoter-cis-regulatory interacting regions is one possible mechanism by which activation of genes essential for the B lineage is controlled. In pro-B cells, we combined Hi-C promoter-cis-regulatory interactions with

EBF1/Pax5 ChIP-seq data. Importantly, in pro-B cells, 57.2% of promoters and 51.2% of cis-regulatory elements (enhancers) engaged in long-range interactions are bound by EBF1 alone or in combination with Pax5. De novo motif analysis (TFs) was performed on putative cis-regulatory areas for genes with PCA-determined chromatin states. Satb2 and Pou2af1 are our candidate genes to test this notion. Each gene has three potential enhancers, and all six were cloned into the PGL3 Promoter vector. Reporter assays were performed on 38B9 cells after transfection. Satb2's E1 activity was highest, while Pou2af1's E2 activity was highest. These results imply that EBF1 regulates chromatin compartmentalization by binding to differently expressed gene promoter and enhancer regions.

After demonstrating EBF1's role in chromatin remodeling, we examine its functional evolution during B-cell development. We performed ChIP-seq for H3K4me1, an epigenetic mark associated with enhancer regions, using Ebf1-/- progenitors and Rag2-/- pro-B cells expressing EBF1. When cells transitioned from multipotent progenitor to committed EBF1-expressing state, H3K4me1-enriched regions (2071) increased. These data suggest that EBF1 expression may cause H3K4me1 acquisition at particular genomic loci.

Hi-C data determined the genome-wide promoter cis-regulatory interactions of pre-pro-B and pro-B cells. The promoter cis-regulatory interactome undergoes considerable rewiring during B-cell destiny determination and is linked to a B lineage-specific gene expression pattern. Cd24a and Cd79b, which are abundantly expressed in pro-B cells, have more cis-interactions than pre-pro-B cells. Quantitative RT-PCR analysis showed that pro-B cells had more Cd79b and Cd24a transcripts than pre-pro B cells. Six and three Cd24a and Cd79b putative enhancers were selected to assess enhancer activity. Reporter assays suggest Ebf1 established the B lineage's lineage-specific cis-regulatory interactome. MPPs' B cell fate may be determined by EBF1's chromatin compartmentalization and promoter cis-regulatory interactions.

EBF1's significance in B cell fate decisions over alternative lineage genes was comprehensively explored using gain-of-function assays. FACS was used to evaluate MPPs transduced with EBF1 and PAX5 and cultured in B and T cells for seven days. When EBF1 matured MPPs into B cells, CD19-positive cells were detected as expected. EBF1 transforms these MPPs into CD19-positive B cells more than PAX5. EBF1 drove MPPs into CD19-positive B cells at the expense of T lineage selection when grown under T cell conditions. Gene expression investigation showed that CD19-positive B cells produced under T cell conditions expressed early B lineage-specific genes, supporting FACS analysis. EBF1-transduced MPPs that differentiated into CD19-positive B cells had efficient VDJ recombination despite being cultured under T lineage-favoring conditions. These data suggest that EBF1 inhibits alternative lineage choice and determines B cell fate.

Due to their significance in B lineage determination and repression of alternate lineage genes, we were intrigued in studying the effect of EBF1 domains on B cell proliferation and relocalization. It is unclear how each domain of EBF1 affects B lineage-specific gene expression during B cell development. This study found that all EBF1 domains except the C-terminal domain activate early B lineage genes. In addition, most EBF1 deletion mutants partially affect the expression of the majority of differentially switched genes.

CONCLUSION

- During the development of MPPs into pro-B cells, EBF1 induces the relocalization of Igh alleles from the nuclear periphery to the center.
- A specific set of genomic loci undergo differential chromatin compartmentalization during B-cell fate specification.
- Gene expression patterns specific to the B lineage are associated with differential switching of certain genomic loci between the A and B compartments.
- 3D FISH analysis confirms the differential compartmentalization of a specific set of genomic loci during the differentiation of pre-proper-pro B cells to pro B cells.
- EBF1 binds to promoter and cis-regulatory regions of target genes, which may facilitate in B lineage-specific chromatin compartmentalization.
- We observed that EBF1 binding is associated with an increase in regions enriched for H3K4me1 as cells transitioned from a multipotential progenitor state to a committed state.
- We observed that the transcriptional activity of early B-lineage genes is positively connected with the number of *cis*-regulatory interactions.
- We found that despite culturing under T cell conditions which is OP9 DL1, EBF1 was able to drive MPPs into CD19 positive B cells at expense of T lineage choice.

- We showed that EBF1 transduced MPPs that are differentiated into CD19 positive B cells showed successful VDJ recombination despite culturing under T lineage conditions.
- We found that except for the C terminal domain all other domains of EBF1 are indispensable for the activation of early B lineage genes.

CHAPTER-6:
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DETERMINING THE ARCHITECTURAL ROLES OF EBF1 IN THE TOPOLOGICAL ORGANIZATION OF THE GENOME AND ITS FUNCTIONAL CHARACTERIZATION DURING B CELL FATE DETERMINATION

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A regulatory network of microRNAs confers lineage commitment during early developmental trajectories of B and T lymphocytes

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The commitment of hematopoietic multipotent progenitors (MPPs) toward a particular lineage involves activation of cell type–specific genes and silencing of genes that promote alternate cell fates. Although the gene expression programs of early–B and early–T lymphocyte development are mutually exclusive, we show that these cell types exhibit significantly correlated microRNA (miRNA) profiles. However, their corresponding miRNA targetomes are distinct and predominated by transcripts associated with natural killer, dendritic cell, and myeloid lineages, suggesting that miRNAs function in a cell-autonomous manner. The combinatorial expression of miRNAs miR-186-5p, miR-128-3p, and miR-330-5p in MPPs significantly attenuates their myeloid differentiation potential due to repression of myeloid-associated transcripts. Depletion of these miRNAs caused a pronounced de-repression of myeloid lineage targets in differentiating early–B and early–T cells, resulting in a mixed-lineage gene expression pattern. De novo motif analysis combined with an assay of promoter activities indicates that B as well as T lineage determinants drive the expression of these miRNAs in lymphoid lineages. Collectively, we present a paradigm that miRNAs are conserved between developing B and T lymphocytes, yet they target distinct sets of promiscuously expressed lineage-inappropriate genes to suppress the alternate cell-fate options. Thus, our studies provide a comprehensive compendium of miRNAs with functional implications for B and T lymphocyte development.

Ago2 RNA immunoprecipitation and sequencing | B- and T-cell development | lymphocyte commitment | microRNAs | lineage differentiation

The differentiation of multipotent hematopoietic progenitors into mature cell types of the immune system has been shown to be under the control of gene regulatory networks involving transcription factors, cytokine signals, and other epigenetic mechanisms (1–5). The LMMPs (lymphoid-primed multipotent progenitors), which are the earliest predecessors of B and T lymphocytes (6, 7), predominantly differentiate toward T lineage upon entering thymus under the influence of Notch1 signaling in addition to TFs like TCF1, GATA-3, and BCL11B (8–11). Correspondingly, expression of active Notch1 in the bone marrow induces thymus-independent differentiation of early lymphoid progenitors into T cells at the expense of B lymphopoiesis (12). Studies from our laboratory and others have demonstrated that transcription factor EBF1 functions as the primary B lineage determinant and acts in conjunction with its downstream target, PAX5, to establish the B lineage identity of LMPPs (13–15). Targeted disruption of EBF1 results in a complete arrest of B cell development at CLP (pre–proB) stage, and the mutant $Ebf1^{-/-}$ progenitors exhibit developmental plasticity toward alternative lineages, including myeloid, dendritic cell (DC), natural killer (NK), and T cells, despite being maintained under B-lymphoid conditions (14, 16). Thus, development of B and T lymphocytes involves distinct transcriptional programs orchestrated and tightly regulated by key lineage determinants.

Multilineage transcriptional priming in progenitors has been shown to allow promiscuous expression of genes associated with divergent cell fates, thus allowing them to be “primed” for differentiation toward various hematopoietic lineages (17, 18). Besides driving the lineage-specific program, the primary cell-fate determinants also have an obligate role to repress the lineage-inappropriate genes in order to enable cell-fate commitment. Therefore, EBF1 and Notch1 may not only exert a direct regulatory control at transcriptional level but also employ an additional layer of control at the posttranscriptional level to effectively regulate gene expression during differentiation. To achieve this, we hypothesized that these primary determinants potentially induce microRNAs (miRNAs) to repress lineage-inappropriate genes and to fine-tune the random fluctuations in transcript abundance (19), thereby providing robustness to the gene expression programs that govern B and T lymphocyte differentiation.

miRNAs are small (22 nt) noncoding RNAs that bind to the target mRNAs (messenger RNAs) within the Ago2–RISC complexes. This interaction results in destabilization and subsequent degradation of the target mRNA molecules, thereby

Significance

MicroRNAs (miRNAs) are a family of noncoding RNAs that regulate gene expression by posttranscriptional mechanisms. They are important for cell differentiation and were found to be misregulated in several immune disorders. However, the genes regulated by miRNAs during lymphocyte development remain largely unknown. Here, we show that miRNAs are conserved between early–B and early–T cells, but they target genes associated with natural killer, dendritic cell, and myeloid lineages in a cell type–specific manner. Knockdown and overexpression studies show miRNAs play an important role in the sustenance of lineage-specific gene expression programs and thereby enforce lymphoid cell-fate commitment. These studies provide insights into the role of miRNAs in the maintenance of lineage identity and regulation of cell-fate decisions.

Author contributions: S.N. and J.M.R.P. designed research; S.N., A.P., and P.K.N. performed research; S.N., D.P., and C.M. contributed new reagents/analytic tools; S.N. and A.D.Y. analyzed data; and S.N., A.D.Y., and J.M.R.P. wrote the paper.

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